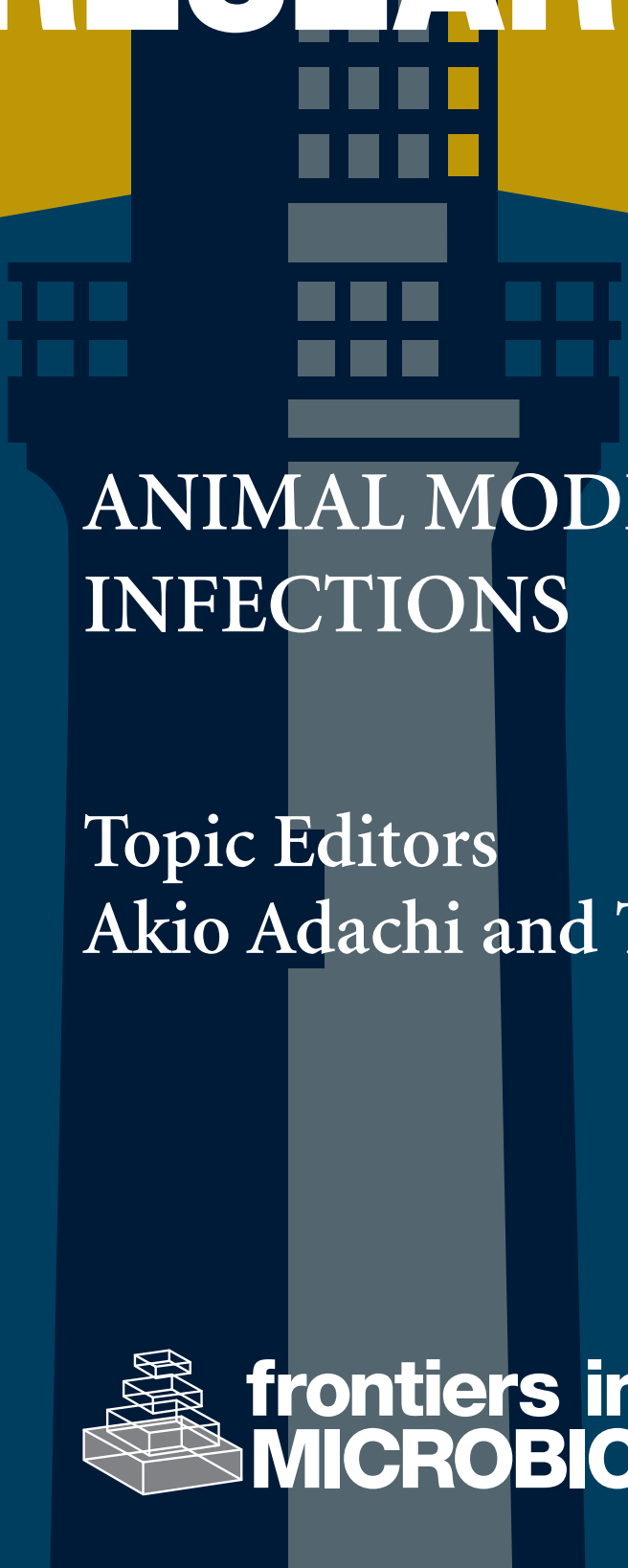
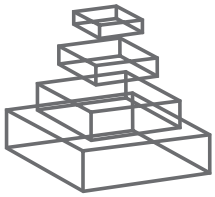
frontiers
RESEARCH TOPICS



ANIMAL MODEL STUDIES ON VIRAL INFECTIONS

Topic Editors
Akio Adachi and Tomoyuki Miura

frontiers in
MICROBIOLOGY



frontiers

Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-456-8
DOI 10.3389/978-2-88919-456-8

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

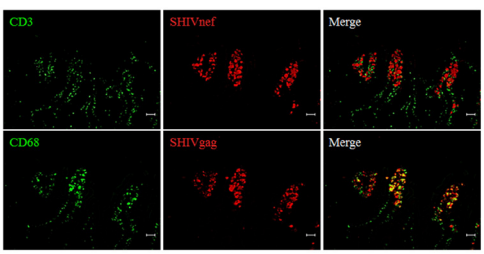
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ANIMAL MODEL STUDIES ON VIRAL INFECTIONS

Topic Editors:
Akio Adachi, Tokushima University, Japan
Tomoyuki Miura, Kyoto University, Japan



Multiple immunofluorescence analysis of jejunum from a macaque infected with chimeric simian-human immunodeficiency virus (SHIV). Green, cellular CD3 and CD68 proteins; Red, viral Nef and Gag proteins. Scale bars=50μm.

Understanding viral replication and pathogenicity properties in infected individuals is a major mission of animal virology. Animal models are essential to analyze the in vivo viral characteristics and to develop countermeasures against viruses. To fight against a wide variety of viruses, basic studies with specific and/or common approaches are required. This Research Topic collects articles that describe studies on numerous virus species at various stages toward animal experiments: (i) description/evaluation/new challenges of animal model studies; (ii) experimental material/methods for animal model studies; (iii) observations for upcoming animal model studies.

Numbers of DNA and RNA viruses such as HHV-6, HPV, Ebola virus, HCV, dengue virus, HTLV-1, HIV-1, SIV, and measles virus are covered by this special issue consisting of original research, methods, review, mini-review, and opinion articles. All readers would understand, we believe and hope, that animal model studies are critical for current virology as always.

Table of Contents

frontiers in
MICROBIOLOGY

EDITORIAL
published: 03 December 2014
doi: 10.3389/fmicb.2014.00672

Animal model studies on viral infections

***Akio Adachi*[1]* and *Tomoyuki Miura*[2]**

[1] *Department of Microbiology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan*
[2] *Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, Kyoto, Japan*
**Correspondence: adachi@basic.med.tokushima-u.ac.jp*

Edited by:
Akihide Ryo, Yokohama City University, Japan

Reviewed by:
Yasuo Ariumi, Kumamoto University, Japan
Ayumi Kudoh, Yokohama City University School of Medicine, Japan

Keywords: animal models, viral infections, viral replication, viral pathogenicity, anti-viral strategies

One of the major missions of animal virology is to understand how viruses replicate and cause asymptomatic/symptomatic conditions in individuals (Nomaguchi and Adachi, 2010). It is especially important for virologists who work on viruses pathogenic for humans to elucidate bases underlying the *in vivo* viral characteristics. Toward this end, animal model studies in some ways are necessary to precisely analyze the *in vivo* situation, and also are essential for developing countermeasures against virus infections. Since a full variety of viruses with distinct biological properties exist, we virologists should study "the target virus" in a specialized manner, in addition to common theoretical/experimental approaches. The Research Topic entitled "Animal model studies on viral infections" collects articles that describe the studies on numerous virus species for their animal models, or those at various stages toward animal experiments.

Articles in this Research Topic were written by experts in various research fields, and can be fairly grouped into a few categories: (i) descriptions/evaluations/new challenges of animal model studies for investigating the biology of viruses; (ii) experimental materials/methods for upcoming animal model studies; (iii) observations important for animal model studies. (i) Reynaud and Horvat (2013) have described the animal models for human herpesvirus 6 to better understand its pathogenic property. Studies on filoviruses, classified as biosafety level-4 and represent a serious world-wide problem today, have been reviewed by Nakayama and Saijo (2013). Mailly et al. (2013) have focused on the quest for appropriate animal models for hepatitis C virus. Clark et al. (2013) have discussed about the use of non-human primates as models for dengue hemorrhagic fever/dengue shock syndrome. Ohsugi (2013) has summarized mouse strains transgenic for the *tax* gene of human T-cell leukemia virus type 1 (HTLV-1). Also, a bovine model for HTLV-1 pathogenesis has been described by Aida et al. (2013). Challenging new attempts to establish human immunodeficiency virus type 1 (HIV-1)/macaque infection models have been reviewed by Misra et al. (2013), and also by Saito and Akari (2013). Another approach to understand HIV-1 biology *in vivo* has been described by Matsuyama-Murata et al. (2013). (ii) Kodama et al. (2013) has described a new and simple method to prepare human dendritic cells from peripheral blood mononuclear cells. Doi et al. (2013) have summarized their studies on macaque-tropic HIV-1 clones. Ikeno et al. (2013) has reported a new, sensitive, and quantitative system to monitor measles virus infection in humanized mice. Iwami et al. (2013) have summarized the quantification of viral infection dynamics based on various quantitative analyses. (iii) Tada et al. (2013) have suggested that LEDGF/p75 may be a cellular factor acting as a species-barrier against HIV-1 in mouse cells. Kuwata et al. (2013) have shown that simian immunodeficiency virus may acquire the increased infectivity and resistance to neutralizing antibodies by truncation of its gp41 cytoplasmic tail. Ohsugi et al. (2013) have reported that natural infection status of laboratory mice by murine norovirus. Finally, Kajitani et al. (2013) have described the possible involvement of E1^E4 protein of human papillomavirus type 18 in its differentiation-dependent life cycle.

We are proud to add our "Animal model studies on viral infections" to a series of Research Topic in Frontiers in Microbiology. A wide variety of DNA and RNA viruses are covered by this special issue consisting of original research, review, mini-review, methods, and opinion articles. As we described in the beginning, animal studies are certainly required for understanding virus replicative/pathogenic properties *in vivo* and for overcoming virally-caused infectious diseases. We human virologists should make every effort to fight against numbers of unique pathogenic viruses.

REFERENCES

Aida, Y., Murakami, H., Takagashi, M., and Takeshima, S. (2013). Mechanisms of pathogenesis induced by bovine leukemia virus as a model for huma T-cell leukemia virus. *Front. Microbiol.* 4:328. doi: 10.3389/fmicb.2013.00328

Clark, K., Onlamoon, N., Hsiao, H.-M., Perng, G., and Villinger, F. (2013). Can non-huma primates serve as models for investigating dengue disease pathogenesis? *Front. Microbiol.* 4:305. doi: 10.3389/fmicb.2013.00305

Doi, N., Okubo, A., Yamane, M., Sakai, Y., Adachi, A., and Nomaguchi, M. (2013). Growth potentials of CCR5-tropic/CXCR4-tropic HIV-1mt clones in macaque cells. *Front. Microbiol.* 4:2188. doi: 10.3389/fmicb.2013.00218

Ikeno, S., Suzuki, M., Muhsen, M., Ishige, M., Kobayashi, M., Ohno, S., et al. (2013). Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR. *Front. Microbiol.* 4:298. doi: 10.3389/fmicb.2013.00298

Iwami, S., Koizumi, Y., Ikeda, H., and Kakizoe, Y. (2013). Quantification of viral infection dynamics in animal experiments. *Front. Microbiol.* 4:264. doi: 10.3389/fmicb.2013.002264

Kajitani, N., Satsuka, A., Yoshida, S., and Sakai, K. (2013). HPV 18 E1^E4 is assembled into aggresome-like compartment and involved in sequestration

of viral oncoproteins. *Front. Microbiol.* 4:251. doi: 10.3389/fmicb.2013.00251

Kodama, A., Tanaka, R., Saito, M., Ansari, A., and Tanaka, Y. (2013). A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive $CD4^+$ T cells in the hu-PBL SCID mice. *Front. Microbiol.* 4:292. doi: 10.3389/fmicb.2013.00292

Kuwata, T., Takaki, K., Enomoto, I., Yoshimura, K., and Matsushita, S. (2013). Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail. *Front. Microbiol.* 4:117. doi: 10.3389/fmicb.2013.00117

Mailly, L., Robinet, E., Meuleman, P., Baumert, T. F., and Zeisel, M. B. (2013). Hepatitis C virus infection and related liver disease: the quest for the best animal model. *Front. Microbiol.* 4:212. doi: 10.3389/fmicb.2013.00212

Matsuyama-Murata, M., Inaba, K., Horiuchi, R., Fukazawa, Y., Ibuki, K., Hayami, M., et al. (2013). Genetic similarity of circulating and small intestinal virus at the end stage of acute pathogenic simian-human immunodeficiency virus infection. *Front. Microbiol.* 4:204. doi: 10.3389/fmicb.2013.00204

Misra, A., Thippeshappa, R., and Kimata, J. T. (2013). Macaques as model hosts for studies of HIV-1 infection. *Front. Microbiol.* 4:176. doi: 10.3389/fmicb.2013.00176

Nakayama, E., and Saijo, M. (2013). Animal models for Ebola and Marburg virus infections. *Front. Microbiol.* 4:267. doi: 10.3389/fmicb.2013.00267

Nomaguchi, M., and Adachi, A. (2010). Virology as biosystematics: towards understanding the viral infection biology. *Front. Microbiol.* 1:2. doi: 10.3389/fmicb.2010.00002

Ohsugi, T. (2013). A transgenic mouse model of huma T cell leukemia virus type 1-associated diseases. *Front. Microbiol.* 4:49. doi: 10.3389/fmicb.2013.00049

Ohsugi, T., Matsuura, K., Kawabe, S., Nakamura, N., Kumar, J. M., Wakamiya, M., et al. (2013). Natural infection of murine norovirus in conventional and specific pathogen-free laboratory mice. *Front. Microbiol.* 4:12. doi: 10.3389/fmicb.2013.00012

Reynaud, J., and Horvat, B. (2013). Animal models for human herpesvirus 6 infection. *Front. Microbiol.* 4:174. doi: 10.3389/fmicb.2013.00174

Saito, A., and Akari, H. (2013). Macaque-tropic human immunodeficiency virus type 1: breaking out of the host restriction factors. *Front. Microbiol.* 4:187. doi: 10.3389/fmicb.2013.00187

Tada, T., Kadoki, M., Yang, L., Tokunaga, K., and Iwakura, Y. (2013). Transgenic expression of the human LEDGF/p75 gene relieves the species barrier against HIV-1 infection in mouse cells. *Front. Microbiol.* 4:377. doi: 10.3389/fmicb.2013.00377

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 November 2014; accepted: 19 November 2014; published online: 03 December 2014.

Citation: Adachi A and Miura T (2014) Animal model studies on viral infections. Front. Microbiol. ***5****:672. doi: 10.3389/fmicb.2014.00672*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 04 July 2013
doi: 10.3389/fmicb.2013.00174

Animal models for human herpesvirus 6 infection

Joséphine M. Reynaud and Branka Horvat*

International Center for Infectiology Research, INSERM U1111, CNRS UMR5308, ENS Lyon, University of Lyon 1, Lyon, France

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Yasuko Mori, Kobe University, Japan
Naoki Inoue, National Institute of Infectious Diseases, Japan

***Correspondence:**
Branka Horvat, International Center for Infectiology Research, INSERM U1111, CNRS UMR5308, ENS Lyon, University of Lyon 1, Tour CERVI, 21 Avenue Tony Garnier, 69365 Lyon, France
e-mail:branka.horvat@inserm.fr

Human herpesvirus (HHV)-6A and HHV-6B are two enveloped DNA viruses of β-herpesvirus family, infecting over 90% of the population and associated with several diseases, including *exanthema subitum* (for HHV-6B), multiple sclerosis and encephalitis, particularly in immunosuppressed patients. Animal models are highly important to better understand the pathogenesis of viral infections. Naturally developed neutralizing antibodies to HHV-6 or a related virus were found in different species of monkeys, suggesting their susceptibility to HHV-6 infection. Both HHV-6 DNA and infectious virus were detected in experimentally infected Cynomolgus and African green monkeys, although most animals remained clinically asymptomatic. Furthermore, HHV-6A infection was shown to accelerate the progression of AIDS (acquired immunodeficiency syndrome) in macaques and to lead to the development of neurological symptoms in the marmoset model. Humanized SCID (severe combined immunodeficiency) mice efficiently replicated HHV-6 and were also susceptible to coinfection with HHV-6 and HIV-1 (human immunodeficiency virus 1). As CD46 was identified as a receptor for HHV-6, transgenic mice expressing human CD46 may present a potentially interesting model for study certain aspects of HHV-6 infection and neuroinflammation.

Keywords: HHV-6, animal model, mouse, monkey, HIV, AIDS, neuroinflammation, CD46

INTRODUCTION

Human herpesvirus (HHV)-6 belongs to the β-Herpesviridae subfamily, together with its closest homolog HHV-7 and human cytomegalovirus (HCMV). The two variants of HHV-6, HHV-6A and HHV-6B, have recently been recognized as two distinct viruses by the international committee on taxonomy of viruses, based mostly on their known genetic and epidemiological features (Braun et al., 1997). Primary infection with HHV-6B was identified as the etiological cause for roseola (*exanthema subitum*), a common febrile illness in infants (Yamanishi et al., 1988), whereas primary infection with HHV-6A has not yet been clearly associated to any specific disease. Like most herpesviruses, HHV-6A and -6B are able to establish asymptomatic long-term persistence in their hosts, and can reactivate under specific conditions. Although the mechanisms of reactivation are not yet completely understood, both viruses are known to reactivate in immunosuppressed patients, causing a variety of complications such as encephalitis, hepatitis, or graft rejection (Dockrell and Paya, 2001; Zerr, 2006). In addition, HHV-6A and -6B have been associated with several neurological diseases in the immunocompetent population. Indeed, numerous clinical studies have established a correlation between HHV-6A and -6B infection and the demyelinating, autoimmune disease-multiple sclerosis (reviewed in Reynaud and Horvat, 2013), and both viruses are thought to be involved in the development of certain cases of encephalitis, meningitis, and epilepsy (Theodore et al., 2008; Yao et al., 2010).

Human herpesvirus-6 has often been isolated from patients with acquired immunodeficiency syndrome (AIDS) and was suggested to play a role in the progression of this disease. Indeed, an active and wide-spread HHV-6 infection was observed in AIDS patients (Knox and Carrigan, 1994; Secchiero et al., 1995) and AIDS was described to progress rapidly after primary HHV-6 infection in children with vertically inherited human immunodeficiency virus (HIV; Kositanont et al., 1999). Both HHV-6 and HIV have a preferential tropism toward $CD4^+$ T cells and can establish simultaneous productive infection with synergistic cytopathic effects (Lusso et al., 1989). Moreover, HHV-6 has a wider range of susceptible cell types than HIV-1 and was shown to induce the expression of the HIV-1 receptor CD4 on immune cells that do not naturally express this molecule, rendering them, thus, susceptible to HIV-1 infection (Lusso et al., 1993, 1995). However, in the context of both AIDS and different other HHV-6-related pathologies, the consequences of coinfection and the potential mechanisms involved in the pathogenesis remain to be elucidated.

A few antiviral drugs have been shown to be efficient against HHV-6 infection *in vitro* (Manichanh et al., 2000; De Clercq et al., 2001; De Bolle et al., 2005b) and were successfully used for the treatment of patients suffering from encephalitis following viral reactivation (reviewed in De Bolle et al., 2005b). However, these treatments are often associated with strong adverse effects and fully controlled specific clinical studies demonstrating their *in vivo* efficiency are still missing. Animal models represent very useful tools for preclinical analyses of potential antiviral drugs and for the study of viral pathogenesis. Here, we review the different animal models developed for the study of HHV-6A and/or HHV-6B infection (**Table 1**) and discuss the data obtained. In particular, the use of animal models has brought new evidence of the capacity of HHV-6A to induce neuropathology and has allowed the study of the interactions between HHV-6 and immunodeficiency viruses, showing a role of HHV-6A in AIDS progression and providing potential explanations for the impact of HHV-6A on the course of HIV infection.

Table 1 | Described animal models for HHV-6 infection.

Species	Genetic modification	Virus (strain)	Route of inoculation	Clinical signs	Virological data	Reference
African green monkey (*Cercopithecus aethiops*)	none	HHV-6B (HST)	s.c./i.v	Skin rash (1 animal)	IgG response, viral DNA (PBMC, l.n.)	Yalcin et al. (1992)
Cynomolgus macaque (*Macaca fascicularis*)	none	HHV-6B (HST)	s.c./i.v.	none	IgG response, viral DNA (PBMC, spleen)	Yalcin et al. (1992)
Pig-tailed macaque (*Macaca nemestrina*)	none	HHV-6A (GS)	i.v.	fever, nasal discharge, splenomegaly, lymphadenopathy, abdominal rash	IgG response, plasma viremia, viral transcripts (l.n.)	Lusso et al. (2007)
Marmoset (*Callithrix jacchus*)	none	HHV-6A (U1102)	i.v.*	motor weaknesses, sensorial abnormalities, facial palsy, lesions in the corpus callosum at MRI	IgG and IgM response, viral DNA (brain, spleen, l.n., heart, kidney, liver)	Leibovitch et al. (2013)
	none	HHV-6B (Z29)	i.v.*	none	IgG response, viral DNA (brain)	Leibovitch et al. (2013)
	none	HHV-6A (U1102)	i.n.*	none	Plasma viremia, viral DNA (saliva, PBMC)	Leibovitch et al. (2013)
Mouse *(Mus musculus)*	none	HHV-6B (Z29)	i.p.	none	IgG response	Svensson et al. (2010)
	none	HHV-6A	N/A	none	N/A	Lusso (1996)
	Hu SCID (Thy/liv)	HHV-6A (GS) HHV-6B (PL-1)	in the implant	N/A	N/A	Gobbi et al. (1999)

** Inoculation performed using several (three or four) injections, s.c., subcutaneous; i.v., intravenous; i.n., intanasal; i.p., intraperitoneal; l.n., lymph nodes; N/A, not available.*

SIMIAN MODELS

NATURAL INFECTION IN MONKEYS

Shortly after the discovery of HHV-6, several groups have searched for evidence of natural infection by HHV-6 in monkeys. Initial studies first reported an absence of specific antibodies in several species of new- and old-world non-human primates (Salahuddin et al., 1986; Lusso et al., 1990). In contrast, another study carried out on 10 different species of monkeys revealed the presence of HHV-6-reactive antibodies, suggesting a previous infection either by HHV-6 or a closely related virus (Higashi et al., 1989). Among the tested species, eight were positive in immunofluorescence assay and seroneutralization. African green monkeys, squirrel monkeys, chimpanzees, and orangutan appeared to be the most frequently infected, with 75–100% of prevalence. Furthermore, several groups of monkeys of the same species but from different locations exhibited similar prevalence rates, thus suggesting that the susceptibility to HHV-6 infection may be species-dependent.

More recently, a simian homolog of HHV-6 was isolated from blood samples from chimpanzees (*Pan troglodytes*; Lacoste et al., 2005). This new member of the β-herpesvirus group, called PanHV6, was found to be particularly close to the Z29 strain of HHV-6B. It was detected in several different subspecies of wild-caught chimpanzees from Cameroon and Gabon, but also in animals born in captivity in the Netherlands, indicating that this virus is present in different populations of chimpanzees. Several simian homologs of other human herpesviruses, including HCMV and Epstein–Barr virus (EBV; Davison et al., 2003; Ehlers et al., 2003) have been identified, which supports the theory that these viruses might have co-evolved with their host species. The natural susceptibility of some species of monkeys to infection with HHV-6 or a simian counterpart indicates that monkeys may represent an appropriate model for the study of HHV-6A and/or -6B pathogenesis.

EXPERIMENTAL INFECTION IN SIMIAN MODELS

Infection of simian cells

Analyses performed on *in vitro*-infected peripheral blood mononuclear cells (PBMCs) from several species of monkeys, indicated that cells from chimpanzees and macaques (*Macaca nemestrina* and *M. mulatta*) are the most susceptible to infection by HHV-6 (Lusso et al., 1990, 1994). The infection of PBMCs led to the production of viral proteins and viral particles, observed by immunofluorescence and electron microscopy. Infection seemed highly variable among the species of monkeys tested and depended on the virus used (A or B). PBMCs from rhesus macaques (*M. mulatta*) were found to be susceptible to HHV-6B infection only, while PBMCs from pig-tailed macaques (*M. nemestrina*) were infected with similar efficiency by both HHV-6A and -6B.

African green monkeys and cynomolgus macaques

The first experiments of *in vivo* HHV-6 infection in monkeys were conducted using African green monkeys (*Cercopithecus aethiops*) and cynomolgus macaques (*M. fascicularis*; Yalcin et al., 1992). Four animals from each species were inoculated with the HST strain of HHV-6B. Monkeys received a single subcutaneous (s.c.) or intravenous (i.v.) injection of 10^5 half maximal tissue culture infective doses ($TCID_{50}$), and were monitored for 33 days. Following virus inoculation, a specific antibody response was detected, as well as the presence of viral DNA in the PBMCs and in the spleen and lymph nodes of some animals. However, the infection remained asymptomatic in all animals, except for one African green monkey, which developed a skin rash on the trunk (**Table 1**).

Pig-tailed macaques

Infection with HHV-6A was later described in pig-tailed macaques (*M. nemestrina*; Lusso et al., 2007). After i.v. inoculation with HHV-6A (GS strain), clinical symptoms of mild to moderate intensity were observed, including fever, nasal discharge, splenomegaly, generalized lymphadenopathy and abdominal rash (in one animal). Moreover, in this model, systemic infection was confirmed by the detection of viral DNA in the plasma, the development of an antibody response, and the presence of viral transcripts in the lymph nodes.

Furthermore, this model has been used to analyze coinfection with HHV-6A and simian immunodeficiency virus (SIV), a simian counterpart of HIV-1 typically used for experimental infection in macaques (Lusso et al., 2007). This approach provided the first *in vivo* data showing that HHV-6A infection can accelerate AIDS progression. Indeed, although HHV-6A infection did not seem to have any effect on SIV spreading, dually infected animals exhibited faster depletion in $CD4^+$ T cells than the singly SIV-infected ones. Interestingly, HHV-6A infection also resulted in a faster decrease in $CD8^+$ T cell count, which could be due to HHV-6A-induced *de novo* expression of CD4 in these cells.

A potential mechanism explaining the enhancement of AIDS by HHV-6A was suggested following the analysis of the virus isolated from monkeys receiving either single SIV infection or HHV-6A/SIV coinfection (Biancotto et al., 2009). *In vitro* replication of viral isolates obtained from singly infected monkeys was inhibited by coinfection with HHV-6A, and treatment with the chemokine CCL5 (regulated upon activation normal T cell expressed and secreted, RANTES) had similar effects. In contrast, all isolates from dually infected monkeys appeared to be resistant to both CCL5 treatment and HHV-6A infection, suggesting that *in vivo* coinfection with HHV-6A probably directs SIV evolution toward CCL5 resistance. Thus HHV-6A infection could create a high-CCL5 environment *in vivo*, in which CCL5-resistance would be advantageous for efficient SIV replication.

The marmoset model: evidence for HHV-6A-induced neurological disease

A recent study described a new model of infection by both HHV-6A and -6B using common marmosets (*Callithrix jacchus*), which represents so far the only model of HHV-6A infection associated with the more important clinical signs (Leibovitch et al., 2013). Indeed, HHV-6A-infected monkeys that received several monthly i.v. injections developed clear neurological symptoms, including motor weakness and sensory abnormalities. Moreover, magnetic resonance imaging (MRI) analyses revealed the presence of hyperintense lesions in the brain of one animal. This study provided the first *in vivo* evidence that HHV-6A infection is able to trigger

neurological disease. In humans, both HHV-6A and -6B DNA was shown to be present in the brain of healthy people, indicating that both species have similar neuroinvasive properties. In marmosets, viral DNA was also occasionally detected in the brain of HHV-6A- and -6B-infected animals, which confirmed the capacity of both viruses to reach the brain. However, while HHV-6A infection led to evident neurological symptoms, infection with HHV-6B remained asymptomatic, thus showing an important difference between HHV-6A and -6B in their ability to infect marmosets (**Table 1**).

Interestingly, an additional group of marmosets was infected with HHV-6A through the intranasal (i.n.) route of inoculation, which resulted in radically different clinical outcomes. Based on histological data, the i.n. pathway was proposed as a possible route of transmission and access to the brain for HHV-6A in humans (Harberts et al., 2011). Contrary to i.v.-injected marmosets, i.n.-injected animals did not exhibit any sign of disease. Moreover, i.n.-inoculated animals rarely developed antibody responses and maintained plasma viremia, whereas i.v. injection led to the development of HHV-6-specific antibody responses and clearance of viral DNA in the plasma. These results suggested that the neurological symptoms observed with i.v. injection might be due to the immune response developed against the virus rather than to the direct consequences of viral infection and spreading. This model therefore emphasizes the importance of the route of inoculation in viral pathogenesis, and provides a clear *in vivo* demonstration that HHV-6A can cause a neurological disease with MS-like symptoms. The marmoset model thus appears as an appropriate model for the analysis of HHV-6A-induced neurological disease, and confirms the correlation between HHV-6 infection and the development of multiple sclerosis.

MURINE MODELS

The possibility of using a murine model to study HHV-6 infection has been attractive to the scientific community since the discovery of HHV-6. However, mice were initially described to be resistant to HHV-6 infection (Lusso, 1996). Nevertheless, a few studies have described the use of *in vitro* or *in vivo* murine models for the study of HHV-6.

SUSCEPTIBILITY OF MURINE CELLS TO HHV-6 INFECTION

The susceptibility to infection by HHV-6A and HHV-6B of several human and non-human cell lines was analyzed in a few studies. Both viruses failed to replicate in the murine mammary carcinoma cell line FM3A and viral transcripts were not detected in these cells, suggesting that murine cells are not permissive to infection by HHV-6 (De Bolle et al., 2005a). However, another study showed that infection by HHV-6A and HHV-6B enables the transcription of viral genes in murine primary oligodendrocyte precursors, although viral replication was not observed (Mock et al., 2006), suggesting that the susceptibility to HHV-6 infection may, to some extent, depend on the cell type analyzed. In addition, both HHV-6A and HHV-6B could induce cell cycle arrest in these cells, similarly, to what was previously observed in human oligodendrocyte precursor cells (Dietrich et al., 2004), indicating that some murine cell types could be used as a model to study certain aspects of HHV-6 infection *in vitro*.

IN VIVO MURINE MODELS FOR THE STUDY OF HHV-6 INFECTION

It has been reported that natural resistance of mice to herpesvirus infection, particularly against herpes simplex virus (HSV) is genetically determined and linked to major histocompatibility complex (MHC) genes (Lopez, 1975). Balb/c mice were among the most susceptible lines and were recently used to analyze the link between HHV-6B infection and allergy (Svensson et al., 2010). Although systemic infection was not observed, inoculation of UV-inactivated virus resulted in the development of specific IgG responses and had protective effects against the development of allergy by limiting the inflammation in lungs, thus suggesting the immunosuppressive effects of HHV-6B *in vivo*.

To overcome natural resistance of mice to HHV-6 infection another approach using immunodeficient mice was developed, aiming to provide an *in vivo* environment for the study of human tissue rather than to analyze the infection in mice. For this purpose, severe combined immunodeficiency (SCID) mice were used. These mice carry a mutation which provokes profound T and B lymphopenia, allowing the engraftment of heterologous tissues (McCune, 1996). SCID mice were humanized by coimplanting human fetal thymus and liver under the murine kidney capsule, permitting the growth of a unique thy/liv organ which histologically resembles human thymus. Mice carrying thy/liv organ support human lymphopoiesis, thus allowing the study of human lymphoid cells in an *in vivo* context, and were used for the study of other human viruses, especially for human immunodeficiency virus (HIV; Van Duyne et al., 2009). Inoculation with HHV-6A or -6B was performed by direct injection in the implant and led to productive infection of human thymic cells, associated with a strong thymic depletion (Gobbi et al., 1999). That study demonstrated that HHV-6 infection is able to induce immunosuppression in an *in vivo* context, which may explain how HHV-6 could enhance the progression of immunodeficiency in AIDS patients. In this model, HHV-6 seemed to exhibit a particular tropism toward intra-thymic T progenitor cells (ITTPs), a rapidly dividing subset of thymic cells which gives rise to other thymocytes at later stages of maturation. This study suggested that lytic infection of ITTPs may play an important role in the HHV-6-induced thymic depletion.

Coinfection with HHV-6A or-6B and HIV-1 was later performed using the same model (Gobbi et al., 2000). Both viruses were found to be able to simultaneously infect the engrafted human tissue, yet infection with either virus did not seem to have any impact on the replication or virulence of the other.

TOWARD NOVEL TRANSGENIC MURINE MODELS

Other models of humanized mice are currently under investigation. A model of rag2$^{-/-}\gamma c^{-/-}$ mice, deficient for T and B lymphocytes and NK cells and engrafted with human hematopoietic stem cells (Chicha et al., 2005) is being developed for the analysis of HHV-6 (Tanner et al., 8th International Conference on HHV-6&7, April 2013). The use of this model has allowed numerous advances in the field of retrovirology (Van Duyne et al., 2009) and will certainly help in the understanding of HHV-6 immunopathogenesis.

Human herpesvirus-6 was shown to use the human protein CD46 as a cellular receptor for viral entry (Santoro et al.,

1999). This transmembrane protein is involved in the protection of host cells against complement lysis (Liszewski et al., 1991), through binding to C3b and C4b components of the complement (**Figure 1**), and was identified as the receptor for a variety of pathogens, including measles virus (vaccine strains), several serotypes of adenovirus and some pathogenic bacteria (Riley-Vargas et al., 2004). Moreover, it was recently found to bind the immunoregulatory molecule Jagged 1, a member of the Notch system (Le Friec et al., 2012; **Figure 1**). CD46 is ubiquitously expressed in humans and is mostly conserved in other primates (Seya et al., 1998). As viral tropism is determined by the pattern of expression of virus-specific cellular receptors, these molecules are key players in viral infection. Contrary to most primate CD46 proteins, murine CD46 has a lower percentage of identity with the human protein and its expression is restricted to the testis, which may account for the resistance of mice to infection. Therefore the generation of transgenic mice expressing human CD46 with a ubiquitous distribution, as in humans, could provide new perspectives for the development of animal models for HHV-6 infection. We have produced several lines of CD46 transgenic mice (Horvat et al., 1996; Marie et al., 2002) and used them to analyze the pathogenesis of HHV-6 infection. HHV-6A seemed to establish long-term persistence in the brain of these mice, and to induce leukocyte infiltration (Reynaud et al., 8th International Conference on HHV-6&7, April 2013). Thus, CD46 transgenic mice may represent a potential new small animal model for the study of HHV-6A-induced neuroinflammation. Some studies have suggested that CD46 may not be the only receptor for HHV-6B entry (Mori et al., 2002, 2004), opening thus, perspectives for the development of additional transgenic models for this virus. The availability of many experimental tools for murine models should facilitate further studies of virus–host interaction and HHV-6 pathogenesis.

CONCLUSION

The development of relevant animal models is critical for a better understanding of viral pathogenesis, generating new diagnostic tools and assessing antiviral therapeutics and vaccines. Although animal models usually do not mimic all the aspects of the human

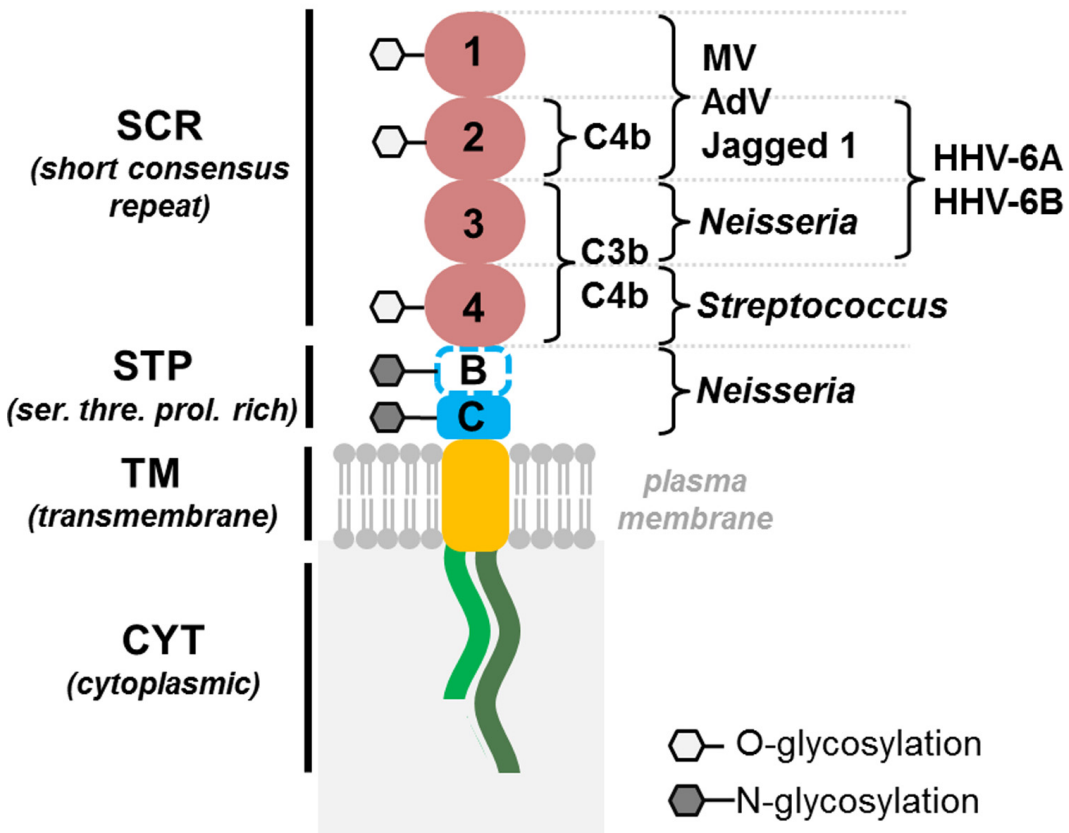


FIGURE 1 | Schematic representation of human HHV-6 receptor CD46, expressed in transgenic mice. CD46 consists of an extracellular part, with indicated binding site for different known CD46 ligands, transmembrane domain and one of two cytoplasmic tails: short, Cyt-1 or long, Cyt-2. MV, Measles Virus; AdV, Adenovirus.

disease, they do reproduce at least some of them and could thus help in a better understanding of certain aspects of viral pathogenesis. The number of animal models to study HHV-6 infection is still rather limited and mainly includes non-human primates. Utilization of pig-tailed macaques provided evidence for the HHV-6-induced acceleration of AIDS and recently HHV-6A infection in marmosets has strongly suggested a link with neurological diseases. The latent nature of HHV-6 infection makes most *in vivo* studies often difficult to carry out. Recent advances in the development of murine models for HHV-6 infection, with numerous and powerful tools available, should be of critical help for in-depth immunobiological and genetic studies of HHV-6 infection.

ACKNOWLEDGMENTS

This work was supported by INSERM and ARSEP and Joséphine M. Reynaud was supported by a doctoral fellowship from the French ministry of research.

REFERENCES

Biancotto, A., Grivel, J.-C., Lisco, A., Vanpouille, C., Markham, P. D., Gallo, R. C., et al. (2009). Evolution of SIV toward RANTES resistance in macaques rapidly progressing to AIDS upon coinfection with HHV-6A. *Retrovirology* 6, 61. doi: 10.1186/1742-4690-6-61

Braun, D. K., Dominguez, G., and Pellett, P. E. (1997). Human herpesvirus 6. *Clin. Microbiol. Rev.* 10, 521–567. doi: 10.1086/322691

Chicha, L., Tussiwand, R., Traggiai, E., Mazzucchelli, L., Bronz, L., Piffaretti, J.-C., et al. (2005). Human adaptive immune system Rag2−/−gamma(c)−/− mice. *Ann. N. Y. Acad. Sci.* 1044, 236–243. doi: 10.1196/annals.1349.029

Davison, A. J., Dolan, A., Akter, P., Addison, C., Dargan, D. J., Alcendor, D. J., et al. (2003). The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. *J. Gen. Virol.* 84, 17–28. doi: 10.1099/vir.0.18606-0

De Bolle, L., Van Loon, J., De Clercq, E., and Naesens, L. (2005a). Quantitative analysis of human herpesvirus 6 cell tropism. *J. Med. Virol.* 75, 76–85. doi: 10.1002/jmv.20240

De Bolle, L., Naesens, L., and De Clercq, E. (2005b). Update on human herpesvirus 6 biology, clinical features, and therapy. *Clin. Microbiol. Rev.* 18, 217–245. doi: 10.1128/CMR.18.1.217-245.2005

De Clercq, E., Naesens, L., De Bolle, L., Schols, D., Zhang, Y., and Neyts, J. (2001). Antiviral agents active against human herpesviruses HHV-6, HHV-7 and HHV-8. *Rev. Med. Virol.* 11, 381–395. doi: 10.1002/rmv.336

Dietrich, J., Blumberg, B. M., Roshal, M., Baker, J. V., Hurley, S. D., Mayer-Pröschel, M., et al. (2004). Infection with an endemic human herpesvirus disrupts critical glial precursor cell properties. *J. Neurosci.* 24, 4875–4883. doi: 10.1523/JNEUROSCI.5584-03.2004

Dockrell, D. H., and Paya, C. V. (2001). Human herpesvirus-6 and -7 in transplantation. *Rev. Med. Virol.* 11, 23–36. doi: 10.1002/rmv.299

Ehlers, B., Ochs, A., Leendertz, F., Goltz, M., Boesch, C., and Mätz-Rensing, K. (2003). Novel simian homologues of Epstein–Barr virus. *J. Virol.* 77, 10695–10699. doi: 10.1128/JVI.77.19.10695-10699.2003

Gobbi, A., Stoddart, C. A., Locatelli, G., Santoro, F., Bare, C., Linquist-Stepps, V., et al. (2000). Coinfection of SCID-hu Thy/Liv mice with human herpesvirus 6 and human immunodeficiency virus type 1. *J. Virol.* 74, 8726–8731. doi: 10.1128/JVI.74.18.8726-8731.2000

Gobbi, A., Stoddart, C. A., Malnati, M. S., Locatelli, G., Santoro, F., Abbey, N. W., et al. (1999). Human herpesvirus 6 (HHV-6) causes severe thymocyte depletion in SCID-hu Thy/Liv mice. *J. Exp. Med.* 189, 1953–1960. doi: 10.1084/jem.189.12.1953

Harberts, E., Yao, K., Wohler, J. E., Maric, D., Ohayon, J., Henkin, R., et al. (2011). Human herpesvirus-6 entry into the central nervous system through the olfactory pathway. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13734–13739. doi: 10.1073/pnas.1105143108

Higashi, K., Asada, H., Kurata, T., Ishikawa, K., Hayami, M., Spriatna, Y., et al. (1989). Presence of antibody to human herpesvirus 6 in monkeys. *J. Gen. Virol.* 70(Pt 12), 3171–3176. doi: 10.1099/0022-1317-70-12-3171

Horvat, B., Rivailler, P., Varior-Krishnan, G., Cardoso, A., Gerlier, D., and Rabourdin-Combe, C. (1996). Transgenic mice expressing human measles virus (MV) receptor CD46 provide cells exhibiting different permissivities to MV infections. *J. Virol.* 70, 6673–6681.

Knox, K. K., and Carrigan, D. R. (1994). Disseminated active HHV-6 infections in patients with AIDS. *Lancet* 343, 577–578. doi: 10.1016/S0140-6736(94)91524-5

Kositanont, U., Wasi, C., Wanprapar, N., Bowonkiratikachorn, P., Chokephaibulkit, K., Chearskul, S., et al. (1999). Primary infection of human herpesvirus 6 in children with vertical infection of human immunodeficiency virus type 1. *J. Infect. Dis.* 180, 50–55. doi: 10.1086/314826

Lacoste, V., Verschoor, E. J., Nerrienet, E., and Gessain, A. (2005). A novel homologue of Human herpesvirus 6 in chimpanzees. *J. Gen. Virol.* 86, 2135–2140. doi: 10.1099/vir.0.81034-0

Le Friec, G., Sheppard, D., Whiteman, P., Karsten, C. M., Shamoun, S. A.-T., Laing, A., et al. (2012). The CD46-Jagged1 interaction is critical for human TH1 immunity. *Nat. Immunol.* 13, 1213–1221. doi: 10.1038/ni.2454

Leibovitch, E., Wohler, J. E., Cummings Macri, S. M., Motanic, K., Harberts, E., Gaitán, M. I., et al. (2013). Novel marmoset (*Callithrix jacchus*) model of human Herpesvirus 6A and 6B infections: immunologic, virologic and radiologic characterization. *PLoS Pathog.* 9:e1003138. doi: 10.1371/journal.ppat.1003138

Liszewski, M. K., Post, T. W., and Atkinson, J. P. (1991). Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu. Rev. Immunol.* 9, 431–455. doi: 10.1146/annurev.iy.09.040191.002243

Lopez, C. (1975). Genetics of natural resistance to herpesvirus infections in mice. *Nature* 258, 152–153. doi: 10.1038/258152a0

Lusso, P. (1996). Human herpesvirus 6 (HHV-6). *Antiviral Res.* 31, 1–21. doi: 10.1016/0166-3542(96)00949-7

Lusso, P., Crowley, R. W., Malnati, M. S., Di Serio, C., Ponzoni, M., Biancotto, A., et al. (2007). Human herpesvirus 6A accelerates AIDS progression in macaques. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5067–5072. doi: 10.1073/pnas.0700929104

Lusso, P., Ensoli, B., Markham, P. D., Ablashi, D. V., Salahuddin, S. Z., Tschachler, E., et al. (1989). Productive dual infection of human CD4+ T lymphocytes by HIV-1 and HHV-6. *Nature* 337, 370–373. doi: 10.1038/337370a0

Lusso, P., Garzino-Demo, A., Crowley, R. W., and Malnati, M. S. (1995). Infection of gamma/delta T lymphocytes by human herpesvirus 6: transcriptional induction of CD4 and susceptibility to HIV infection. *J. Exp. Med.* 181, 1303–1310. doi: 10.1084/jem.181.4.1303

Lusso, P., Malnati, M. S., Garzino-Demo, A., Crowley, R. W., Long, E. O., and Gallo, R. C. (1993). Infection of natural killer cells by human herpesvirus 6. *Nature* 362, 458–462. doi: 10.1038/362458a0

Lusso, P., Markham, P. D., DeRocco, S. E., and Gallo, R. C. (1990). In vitro susceptibility of T lymphocytes from chimpanzees (*Pan troglodytes*) to human herpesvirus 6 (HHV-6): a potential animal model to study the interaction between HHV-6 and human immunodeficiency virus type 1 in vivo. *J. Virol.* 64, 2751–2758.

Lusso, P., Secchiero, P., and Crowley, R. W. (1994). In vitro susceptibility of *Macaca nemestrina* to human herpesvirus 6: a potential animal model of coinfection with primate immunodeficiency viruses. *AIDS Res. Hum. Retroviruses* 10, 181–187. doi: 10.1089/aid.1994.10.181

Manichanh, C., Grenot, P., Gautheret-Dejean, A., Debré, P., Huraux, J. M., and Agut, H. (2000). Susceptibility of human herpesvirus 6 to antiviral compounds by flow cytometry analysis. *Cytometry* 40, 135–140. doi: 10.1002/(SICI)1097-0320(20000601)40:2

Marie, J. C., Astier, A. L., Rivailler, P., Rabourdin-Combe, C., Wild, T. F., and Horvat, B. (2002). Linking innate and acquired immunity: divergent role of CD46 cytoplasmic domains in T cell induced inflammation. *Nat. Immunol.* 3, 659–666. doi: 10.1038/ni810

McCune, J. M. (1996). Development and applications of the SCID-hu mouse model. *Semin. Immunol.* 8, 187–196. doi: 10.1006/smim.1996.0024

Mock, D. J., Strathmann, F., Blumberg, B. M., and Mayer-Proschel, M. (2006). Infection of murine oligodendroglial precursor cells with Human Herpesvirus 6 (HHV-6) – establishment of a murine in vitro model. *J. Clin. Virol.* .37(Suppl. 1), S17–S23. doi: 10.1016/S1386-6532(06)70006-3

Mori, Y., Akkapaiboon, P., Yonemoto, S., Koike, M., Takemoto, M.,

Sadaoka, T., et al. (2004). Discovery of a second form of tripartite complex containing gH-gL of human herpesvirus 6 and observations on CD46. *J. Virol.* 78, 4609–4616. doi: 10.1128/JVI.78.9.4609-4616.2004

Mori, Y., Seya, T., Huang, H. L., Akkapaiboon, P., Dhepakson, P., and Yamanishi, K. (2002). Human herpesvirus 6 variant A but not variant B induces fusion from without in a variety of human cells through a human herpesvirus 6 entry receptor, CD46. *J. Virol.* 76, 6750–6761. doi: 10.1128/JVI.76.13.6750-6761.2002

Reynaud, J. M., and Horvat, B. (2013). Human herpesvirus 6 and neuroinflammation. *ISRN Virol.* 2013, 11.

Riley-Vargas, R. C., Gill, D. B., Kemper, C., Liszewski, M. K., and Atkinson, J. P. (2004). CD46: expanding beyond complement regulation. *Trends Immunol.* 25, 496–503. doi: 10.1016/j.it.2004.07.004

Salahuddin, S. Z., Ablashi, D. V., Markham, P. D., Josephs, S. F., Sturzenegger, S., Kaplan, M., et al. (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 234, 596–601. doi: 10.1126/science.2876520

Santoro, F., Kennedy, P. E., Locatelli, G., Malnati, M. S., Berger, E. A., and Lusso, P. (1999). CD46 is a cellular receptor for human herpesvirus 6. *Cell* 99, 817–827. doi: 10.1016/S0092-8674(00)81678-5

Secchiero, P., Carrigan, D. R., Asano, Y., Benedetti, L., Crowley, R. W., Komaroff, A. L., et al. (1995). Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. *J. Infect. Dis.* 171, 273–280. doi: 10.1093/infdis/171.2.273

Seya, T., Nomura, M., Murakami, Y., Begum, N. A., Matsumoto, M., and Nagasawa, S. (1998). CD46 (membrane cofactor protein of complement, measles virus receptor): structural and functional divergence among species (review). *Int. J. Mol. Med.* 1, 809–816.

Svensson, A., Almqvist, N., Chandy, A. G., Nordström, I., and Eriksson, K. (2010). Exposure to human herpes virus type 6 protects against asthma in mice. *J. Allergy Ther.* 1, 101.

Theodore, W. H., Epstein, L., Gaillard, W. D., Shinnar, S., Wainwright, M. S., and Jacobson, S. (2008). Human herpes virus 6B: a possible role in epilepsy? *Epilepsia* 49, 1828–1837. doi: 10.1111/j.1528-1167.2008.01699.x

Van Duyne, R., Pedati, C., Guendel, I., Carpio, L., Kehn-Hall, K., Saifuddin, M., et al. (2009). The utilization of humanized mouse models for the study of human retroviral infections. *Retrovirology* 6, 76. doi: 10.1186/1742-4690-6-76

Yalcin, S., Mukai, T., Kondo, K., Ami, Y., Okawa, T., Kojima, A., et al. (1992). Experimental infection of cynomolgus and African green monkeys with human herpesvirus 6. *J. Gen. Virol.* 73(Pt 7), 1673–1677. doi: 10.1099/0022-1317-73-7-1673

Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y., et al. (1988). Identification of human herpesvirus-6 as a causal agent for *exanthem subitum*. *Lancet* 1, 1065–1067. doi: 10.1016/S0140-6736(88)91893-4

Yao, K., Crawford, J. R., Komaroff, A. L., Ablashi, D. V., and Jacobson, S. (2010). Review part 2: human herpesvirus-6 in central nervous system diseases. *J. Med. Virol.* 82, 1669–1678. doi: 10.1002/jmv.21861

Zerr, D. M. (2006). Human herpesvirus 6 and central nervous system disease in hematopoietic cell transplantation. *J. Clin. Virol.* 37(Suppl. 1), S52–S56. doi: 10.1016/S1386-6532(06)70012-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2013; accepted: 11 June 2013; published online: 04 July 2013.
Citation: Reynaud JM and Horvat B (2013) Animal models for human herpesvirus 6 infection. Front. Microbiol. ***4****:174. doi: 10.3389/fmicb.2013.00174*
This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 05 September 2013
doi: 10.3389/fmicb.2013.00267

Animal models for Ebola and Marburg virus infections

Eri Nakayama and Masayuki Saijo*

Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Stefan Pöhlmann, German Primate Center, Germany
Takeo Ohsugi, Kumamoto University, Japan

***Correspondence:**
Masayuki Saijo, Department of Virology 1, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
e-mail: msaijo@nih.go.jp

Ebola and Marburg hemorrhagic fevers (EHF and MHF) are caused by the Filoviridae family, *Ebolavirus* and *Marburgvirus* (ebolavirus and marburgvirus), respectively. These severe diseases have high mortality rates in humans. Although EHF and MHF are endemic to sub-Saharan Africa. A novel filovirus, Lloviu virus, which is genetically distinct from ebolavirus and marburgvirus, was recently discovered in Spain where filoviral hemorrhagic fever had never been reported. The virulence of this virus has not been determined. Ebolavirus and marburgvirus are classified as biosafety level-4 (BSL-4) pathogens and Category A agents, for which the US government requires preparedness in case of bioterrorism. Therefore, preventive measures against these viral hemorrhagic fevers should be prepared, not only in disease-endemic regions, but also in disease-free countries. Diagnostics, vaccines, and therapeutics need to be developed, and therefore the establishment of animal models for EHF and MHF is invaluable. Several animal models have been developed for EHF and MHF using non-human primates (NHPs) and rodents, which are crucial to understand pathophysiology and to develop diagnostics, vaccines, and therapeutics. Rhesus and cynomolgus macaques are representative models of filovirus infection as they exhibit remarkably similar symptoms to those observed in humans. However, the NHP models have practical and ethical problems that limit their experimental use. Furthermore, there are no inbred and genetically manipulated strains of NHP. Rodent models such as mouse, guinea pig, and hamster, have also been developed. However, these rodent models require adaptation of the virus to produce lethal disease and do not mirror all symptoms of human filovirus infection. This review article provides an outline of the clinical features of EHF and MHF in animals, including humans, and discusses how the animal models have been developed to study pathophysiology, vaccines, and therapeutics.

Keywords: Ebola virus, Marburg virus, filovirus, animal models, viral hemorrhagic fever

INTRODUCTION

The Family *Filoviridae* includes three accepted genera, *Ebolavirus* (ebolavirus), *Marburgvirus* (marburgvirus), and *Cuevavirus* (**Figure 1**) (Kuhn et al., 2011, 2013). Filoviruses are classified as biosafety level 4 (BSL-4) agents because they cause severe hemorrhagic fevers in humans and non-human primates (NHPs) with high case-fatality rates, ranging between 23 and 90% (Sanchez et al., 2007). Each of the *Marburgvirus* and *Cuevavirus* genera consists of a single species, *Marburg marburgvirus* and *Lloviu cuevavirus*, respectively. The genus *Marburgvirus* has two subspecies: Marburg virus (MARV) and Ravn virus (RAVV). The genus *Ebolavirus* is divided into five distinct species, *Zaire ebolavirus* (Ebola virus, EBOV), *Sudan ebolavirus* (Sudan virus, SUDV), *Tai Forest ebolavirus* (Tai Forest virus, TAFV), *Bundibugyo ebolavirus* (Bundibugyo virus, BDBV), and *Reston ebolavirus* (Reston virus, RESTV; Kuhn et al., 2013). EBOV is highly virulent to humans and NHPs with a mortality rate of up to 90% in African epidemics. The case fatality rate of SUDV and BDBV is ∼50 and 25%, respectively; the only person known to have been infected with TAFV survived. RESTV has been known to cause symptomatic disease in NHPs but not in humans. Lloviu virus belonging to the genus *Cuevavirus* was identified in the absence of replicating isolates during an investigation of die-off bats in Spain and the virulence for humans and NHPs has not been assessed (Negredo et al., 2011).

Although there has been an increasing frequency of filovirus outbreaks reported from endemic regions of Africa and Asia in recent years, there are no licensed vaccines or effective therapeutics for filovirus hemorrhagic fever.

The primary source of patients with filovirus hemorrhagic fever was mainly linked to exposure to animal carcasses found in the forest or to the putative bat reservoir, resulting in subsequent transmission through direct person-to-person contact (Leroy et al., 2004, 2009). Filoviruses enter the body via direct contact with infectious blood and/or body fluids. After an incubation period of 2–21 days, non-specific initial symptoms such as fever, chills, fatigue, headache, and myalgia appear. About 5–7 days after onset, a maculopapular rash usually develops on the face, buttocks, trunk, and/or arms and later generalizes over the entire body. As disease progresses, systemic (prostration, lethargy), gastrointestinal (anorexia, vomiting, abdominal pain, diarrhea), respiratory (chest pain, breath shortness, cough, nasal discharge), vascular (conjunctival injection, postural hypotension, edema), and neurological (headache, confusion, coma)

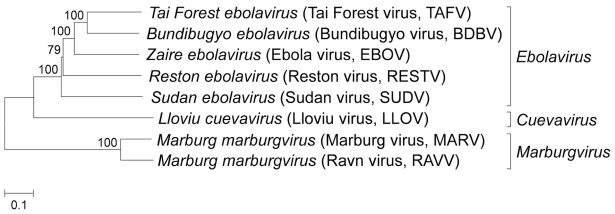


FIGURE 1 | Phylogenetic analysis of filovirus based on nucleotide sequence. The phylogenetic tree based on complete viral genome sequences was constructed by using the neighbor-joining method. Numbers at branch points indicate bootstrap values (1000 replicates). The GenBank accession numbers of Tai Forest virus (TAFV), Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), Lloviu virus (LLOV), Marburg virus (MARV), and Ravn virus (RAVV) are FJ217162, FJ217161, AF086833, AB050936, AY729654, JF828358, DQ217792, and EF446131, respectively.

manifestations are observed. Some patients develop multiple foci of mucosal hemorrhage, which is especially evident in conjunctiva and gingiva together with bleeding from venipuncture sites. Hemorrhagic symptoms observed during the peak of the illness include petechiae, ecchymoses, epistaxis, mucosal hemorrhages, and/or visceral hemorrhagic effusions. In fatal cases, patients die with hypovolemic shock and multiple organ failure between Day 6 and 16.

Animal models of filovirus infection have been developed in mice, guinea pigs, hamsters, and NHPs (Connolly et al., 1999; Bente et al., 2009; Bradfute et al., 2012; Wahl-Jensen et al., 2012). The development of animal models that accurately reflect human disease is critical to understanding the pathogenesis of Ebola and Marburg hemorrhagic fevers (EHF and MHF, respectively), because filoviral outbreaks in humans are sporadic and there is limited clinical data and access to human tissue. Since the wild-type virus replicates to high titers in NHPs and the virus causes symptoms, including hemorrhage and shock, which are similar to those of patients with EHF and MHF, NHP models may be the most useful to evaluate the efficacy of candidate vaccines and treatment measures. However, small animal models are also needed for preliminary evaluation of vaccines and therapeutic interventions against filovirus diseases, because of the ethical and handling issues related to using NHPs.

Here, we summarize and discuss the animal models developed for the study of hemorrhagic fever caused by filoviruses.

MOUSE MODELS

In contrast to the development of the NHP and guinea pig models, as described in later sections, development of a mouse model of filovirus infection has been unsuccessful due to the fact that adult immunocompetent mice were resistant to wild-type filovirus infection. The intraperitoneal or intracerebral inoculation of newborn mice and 4-day-old suckling mice with non-mouse adapted EBOV was shown to cause lethal infections, but 8-day-old or older mice did not show any symptoms (Johnson et al., 1995; Bray, 2001). Serial passage of wild-type EBOV in suckling mice was needed for adaptation, in which the virus acquired lethal virulence to adult immunocompetent mice (Bray, 2001). Intraperitoneal inoculation of mouse-adapted EBOV with a 1–100 plaque forming unit (pfu) dose (30–3000 times the median lethal dose) caused lethal infection to 5-week-old BALB/c, C57BL/6 and ICR (CD-1) mice, but subcutaneous inoculation of the virus at a dose of 10^6 pfu did not cause symptomatic illness in 3-week-old adult mice (Bray, 2001). This phenomenon is not observed in NHP and guinea pig models, which are susceptible to wild-type EBOV infection through any route of inoculation. $CD8^+$ T cells and perforin, but not B cells and $CD4^+$ T cells, are required for resistance to subcutaneous inoculation of EBOV (Gupta et al., 2005). It is supposed that the presence of regional lymph nodes and/or Langerhans cells in the skin contributes to protection from filoviral subcutaneous infection via activation of $CD8^+$ cells, however, there are no reports to prove this hypothesis.

It has been shown that mouse-adapted filovirus is fatal over a broad range of ages in BALB/c mice. Infected mice became acutely ill with symptoms of ruffled fur, reduced activity, and weight loss on Day 3–4 post-infection and died on Days 5–7, although these lengths differed depending on the challenge dose (Bray et al., 1999; Warfield et al., 2009). Virus titer in the liver and spleen exceeded 10^7 pfu/g within 3 days after infection and then reached a maximum of over 10^9 pfu/g at Day 5 post-inoculation. These titers exceeded the peak viral concentrations in the liver and spleen of infected guinea pigs (about 10^6 pfu/g) and NHPs (about 10^7 pfu/g; Bray et al., 1999). The virus is generally undetectable in serum on Day 1, but by Day 3 the viremia level peaks at approximately 10^7 pfu/ml, which was comparable to that in NHPs and exceeds that in guinea pigs (10^{4-5} pfu/ml; Bray et al., 1999).

As seen in the NHP model, the systemic viral spread results in extensive infection and necrosis of the liver, spleen, and other organs (Bray et al., 1999; Warfield et al., 2009). In liver from mice infected with mouse-adapted EBOV or RAVV, viral replication was observed in hepatocytes, Kupffer cells, and sinusoidal endothelial lining cells. Histological lesions were observed by Day 4 after inoculation, including coalescing, foci of hepatocellular vacuolar change, degeneration, and necrosis of hepatocytes. In the spleen, viral antigen was detected on Day 2 after infection, at which point coagulopathy, such as disseminated intravascular coagulation (DIC) accompanied by prolongation of prothrombin time (PT) and activated partial thromboplastin time (aPTT), was not observed in the moribund mice (Bray et al., 2001; Warfield et al., 2009).

Mouse-adapted EBOV initially infects macrophages and other mononuclear phagocytes at the site of invasion and in regional lymph nodes. The major target cells of infection are as the same as those in humans, NHPs, and guinea pigs (Davis et al., 1997; Connolly et al., 1999; Zaki and Goldsmith, 1999; Gibb et al., 2001). Viral replication in mononuclear phagocytes in the lymph node, spleen, and thymus and an increase in the number of virus-infected Kupffer cells in the liver were observed by Day 3 after infection. Most mononuclear phagocytes throughout the body appear to be infected and the mice died by Days 5–6.

The adaptation of EBOV to adult mice resulted in 8 amino acid changes in both the coding and non-coding regions of the virus

genome compared to the original wild-typed virus (Ebihara et al., 2006). Nucleotide substitutions leading to amino acid changes were found in VP35, VP24, NP, and L viral proteins. VP24 and VP35 are known as type I interferon (IFN) antagonists and interfere with type I IFN-mediated antiviral response *in vitro* (Bowen et al., 1980; Basler et al., 2000; Bente et al., 2009; Halfmann et al., 2011). VP24 functions as an IFN antagonist by binding karyopherin α and blocking nuclear accumulation of signal transducer and activator of transcription 1 (STAT1; Reid et al., 2007). VP35 is also implicated in blocking type I IFN responses by inhibiting phosphorylation of interferon regulatory factor (IRF) 3 and 7 by the Tank binding kinase-1 and I-Kappa-B kinase epsilon, and sequestering the viral RNA from detection by RIG-I like receptor (Ramanan et al., 2011, 2012). It is considered that there is a significant relationship between filoviral virulence and the ability of the virus to evade the type I IFN-induced antiviral response (van der Groen et al., 1979). The mutations in NP and VP24 genes were found to be critical for acquisition of EBOV virulence in adult mice, but not VP35 mutations (Ebihara et al., 2006). NP is tightly coupled with viral RNA and forms the nucleocapsid complex together with L, VP30, and VP35. Although it is unclear how NP is involved in the IFN response, NP is thought to confer evasion from the IFN-stimulated antiviral responses directly or indirectly in infected mice.

The mutations identified for adaptation of marburgvirus to mice differed from those required for that of ebolavirus. The amino acid mutations were found in VP40, VP35, NP, and VP30 in mouse-adapted RAVV compared to those of the wild-type virus derived from a patient (Warfield et al., 2009). It is still unclear what is the role and necessity of each of the mutations in mouse-adapted RAVV. Further experiments are required to clarify which mutations are critical for adaptation to mice.

Adult mice treated with antibodies against IFN-α/β became susceptible to infections with non-adapted EBOV or SUDV infected via the intraperitoneal route, and to mouse-adapted virus infected via the subcutaneous route (Bray, 2001). Furthermore, non-adapted EBOV, SUDV, MARV, or RAVV caused illness in KO mice lacking type I IFN receptors or the STAT1 protein (Bray, 2001). These results suggest that inhibition of type I IFN response against filovirus infections is critical for pathogenesis in mouse models.

Mice infected with the mouse-adapted filovirus are different from humans and NHPs infected with the original filoviruses in terms of a lack of severe coagulation disorder and fibrin deposition. Mouse models are useful tools for studying basic aspects of replication, pathogenesis, and immune responses and also serve as an irreplaceable platform for evaluating the efficacy of the wide range of the candidate vaccines and therapeutic agents.

GUINEA PIG MODELS

Guinea pigs are susceptible to several arenaviruses, Lassa fever virus, Junin virus, and Guanarito virus and used as animal models for human viral hemorrhagic fevers caused by these viruses (Bowen et al., 1977; Jahrling et al., 1982; Kenyon et al., 1990; Hall et al., 1996). However, infection of guinea pigs with wild-type filovirus usually causes only a transient febrile illness (Simpson et al., 1968; Robin et al., 1971; Bowen et al., 1977). Filoviruses need to be serially passaged in guinea pigs to acquire the ability to cause lethal infection in guinea pigs (Simpson et al., 1968; Robin et al., 1971; Ryabchikova et al., 1996). Guinea pigs inoculated with guinea pig (GP)-adapted virus showed similar symptoms such as fever, anorexia, and dehydration, to those reported in humans and NHPs infected with wild-type filovirus (Simpson et al., 1968; Robin et al., 1971; Connolly et al., 1999). GP-adapted EBOV-infected guinea pigs showed fibrin deposition coincident with decreases in platelet count during the late stage of infection (Connolly et al., 1999). GP-adapted EBOV replicated to high titers in the spleen, liver, adrenal gland, and lung, resulting in viremia in guinea pig models, although the peak titers were less than those demonstrated in NHPs. Viremia in guinea pigs developed within 2 days after inoculation and increased during the course of the disease, reaching a peak on Day 7 ($>10^4$ pfu/ml; Connolly et al., 1999; Subbotina et al., 2010). The guinea pigs infected with the GP-adapted filovirus died on 7–9 days after infection (Simpson et al., 1968; Robin et al., 1971; Connolly et al., 1999; Subbotina et al., 2010).

Histopathological changes in the liver of guinea pigs infected with GP-adapted filoviruses included replication of the viruses in Kupffer cells, multifocal necrosis of hepatocytes, and congestion and destruction of the sinusoid wall, which were also similar to those reported in humans and NHPs infected with wild-type filoviruses (Korb and Slenczka, 1971; Connolly et al., 1999; Ryabchikova et al., 2003). However, infiltrations of inflammatory cells in the liver and other organs were mild or absent (Connolly et al., 1999; Ryabchikova et al., 2003). Lymphoid necrosis was observed in the spleen and lymph nodes of guinea pig models.

Neutrophilia and lymphopenia became detectable in the guinea pig model as early as 2 days after infection and the severity continued to increase over the course of infection (Connolly et al., 1999; Subbotina et al., 2010). However, lymphocyte bystander apoptosis, an important feature in NHPs and mice, was not prominent in guinea pigs (Bray et al., 1999; Connolly et al., 1999; Bradfute et al., 2007). Thrombocytopenia was marked during the late stages of the disease when guinea pigs became moribund and platelets fell from a mean of ∼500,000 to <50,000/μl. Fibrin deposition was a late event, beginning only modestly in the liver and spleen on Day 4, with increases in distribution and amount on Days 7–9, coincident with decreases in platelet counts.

Comparative sequence analysis of the complete genomes of the GP-adapted EBOV and wild-type virus showed 8 nucleotide differences, which led to 5 amino acid substitutions; single amino acid mutations in NP and L and 3 mutations in VP24 (Volchkov et al., 2000). Using a reverse genetics approach, it was shown that VP24 had a critical role in the pathogenesis and the amino acid changes in VP24 were essential to achieve EBOV virulence in guinea pigs. VP24 was demonstrated to antagonize IFN signaling by binding host karyopherin α proteins and prevent transport of the tyrosine phosphorylated transcription factor STAT-1 to the nucleus (Reid et al., 2007; Mateo et al., 2010; Zhang et al., 2012). One of the substitutions in VP24 of GP-adapted EBOV

was located in the proximal domain, which was recently shown to be involved in karyopherin binding and required for efficient control of the IFN response (Mateo et al., 2010). However, the mutations associated with EBOV adaptation to the guinea pigs did not affect the ability of VP24 to inhibit IFN signaling (Mateo et al., 2011). VP24 participates in the assembly and/or proper formation of viral nucleocapsids. The lack of virulence of wild-type virus in guinea pigs was associated with an inability of the virus to replicate in and/or be released from hepatocytes and macrophages efficiently (Mateo et al., 2011). Wild-type VP24 is somehow incapable of participating in assembly of viral nucleocapsids in guinea pigs. Mutations in VP24 for adaption to guinea pigs recovered the ability of EBOV to replicate in both macrophages and hepatocytes and to facilitate the systemic spread of the virus.

SYRIAN GOLDEN HAMSTER MODELS

The pathogenesis of rodent-adapted filoviruses differs in some aspects from those of humans and NHPs infected with wild-typed virus (**Table 1**). Fever and cutaneous rash, which are major clinical signs of EHF and MHF in humans and NHPs, are absent in mice infected with mouse-adapted virus. Fever appears in guinea pigs infected with GP-adapted virus, but rash does not develop in these animals. Mice infected with mouse-adapted virus do not consistently display coagulation abnormalities. Compared to mice, guinea pigs infected with GP-adapted virus develop coagulation defects, including a drop in platelet counts and an increase in coagulation time, but coagulopathy (i.e., DIC) are not as marked as that observed in NHPs. Furthermore, lymphocyte apoptosis observed in humans, NHPs, and mice was not determined in the guinea pig model. Because of these differences in the rodent models, some vaccines (e.g., irradiated virion) and therapeutics (e.g., passive immunization with antiserum) that were effective in rodents challenged with adapted virus fail to protect NHPs challenged with wild-type virus (Wahl-Jensen et al., 2012).

Moreover, in the guinea pig model, the lack of available reagents and tools, such as quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and ELISA for cytokine profiling, makes the guinea pig model less desirable. Therefore, the development of other rodent models that better recapitulate EHF and MHF in humans was desired for more relevant pathogenesis studies and high throughput screening of prophylactic and post-exposure treatment prior to their testing in NHPs.

Syrian golden hamster (*Mesocricetus auratus*) is broadly used in animal models for human infectious diseases (Zivcec et al., 2011). Suckling hamsters were susceptible to wild-type MARV but disease was evident in only 40–80% of the animals following either intracerebral or intraperitoneal inoculation (Zlotnik, 1971). The symptoms and the mortality which was up to 90% were observed by inoculation with ninth passage materials in 5–6-week-old hamsters. While the pathological changes in the hamsters are similar to those observed

Table 1 | Comparison of pathological features of different animal models of filovirus infection.

	Mouse	Guinea pig	Hamster	NHP	Human
Virus adaptation	Adapted	Adapted	Adapted	Wild-type	Wild-type
Viremia	High	High	High	High	High[a]
Virulence	High	High	High	High	High[b]
Weight loss	Severe	Severe	No	Severe	Severe[c]
Rash	No	No	No	Yes	Yes[d]
Thrombocytpenia	Yes	Yes	Yes	Yes	Yes[e]
Lymphocyte apoptosis	Yes	Limited	Yes	Yes	Yes[f]
Cytokine response	Yes	Yes	Yes	Yes	Yes[g]
PT	Remained	Increased	Increased	Increased	Increased[h]
PTT/aPTT	Remained	Increased	Increased	Increased	Increased[i]
TT	ND	ND	Increased	Increased	ND
Fibrin deposition in organs	Little	Moderate	Little	Abundant	Observed[j]
Protein C activity	ND	ND	Decreased	Decreased	ND

[a] *Ksiazek et al., 1999; Ndambi et al., 1999; Sanchez et al., 2004; Towner et al., 2004; Kuhn, 2008.*

[b] *Isaacson et al., 1978; Piot et al., 1978; Smith et al., 1978; Bwaka et al., 1999; Sanchez et al., 2007; Kuhn, 2008.*

[c] *Bwaka et al., 1999; Kuhn, 2008.*

[d] *Isaacson et al., 1978; Smith et al., 1978; Bwaka et al., 1999; Sanchez et al., 2007; Kuhn, 2008.*

[e] *Sanchez et al., 2007; Kuhn, 2008.*

[f] *Baize et al., 1999.*

[g] *Baize et al., 1999, 2002; Sanchez et al., 2007.*

[h] *Sanchez et al., 2007; Kuhn, 2008.*

[i] *Sanchez et al., 2007.*

[j] *Dietrich et al., 1978.*

Abbreviation; PT, prothrombin time; PTT, partial thromboplastin time; APTT, activated partial thromboplastin time; TT, thrombin time ; ND, no data.

in other animal models including patients, encephalitis, which is not observed in other animals, were constantly demonstrated in all suckling hamsters, irrespective of route of inoculation, and in adult hamsters, when the virus was inoculated intracerebrally.

The Syrian golden hamster model was developed for EHF based on infection with mouse-adapted EBOV. Six-week-old hamsters infected intraperitoneally with 10^3 focus forming unit (ffu) of mouse-adapted EBOV started to show clinical signs of disease, including ruffled fur and decreased activity, by Day 3 after infection and succumbed to disease within 4–5 days after infection. When inoculated subcutaneously, mouse-adapted virus failed to produce lethal infection in hamsters in the same way as the mouse model. Mouse-adapted EBOV-infected hamsters showed severe coagulopathy with prolonged PT, aPTT, and thrombin time (TT), in the late stage of infection (Ebihara et al., 2013). Other factors, including increased fibrinogen, decreased protein C activity, thrombocytopenia, and coagulation disorder were observed in the hamster model. The target organs were the mesenteric lymph node, spleen, liver, and adrenal gland. In the mesenteric lymph node, the target cells were the macrophages and dendritic cells (DCs). The viral antigens were found in macrophages, marginal reticular-like cells, and DC-like cells in the spleen, and Kupffer cells and hepatocytes in the liver. Histopathological changes, including inflammatory cell infiltration, cellular necrosis, and apoptosis, were mainly noted in the lymphoid organs (spleen and mesenteric lymph node) and liver. These pathological changes were similar to those demonstrated in NHPs and other rodent models (Baskerville et al., 1978; Fisher-Hoch et al., 1992; Jahrling et al., 1996; Ryabchikova et al., 1996, 1999; Bray et al., 1999; Connolly et al., 1999; Warfield et al., 2009). Fibrin deposits in liver sinuses, which are a hallmark of EBOV infection in humans and NHPs, were detected to a lesser extent in the hamster model (Ebihara et al., 2013). Suppression of type I IFN that enhances viral replication in target cells and contributes to lethal disease was observed (Ebihara et al., 2013).

It has been demonstrated that the mouse-adapted EBOV-based Syrian golden hamster model shows the most similar clinical and pathological features, including coagulation abnormalities, to those observed in humans and NHPs infected with wild-type EBOV.

NON-HUMAN PRIMATE MODELS

Although guinea pigs, mice, and hamsters models have been developed to study EHF and MHF as stated above, the rodent models are not ideal because mice and guinea pigs, except a novel hamster model, do not entirely exhibit coagulation disorders that are associated with human and NHPs filovirus infections (**Table 1**). Additionally the bystander death of large numbers of uninfected lymphocytes due to apoptosis that are hallmark features in filovirus-infected humans and NHPs is not present in infected guinea pigs. In mouse models, the bystander lymphocyte apoptosis was reported, but the process and morphology of lymphocyte apoptosis was different from those of filovirus-infecting humans and NHPs (Bradfute et al., 2007).

In NHP models, apoptosis was the primary reason for lymphocyte death, but the lymphocyte death in mouse models appeared to occur by apoptosis and apoptosis-like programmed cell death. Furthermore, NHPs are lethally infected with non-adapted filovirus isolates resulting in pathophysiology similar to that demonstrated in humans, although rodent models required serial passages of the virus for adaptation to produce lethal disease. Because of the aforementioned disadvantages and differences in the disease pathology, NHPs remain the most useful and reasonable model of EHF and MHF despite practical and ethical considerations leading to the restriction of experiments.

MARBURGVIRUS INFECTION IN NHPs

The first documented outbreak of MHF was associated with wild-caught African green monkeys (*Chlorocebus aethiops*) in Uganda and imported to Marburg and Frankfurt, Germany, and to Belgrade, Serbia Montenegro, former Yugoslavia, in 1967 (Martini, 1971). Since the first outbreak of MHF originated from the wild-caught African green monkeys, this species was an obvious choice for an animal model of MHF. At that time, rhesus macaques (*Macaca mulatta*) were found to be equally susceptible to infection and showed symptoms after inoculation with MARV (Hass and Maass, 1971). Cynomolgus macaques (*Macaca fascicularis*) were also well characterized as an MHF model (Hensley et al., 2011). After an incubation period of 2–6 days, the monkeys showed febrile illness, anorexia, diarrhea, skin rash, and hemorrhagic manifestations by any routes of MARV-inoculation (Simpson et al., 1968; Simpson, 1969; Murphy et al., 1971; Geisbert et al., 2007; Alves et al., 2010; Hensley et al., 2011). Death occurred by 6–13 days post-infection after a sudden decrease in body temperature and the mortality rate was almost 100%. It was shown that reducing the virus inoculum led to delayed onset of the disease and longer time to death without reduction in mortality rate (Hass and Maass, 1971). In the macaques, petechial rashes on the forehead, chest, axillae, and groins were prominent and resembled the rashes that appeared in patients with MHF, but intriguingly the rashes were not seen in African green monkeys (Simpson, 1969).

A marked lymphocytosis was observed at the beginning of the illness (Simpson et al., 1968; Simpson, 1969; Gonchar et al., 1991; Spiridonov et al., 1992; Johnson et al., 1996; Geisbert et al., 2007). Thrombocytopenia and leukocytosis due to increased neutrophilia were prominent on 5–6 days after infection (Hensley et al., 2011). Changes in coagulation systems, such as a decrease in circulating levels of protein C, an increase in levels of circulating D-dimer and fibrin deposition in tissues were noted at late stages of the disease (Geisbert et al., 2007; Hensley et al., 2011). The pathological changes in liver including multifocal necrosis of the parenchyma cells, and lymphocyte apoptosis in lymphoid tissues were prominent (Geisbert et al., 2000). Monocyte/macrophages and DCs in the lymphoid tissues as well as Kupffer cells and sinusoids lining cells in the liver were the primary target cells for infections with MARV (Hensley et al., 2011). The infection then progressed to parenchymal cells in the liver, adrenal gland, and high endothelial venules in lymphoid tissues. Finally, the infection spread to endothelial cells in a variety

of organ tissues (Hensley et al., 2011). The virus or viral antigen was detected in liver, lymph nodes, spleen, adrenal grand, kidney, and blood in infected cynomolgus macaques. Onset of viremia occurred on Day 3, and in cynomolgus macaques and African green monkeys the maximum titer was 10^{7-8} pfu/ml on Day 8 after infection (Hass and Maass, 1971; Hensley et al., 2011).

Under experimental conditions, the possibility of aerosol transmission of MARV was shown in macaque models, although such a transmission route has not been described in human outbreaks (Pokhodiaev et al., 1991; Alves et al., 2010).

EBOLAVIRUS INFECTION IN NHPs

African green monkeys, cynomolgus macaques, rhesus macaques, and hamadryas baboons (*Papio hamadryas*) have been employed as a model of EBOV infection (Baskerville et al., 1978, 1985; Bowen et al., 1978, 1980; Ellis et al., 1978; Fisher-Hoch et al., 1985, 1992; Johnson et al., 1995; Jaax et al., 1996; Jahrling et al., 1996; Davis et al., 1997; Ryabchikova et al., 1999; Ignatiev et al., 2000; Geisbert et al., 2003b,e). The monkeys infected with EBOV became febrile 3 days after infection with temperatures above 40°C. Pyrexia usually persisted throughout the course of the disease, which usually ended in a decrease in temperature followed by death, which occurred within 5–8 days after infection (Baskerville et al., 1978; Bowen et al., 1978, 1980; Ellis et al., 1978; Fisher-Hoch et al., 1985; Luchko et al., 1995). By Day 4, anorexia developed with a loss of drinking ability, causing severe weight loss and dehydration. Some monkeys that survived until Day 5 had diarrhea, rectal bleeding, and/or intermittent melena. Petechial skin rashes appeared on the forehead, fore and hind limbs, and chest 4–5 days post-infection in macaques, but on Day 7 in baboons (Bowen et al., 1978; Ellis et al., 1978; Luchko et al., 1995; Ignatiev et al., 2000; Geisbert et al., 2003b). African green monkeys did not develop the cutaneous rash as demonstrated in monkeys infected with MARV (Simpson, 1969; Baskerville et al., 1978). Viremia became detectable within 3 days after infection with the maximum virus titer at the level of $10^{6.5-7}$ pfu/ml on Day 4–5 (Bowen et al., 1978; Fisher-Hoch et al., 1992; Jahrling et al., 1996; Geisbert et al., 2003b). The virus was positive in liver, spleen and lung on Day 4 and also appeared to have lower affinity for kidney, adrenal, lung, testis, lymph node, and pancreas (Baskerville et al., 1978, 1985; Bowen et al., 1978; Geisbert et al., 2003b). Mean virus titers in these organs increased progressively and reached the highest level of $10^{5.5-8.6}$ pfu/g on Day 6 (Geisbert et al., 2003b).

Total blood cell counts revealed marked neutrophilia and lymphopenia in the monkeys. Neutrophils and immature neutrophils increased remarkably by Day 4 (Fisher-Hoch et al., 1985; Geisbert et al., 2003b; Ebihara et al., 2011). Coincident with this process, severe lymphopenia due to lymphocyte apoptosis developed by Day 3 (Fisher-Hoch et al., 1985; Geisbert et al., 2003b). Extensive lymphocyte apoptosis, both in the vasculature and in lymphoid tissue, appears to be critical to the pathogenesis of EHF. Especially within the $CD8^+$ subset, the NK cell population dropped dramatically in the early stage of infection (Geisbert et al., 2003b). Lymphocytes were not productively infected and the apoptosis was not associated with direct viral infection (Geisbert et al., 2000). However, the mechanism underlying such apoptosis is unclear. Another characteristic feature was the abnormality of platelet function preceding thrombocytopenia (Fisher-Hoch et al., 1985; Geisbert et al., 2003b). Thrombocytopenia developed between 3 and 4 days and abnormalities in coagulation parameters, including prolonged PT, aPTT, and TT appeared (Geisbert et al., 2003b; Ebihara et al., 2011). Examination of coagulation parameters revealed that decreased protein C coagulation inhibitor activity due to excessive consumption triggered severe coagulopathy as indicated by prolonged coagulation times and decreased fibrinogen levels (Ebihara et al., 2011).

The NHP model has been proven to be valuable in providing new information regarding filoviral pathogenesis. EBOV spreads from the initial infection site via monocytes/macrophages and DCs to regional lymph nodes, likely via lymphatics, and to liver and spleen through the blood stream. Tissue macrophages, including Kupffer cells, DCs, and fibroblastic reticular cells become infected with EBOV at this stage. EBOV activates DCs by upregulating expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is expressed on DCs and mediates their cytotoxic activity (Geisbert et al., 2003b). Such overexpression of TRAIL is enhanced by overexpression of IFN-α in NHPs infected with EBOV and triggers lymphocyte apoptosis. Monocytes/macrophages infected with EBOV release various soluble factors including proinflammatory cytokines to recruit additional target cells to areas of infection. As disease progresses, increased levels of oxygen free radicals (e.g., nitric oxide) released from virus-infecting macrophages at the inflammatory sites trigger apoptosis of NK cells. The lymphocyte apoptosis caused by TRAIL and nitric oxide interferes with the innate immune response, resulting in escape of EBOV infection from mounting an adaptive response. Coagulation abnormalities are not the direct result of EBOV replication-induced cytolysis of endothelial cells, but are likely triggered by immune-mediated mechanisms (Geisbert et al., 2003d,e). Extensive viral replication leads to increased levels of additional proinflammatory cytokines, notably IL-6, which triggers the coagulation irregularities. This is probably through upregulation of tissue factor expression/release from virus-infected monocytes/macrophages. Tissue factor works as the primary cellular inhibitor of coagulation protease cascades. Activation of the coagulation cascade induces the fibrinogenic and fibrinolytic pathways and finally leads to DIC, hemorrhagic shock, thrombosis-related organ failure and death (Arai et al., 2000).

Monkey species-specific disease features of the pathogenesis of EBOV infection were observed, not only in the development of cutaneous rash but also in the impairment of the clotting systems of African green monkeys and baboons infected with EBOV. In African green monkeys, fibrin thrombosis was generalized in all visceral organs, while in baboons hemorrhages were prominent in visceral organs, most notably in the liver and spleen (Ryabchikova et al., 1999; Ignatiev et al., 2000). Genetic differences, even among the same animal species, and the origin of a species may influence disease presentation and progression.

The dose and species of challenge virus affects the progression of disease. Intramuscular inoculation of cynomolgus macaques with 10^3 pfu of EBOV produced a 100% lethal infection, with deaths occurring 6–7 days post-infection (Geisbert et al., 2002). When the challenge dose was lowered to 10 pfu, uniform lethality was still achieved, but deaths occurred 9–12 days post-infection (Geisbert et al., 2003c). Viremia was demonstrated as early as 24 h after subcutaneous infection of rhesus macaques with a high infectious dose (10^5 pfu) of EBOV. In rhesus and cynomolgus macaques infected with 10^3 pfu of EBOV, viremia is first detected by Day 3 after infection. SUDV causes slower disease progression than EBOV and it has been reported that some monkeys infected with SUDV did not die and recovered from the illness (Ellis et al., 1978; Bowen et al., 1980; Fisher-Hoch et al., 1992). One of four rhesus macaques infected with SUDV died on Day 12 but the other macaques survived and remained normal thereafter (Ellis et al., 1978). The dead macaque had small numbers of virus particles in the liver but no virus particles were found in the kidney, spleen, heart, lung, and brain. The liver, lung, and spleen from EBOV infected macaques, which were moribund and killed on Day 6 contained large numbers of virions. The apparent limitation of viral replication in the liver of SUDV-infected host and the contrasting widespread involvement of liver and other organs such as the spleen and lung of EBOV-infected host are similar in patients and macaques. RESTV, which is considered not to be virulent to humans, is clearly less pathogenic than EBOV and SUDV in African green monkeys and cynomolgus macaques (Fisher-Hoch et al., 1992; Jahrling et al., 1996). Only 5 of 16 monkeys infected with EBOV or SUDV survived, whereas 11 of 15 monkeys infected with RESTV survived (Fisher-Hoch et al., 1992). Viremia, clinical signs (temperature rise, anorexia, depression, or evidence of disturbed hemostasis), serum chemistry changes (elevated aspartate aminotransferase and lactate dehydrogenase activities) and pathological changes (necrosis of hepatocytes, adrenalcytes, and lymphoid elements of the spleen and prominent fibrin thrombi and fibrin precipitation) in RESTV infected monkeys developed slower and/or milder than those observed in monkeys infected with EBOV and/or SUDV.

Most human cases are thought to occur by direct contact with blood and/or body secretions from patients or animal cadavers. Aerosol transmission among humans has not been reported. However, evidence of intercage transmission of RESTV was observed in the 1989–1990 epizootic cases of RESTV in the Hazleton facility in Reston, Virginia, and demonstration of high concentrations of ebolavirus in nasal secretions and alveoli in experimental infection implicated the potency of aerosol transmission of ebolavirus (Baskerville et al., 1978, 1985; Bowen et al., 1978; Jahrling et al., 1990, 1996; Dalgard et al., 1992; Jaax et al., 1995; Miranda et al., 1999, 2002). Furthermore, the rhesus macaques experimentally challenged with aerosolized EBOV developed the same disease as macaques infected parenterally (Johnson et al., 1995). Regardless of the route of infection (intramuscular, subcutaneous, conjunctival, and aerosol injections), NHPs are highly susceptible to EBOV infection (Baskerville et al., 1978; Bowen et al., 1978, 1980; Ellis et al., 1978; Baskerville et al., 1985; Fisher-Hoch et al., 1985, 1992; Johnson et al., 1995; Jaax et al., 1996; Jahrling et al., 1996; Davis et al., 1997; Ryabchikova et al., 1999; Ignatiev et al., 2000; Geisbert et al., 2003b,e).

VACCINES

INACTIVATED WHOLE VIRION

The development of filovirus vaccines has been performed based on inactivated whole virion preparations. About half of the rhesus macaques or African green monkeys treated were protected against homologous MARV challenge, when formalin- or gamma-inactivated whole MARV virions were used as vaccine candidates (Ignat'ev et al., 1991; Ignatyev et al., 1996). Vaccination with formalin-inactivated EBOV virions protected 4 of 5 hamadryas baboons (Mikhailov et al., 1994), while other studies suggested that inactivated virus did not induce sufficient immunity to protect baboons against a lethal challenge (Chupurnov et al., 1995). Furthermore, vaccination with gamma-irradiated EBOV virions alone or in a form of liposomes containing lipid A failed to protect cynomolgus macaques against lethal infection (Geisbert et al., 2002). Overall, these vaccine candidates based on inactivated virions did not confer sufficient protection in NHP models. Furthermore, these vaccines are unlikely to be used in humans due to safety risk of incomplete inactivation. However, these results promoted the development of an alternative vaccine platform, such as DNA-based vaccines, recombinant viral vector, or virus-like particles as described below and **Table 2**.

VENEZUELAN EQUINE ENCEPHALITIS VIRUS REPLICON

Venezuelan equine encephalitis virus (VEEV) replicons that express either GP or NP of MARV Musoke protected guinea pigs from viremia and death caused by GP-adapted MARV challenge (Hevey et al., 1998). Cynomolgus macaques administrated with MARV Musoke GP-expressing VEEV replicons alone or in combination with NP were also protected from lethal infection with the homologous Musoke strain, but not from heterologous RAVV (Hevey et al., 1998; Falzarano et al., 2011). additionally, vaccination with NP alone prevented death but not disease onset in two of three monkeys and allowed all animals to become viremic. For EBOV, EBOV GP-expressing VEEV replicons, alone or in combination with EBOV NP-expressing VEEV replicons, protected mice, and guinea pigs from lethal infection, whereas immunization with EBOV NP-expressing VEEV replicons alone protected mice but not guinea pigs (Pushko et al., 2001). Furthermore, vaccination with recombinant VEEV, expressing EBOV GP, NP, or both GP and NP, failed to protect cynomolgus macaques from a lethal EBOV infection (Geisbert et al., 2002). One recent study produced different results, whereby a VEEV-based vaccine was fully protective in cynomolgus macaques against EBOV, SUDV, and MARV (Friedrich et al., 2012). The results obtained from these studies are inconsistent, suggesting that VEEV-based vaccine may be promising although further research is needed.

ADENOVIRUS-BASED VACCINES

Adenovirus (AdV) vectors commonly used are based on serotype 5 (AdV5). A single infection of the recombinant MARV Angola

Table 2 | Efficacy of vaccines in animal models of filovirus infection.

Vaccine	Viral protein including vaccines	Species tested	Strategy		Challenge virus	Survival rate (%)	References
			Dose/Schedule	Route			
VEEV	MARV Musoke GP or NP	Guinea pig	10^6 FFU, 2 or 3 doses	sc	GP-adapted MARV Musoke	100	Hevey et al., 1998
	MARV Musoke GP or GP + NP	NHP	10^7 FFU, 3 doses		MARV Musoke	100	
	MARV Musoke NP					67	
	EBOV NP or GP + NP EBOV GP	Mouse	2×10^6 IU, 2 doses		mouse-adapted EBOV	100 90	Pushko et al., 2001
	EBOV GP or GP + NP EBOV NP	Guinea pig	10^7 IU, 3 doses		GP-adapted EBOV	100 20	
	EBOV GP, NP or GP + NP	NHP	10^7 FFU, 3 doses		EBOV	0	Geisbert et al., 2002
AdV	GPs of MARV (Musoke and Ci67) and RAVV	Guinea pig	$5 \times 10^{7-8}$ PFU, 2 doses	sc	MARV (Musoke or Ci67) or RAVV	100	Wang et al., 2006a
	MARV Angola GP	NHP	10^{11} PU, 1dose	im	MARV Angola	100	Geisbert et al., 2010a
	EBOV GP	Mouse	10^8 PFU, 2 doses	sc	mouse-adapted EBOV	100	Wang et al., 2006b
	EBOV GP + NP	NHP	2×10^{12} particles, 1 or 2 doses	im	EBOV	100	Sullivan et al., 2003
	GPs of EBOV and SUDV, MARV (Musoke and Ci67) and RAVV + NP of EBOV and MARV Musoke		4×10^{10} PFU, 2 doses		EBOV, SUDV or MARV (Musoke or Ci67)	100	Swenson et al., 2008a
DNA	MARV Musoke or RAVV GP	Guinea pig	10 μg, 3 or 4 doses with RIBI aadjuvand	sc	GP-adapted MARV Musoke	100	Riemenschneider et al., 2003
	MARV Musoke GP	NHP	20 μg, 3 doses		MARV Musoke	67	
	MARV Angola GP		4 mg, 4 doses	im	MARV Angola	100	Geisbert et al., 2010a
	EBOV GP	Mouse	0.5 μg, 4 doses 0.5 μg, 1 dose and 1.5 μg, 3 or 4 doses		mouse-adapted EBOV	78 100	Vanderzanden et al., 1998
	EBOV GP or NP	Guinea pig	500 μg, 4 doses		GP-adapted EBOV	100	Xu et al., 1998
DNA + AdV	DNA: GPs of EBOV, SUDV and TAFV + EBOV NP AdV: EBOV GP	NHP	4 mg of DNA, 3 doses and boosted with 10^{10} PFU of AdV	im	EBOV	100	Sullivan et al., 2000
	DNA: MARV Angola GP AdV: MARV Angola GP		4 mg of DNA, 3 doses and boosted with 10^{11} PU of AdV		MARV Angola	100	Geisbert et al., 2010a

(Continued)

Table 2 | Continued

Vaccine	Viral protein including vaccines	Species tested	Strategy			Challenge virus	Survival rate (%)	References
			Dose/Schedule		Route			
HPIV3	EBOV GP or GP + NP	Guinea pig	$10^{5.3}$ PFU		in	GP-adapted EBOV	100	Bukreyev et al., 2006
	EBOV GP, GP + NP, or GP + GM-CSF	NHP	4×10^6 TCID50, 1 dose		in and intracheally	EBOV	83	Bukreyev et al., 2007
	EBOV GP		2×10^7 TCID50, 2 doses				100	
HPIV3/ ΔHN-F	EBOV GP	Guinea pig	4×10^5 PFU, 1 dose		in	GP-adapted EBOV	100	Bukreyev et al., 2009
VSV	MARV Musoke GP	NHP	2×10^7 PFU, 1 dose	28 day before infection	im	MARV (Musoke or Angola) or RAVV	100	Daddario-Dicaprio et al., 2006a
			10^7 PFU, 1 dose	28 or 141 d before infection[a]		MARV Musoke and Popp	100	Jones et al., 2005
	EBOV GP	Mouse	2×10^5 PFU, 1 dose	24 h before infection	ip	mouse-adapted EBOV	100	Feldmann et al., 2007
				30 mpi			100	
				24 hpi			100	
		Guinea pig		24 h before infection		GP-adapted EBOV	67	
				1 hpi			83	
				24 hpi			50	
		NHP	10^7 PFU, 1 dose	28 day before infection	im	EBOV	100	Jones et al., 2005
			10^7 PFU, 1 dose	262 day before infection[b]		SUDV	25	
	EBOV GP + SUDV GP + Musoke GP		3×10^7 PFU, 1 dose	28 day before infection		EBOV, SUDV, TAFV or MARV Musoke	100	Geisbert et al., 2009
	MARV Musoke GP		2×10^7 PFU, 1 dose	24 hpi		MARV Musoke	83	Geisbert et al., 2010c
				48 hpi			33	
			1×10^7 PFU, 1 dose	20–30 mpi			100	Daddario-Dicaprio et al., 2006b
	EBOV GP		2×10^7 PFU, 1 dose, 20–30 mpi			EBOV	50	Feldmann et al., 2007
	SUDV GP					SUDV	100	Geisbert et al., 2008

(Continued)

Table 2 | Continued

Vaccine	Viral protein including vaccines	Species tested	Strategy		Challenge virus	Survival rate (%)	References
			Dose/Schedule	Route			
VLP	MARV Musoke GP + VP40 produced in 293T[c]	Guinea pig	50 μg, 3 doses with RIBI adjuvant	im	GP-adapted MARV (Musoke or Ci67) or RAVV	100	Swenson et al., 2008b
		NHP	1 mg, 3 doses with QS-21 adjuvant		MARV (Musoke or Ci67) or RAVV	100	
	EBOV GP + VP40 + NP produced in 293T[c]	Mouse	50 μg, 2 doses, with QS-21 adjuvant		Mouse-adapted-EBOV	100	Warfield et al., 2007a
	EBOV GP + VP40 produced in 293T[c]		10 μg, 3 doses	im or ip		100	Warfield et al., 2003
	EBOV GP + NP + VP40 produced in 293T[c]	NHP	250 μg, 3 doses, with RIBI adjuvant	im	EBOV	100	Warfield et al., 2007b
	EBOV GP + VP40 produced in insect cells[d]	mouse	50 μg, 2 doses		Mouse-adapted-EBOV	100	Sun et al., 2009
			10 μg, 3 doses			83	
	EBOV GP + VP40 + NP produced in insect cells[d]		10-50 μg, 2 doses, with QS-21 adjuvant			100	Warfield et al., 2007a

[a] *Cynomolgus macaques were immunized by intramuscular injection with a single dose of VSVΔG expressing MARV Musoke GP and subsequently challenged on Day 28 after immunization by intramuscular injection with MARV Musoke strain. The immunized macaques, which were protected from the lethal MARV challenge, were rechallenged with MARV Popp strain 113 days after initial challenge (141 days after immunization).*

[b] *Cynomolgus macaques were immunized by intramuscular injection with a single dose of VSVΔG expressing EBOV GP and subsequently challenged on Day 28 after immunization by intramuscular injection with EBOV. The macaques protected from the lethal EBOV challenge were rechallenged with SUDV 234 days after initial challenge (262 days after immunization).*

[c] *293T cells were cotransfected with plasmid vectors encoding GP and VP40 (and NP) of EBOV or MARV. The VLPs were collected and purified from the cell supernatants.*

[d] *The VLPs were produced by use of recombinant baculovirus constructs expressing GP and VP (and NP) of EBOV or MARV from coinfected insect cells.*

Abbreviations; FFU, focus-forming unit; GP-adapted, guinea pig-adapted; IU, infectious unit; PFU, plaque-forming units; PU, particle units; sc, subcutaneously; im, intramuscularly; in, intranasally; ip, intrapenitoneally; mpi, minutes post-infection; hpi, hours post-infection; dpi, days post-infection.

GP-expressing AdV5 resulted in complete protection of cynomolgus macaques from illness and death by challenge with homologous virus (Geisbert et al., 2010a). Vaccination with a mixture of EBOV GP—expressing AdV5 and EBOV NP—expressing AdV5 have demonstrated 100% protection in cynomolgus macaques against homologous virus challenge (Sullivan et al., 2003). However, the genome insert size in this first generation AdV vector was restricted to as little as a single filovirus GP gene. The second generation AdV vector has the advantage of being able to express multiple antigens in a single construct over the first generation vector. The second generation bivalent AdV vector expressing GPs of EBOV and SUDV led to efficient induction of antibodies specific to EBOV and SUDV (Wang et al., 2006b). A trivalent AdV vector expressing MARV GPs of Ci67, Musoke and RAVV efficiently led to MARV-specific antibodies in mice and guinea pigs and showed complete protection of guinea pigs against MARV and RAVV infections (Wang et al., 2006a). Additionally, vaccination of cynomolgus macaques with second generation AdV vectors, which expressed multiple filovirus GPs of EBOV, SUDV, Ci67, Musoke, and RAVV, induced 100% protection against challenge with EBOV and SUDV and two different strains of MARV (Ci67 and Musoke; Swenson et al., 2008a). Although the AdV-based vaccines showed efficacy, the vaccines have a major obstacle: the prevalence of pre-existing immunity to AdV that may substantially limit their immunogenicity and clinical utility. It is estimated that the prevalence of antibody to AdV5 is up to 60% in the general human population and up to 85% in Africa (Schulick et al., 1997; Piedra et al., 1998). Indeed, macaques pre-immunized against AdV5 and vaccinated with EBOV GP-expressing AdV5 were not protected from lethal challenge with EBOV infection (Geisbert et al., 2010b). AdV serotype 26 and 35 segregated genetically from AdV5 exhibit lower seroprevalence in humans (Vogels et al., 2003; Abbink et al., 2007; Mast et al., 2010). Therefore, AdV serotype 26 and 35 vectors with expression of EBOV or SUDV GPs have been generated and the protective efficacy examined by using the NHP model, but these vectors failed to protect cynomolgus macaques against lethal EBOV challenge (Geisbert et al., 2011).

DNA

The plasmid coding the DNA of GP from MARV Musoke or RAVV demonstrated efficacy in protection of guinea pigs and cynomolgus macaques against lethal infection of each homologous strain (Riemenschneider et al., 2003). All of the guinea pigs vaccinated three or four times with DNA vaccines were aviremic and appeared healthy. In cynomolgus macaques, four of six monkeys immunized with 3 doses of DNA vaccine encoding Musoke GP were protected from homologous challenge with MARV Musoke (Riemenschneider et al., 2003). In a report of DNA vaccines encoding GP of MARV Angola strain, the 4 vaccination doses resulted in protection of all four vaccinated monkeys, but three of the four monkeys showed symptoms and/or lymphopenia (Geisbert et al., 2010a). A combination vaccine regimen (3 times injection with DNA and boost with recombinant Angola GP-expressing AdV vector) protected the monkeys from lethal infections but two of the four monkeys showed rash or lymphopenia. A single inoculation with AdV vaccine induced optimal immune responses to eliminate symptoms and death by itself. These data suggest that DNA vaccines do not optimally control MARV infection (Geisbert et al., 2010a). However, three-plasmid DNA vaccines encoding EBOV GP, SUDV GP, and EBOV NP were evaluated in a phase I trial as safe and immunogenic in humans (Martin et al., 2006). The EBOV DNA vaccine also protected mice and guinea pigs against a lethal challenge (Vanderzanden et al., 1998; Xu et al., 1998; Martin et al., 2006). In one study, cynomolgus macaques, which received 3 injections of DNA vectors encoding EBOV GP, SUDV GP, TAFV GP, and EBOV NP, were boosted with recombinant EBOV GP-expressing AdV (Sullivan et al., 2000). All four monkeys vaccinated survived and showed no symptoms of EBOV infection. This prime-boost strategy provided a sufficient immune response to clear the virus efficiently.

HUMAN PARAINFLUENZA VIRUS

In an outbreak of RESTV in the Hazleton facility in Reston, Virginia, aerosol transmission between NHPs may have occurred (Jahrling et al., 1990; Dalgard et al., 1992; Miranda et al., 1999, 2002). To address the assumed aerosol transmission of filovirus, a vaccine that induces a strong immune response in the respiratory tract was developed. Human parainfluenza virus type 3 (HPIV3), a common respiratory virus, was modified as a form of vaccine vector and used for development of a vaccine against EBOV. The HPIV3 vectors, which express EBOV GP or EBOV GP together with NP, protected guinea pigs and rhesus macaques against EBOV challenge (Bukreyev et al., 2006, 2007). In guinea pigs, a single intranasal inoculation with HPIV3 expressing EBOV GP or both GP and NP showed complete protection against signs of illness and death (Bukreyev et al., 2006). The rhesus macaques were immunized with a single dose of EBOV GP-expressing HPIV3, or EBOV GP and NP-expressing HPIV3, through a combined intranasal and intratracheal inoculation. Five of six monkeys immunized with the HPIV3 based vaccine survived and four of six monkeys did not show any clinical illness (Bukreyev et al., 2007). Two doses of intranasal immunizations showed greater efficacy, including complete protection of all three rhesus macaques against clinical illness and death. However, HPIV3 may not be effective as a vaccine vectors in humans, since HPIV3 is a common childhood pathogen and the majority of the population have pre-existing immunity to HPIV3. To overcome the problem of pre-existing immunity, a chimeric HPIV3, where both HPIV3 surface proteins, HN and F, were deleted and replaced with EBOV GP was developed (Bukreyev et al., 2009). A single immunization with the vaccine completely protected guinea pigs against a lethal infection. It was shown that the HPIV3 based vaccine, which expressed EBOV GP, was immunogenic equally among HPIV3-naïve and HPIV3 antibody-positive subjects and effective when vaccinated twice. However, pre-existing HPIV3-specific immunity in rhesus macaques reduced the replicative capacity of the HPIV3-based vaccine in the respiratory tract (Bukreyev et al., 2010). Nevertheless, this study indicated that the vaccination induced an appropriate antibody response.

VESICULAR STOMATITIS VIRUS

A vaccine to resolve the problem of pre-existing immunity utilized the recombinant vesicular stomatitis virus (VSV) vector, which expresses filovirus GP. VSV is mainly a veterinary pathogen and human infection with VSV is rare and not associated with disease in humans. A single intramuscular vaccination of cynomolgus macaques with recombinant VSV with expression of MARV Musoke GP elicited complete protection against a high dose (10^3 pfu) intramuscular challenge with both homologous Musoke strain and heterologous Popp strain, Angola strain, and RAVV (Jones et al., 2005; Daddario-Dicaprio et al., 2006a). For EBOV, a single immunization of cynomolgus macaques with recombinant VSV vector, which expresses EBOV GP, also elicited complete protection against EBOV challenge (Jones et al., 2005). The surviving macaques from lethal EBOV infection were re-challenged with heterologous SUDV, but the cross-protection was not observed (Jones et al., 2005). Administration of the EBOV GP-expressing VSV vaccine through the oral or intranasal route completely protected cynomolgus macaques from EBOV challenge (Qiu et al., 2009). A blended vaccine consisting of equal amounts of 3 different VSV vectors, which expresses GP of each of EBOV, SUDV, and MARV, generated complete protection of cynomolgus macaques against challenges with EBOV, TAFV, and MARV (Geisbert et al., 2009). Macaques vaccinated with the blended vaccine followed by challenge with SUDV showed mild clinical sign of illness including fever, lymphopenia, and mild anorexia, and the macaques recovered from illness. Importantly, none of the macaques vaccinated with the blended vaccine succumbed to a filovirus challenge. The efficacy of the recombinant VSV vaccine has been evaluated as a post-exposure prophylaxis for filovirus infections. Administration of recombinant VSV with MARV Musoke GP expression to rhesus macaques shortly after a homologous high-dose MARV challenge resulted in complete protection of all subjects from clinical illness and death (Daddario-Dicaprio et al., 2006b). Furthermore, administration of recombinant MARV Musoke GP-expressing VSV at 24 and 48 h following infection resulted in protection of 83 and 33% of rhesus macaques, respectively (Geisbert et al., 2010c). When recombinant EBOV GP-expressing VSV were administrated to mice 24 h prior to challenge, and 1 and 24 h post-challenge, all treated mice survived (Feldmann et al., 2007). In guinea pigs treated with EBOV GP-expressing VSV at 24 h prior to challenge, and 1 or 24 h post-challenge, the survival rates were 67, 83, and 50%, respectively. It was also demonstrated that post-exposure vaccination with the recombinant VSV GP vectors for EBOV and SUDV in rhesus macaques was effective against challenge with homologous viruses, although the protection rate was dependent on the species of ebolavirus. The survival rates of the EBOV- or SUDV-infected monkeys were 50 and 100%, respectively (Feldmann et al., 2007; Geisbert et al., 2008).

VIRUS-LIKE PARTICLE

Virus-like particle (VLPs), which mimic authentic virions structurally but do not contain infectious genetic material, are non-infectious and safer than replicating vaccines. The efficiency of a MARV vaccine consisting of VLPs with MARV Musoke GP and VP40 was assessed in guinea pig and cynomolgus macaque models (Swenson et al., 2008b). The guinea pigs and monkeys immunized three times with MARV-Musoke VLPs with RIBI or QS-21 adjuvant were challenged with Musoke strain, Ci67 strain, or RAVV. All guinea pigs and eight monkeys were protected from death and clinical illness following the lethal challenge, except for a single monkey. The monkey challenged with RAVV, which is the most genetically distinct strain of marburgvirus, developed minor signs of disease without detectable viremia. For ebolavirus, mice vaccinated with EBOV VLP in the presence or absence of adjuvant were protected from lethal EBOV infection in a dose-dependent manner (Warfield et al., 2003, 2007a; Sun et al., 2009). Furthermore, the efficacy of the EBOV VLP, which consists of EBOV GP, NP, and VP40 was evaluated in cynomolgus macaques (Warfield et al., 2007b). All five monkeys that received three injections of the EBOV VLPs with RIBI adjuvant were completely protected against EBOV challenge.

There are some other vaccine candidates, including an EBOV lacking VP30 (which encodes the essential transcription factor), an Fc portion of a human IgG fused to EBOV-GP, a bean yellow dwarf virus-derived replicon system, and a cytomegalovirus-based vaccine encoding an EBOV NP CTL epitope (Halfmann et al., 2009; Konduru et al., 2011; Phoolcharoen et al., 2011; Tsuda et al., 2011a). However, the immunogenic efficacy of these vaccines has only been confirmed in the rodent models and further studies are needed to evaluate the protective efficacy and safety in NHPs.

TREATMENTS

RECOMBINANT NEMATODE ANTICOAGULANT PROTEIN C2

Coagulation abnormalities are one of the most prominent hallmarks of filovirus infection. It has been suggested that tissue factor plays an important role in triggering the hemorrhagic complications in NHPs infected with filoviruses (Geisbert et al., 2003d). Overexpression of tissue factor that performs as the primary cellular inhibitor of the coagulation protease cascades is one of the causes of DIC and thrombosis-related organ failure. The effect of blocking the pathway leading from the complex of activated factor VII and tissue factor to thrombin was examined in filovirus infection. Recombinant nematode anticoagulant protein c2 (rNAPc2), which directly inhibits factor VII and tissue factor, provided partial post-exposure protection to rhesus macaques infected with filovirus (Geisbert et al., 2003a, 2009). In rNAPc2-treated rhesus macaques, the mean survival time (11.7 days) was longer than that in untreated control monkeys (8.3 days) and 33% of EBOV-infected macaques survived. In MARV Angola-infected rhesus macaques treated with rNAPc2, 1 of 6 (17%) monkeys survived and the mean survival time for the five dead monkeys was significantly prolonged compared with that of the untreated control monkeys. rNAPc2 demonstrated a clear improvement in terms of survival rate and an increase in mean survival time in a normally 100% lethal model of filovirus infection.

RECOMBINANT HUMAN ACTIVATED PROTEIN C

Activated protein C (APC) is generated from the protein C, which is a vitamin K-dependent plasma protein and inactivates factors V and VIII to down-regulate thrombin generation. It has

Table 3 | Efficacy of post-exposure treatment in animal models of filovirus infection.

Treatment	Mechanism/target viral protein		Species tested	Strategy			Challenge virus	Survival rate (%)	References
				Dose	Route	Dose schedule			
rNAPc2	Blocks TF: FVIIa mediated activation of factor X		NHP	30 μg/kg bw	sc	10 mpi and administraion daily for 14 days	EBOV	33	Geisbert et al., 2003a
						24 hpi and administraion daily for 8 days		33	
						10 mpi and administraion daily for 14 days	MARV Angola	17	Geisbert et al., 2007
APC	Anti-thrombotic: cleaves and inhibits coagulation cofactors FVIIIa and Fva		NHP	2 mg/m^2/h	iv	30–60 mpi and administration for 7 days	EBOV	18	Hensley et al., 2007
PMO	Targets viral mRNA to block transcription	EBOV VP24, VP35 and L	Mouse	500 μg	ip	twice at 24 h and 4 h before infection	mouse-adapted EBOV	100	Warfield et al., 2006
			Guinea pig			single dose at 24 hpi	GP-adapted EBOV	100	
						single dose 24 h before infection		>25	
						single dose 24 hpi		25–50	
						single dose 96 hpi		50–75	
			NHP	12.5–200 mg	im	2 day before challenge and administration for 9 days	EBOV	75	
PMO plus		EBOV VP24 and VP35 (AVI-6002)	NHP	40 mg/kg bw	sc and ip	30–60 mpi and administration daily for 10 or 14 days	EBOV	63	Warren et al., 2010
				28 or 40 mg/kg bw	iv	30-60 mpi and administration daily for 14 dpi		60	
				4 mg/kg bw				0	
				16 mg/kg bw				20	

(Continued)

Table 3 | Continued

Treatment	Mechanism/target viral protein	Species tested	Strategy			Challenge virus	Survival rate (%)	References
			Dose	Route	Dose schedule			
	MARV Musoke VP24 and NP (AVI-6003)	NHP	30 or 40 mg/kg bw	sc and ip		MARV Musoke	100	
			40 mg/kg	sc or iv			100	
			30 mg/kg	iv			100	
			7.5 or 15 mg/kg				60	
siRNA	Targets viral mRNA to block transcription	EBOV L	guinea pig	PEI-mixed, 8 mg/kg	ip	3 h before infection and 24, 48 and 96 hpi	GP-adapted EBOV	25	Geisbert et al., 2006
				SNALP-formulated, 1 mg/kg		1, 24, 48, 72, 96, 120 and 144 hpi		60[a]	
				SNALP-formulated, 0.75 mg/kg				100	
		EBOV L, VP24 and VP35	NHP	SNALP-formulated, 2 mg/kg	iv	30 mpi, 1, 3 and 5 dpi	EBOV	66	Geisbert et al., 2010d
		EBOV L, VP24 and VP35				30 mpi, 1, 2, 3, 4, 5 and 6 dpi		100	

[a] *Two of five guinea pigs received the siRNAs using the SNALP delivery systems died but the death could not be attributed to viral replication.*

Abbreviation; bw, bodyweight; sc, subcutaneously; iv, intravenously; ip, intrapenitoneally; im, intramuscularly; mpi, minutes post-infection; hpi, hours post-infection; dpi, days post-infection.

been reported that circulating levels of protein C were rapidly and significantly reduced in cynomolgus macaques and rhesus macaques during EBOV infections, because the protein C might be produced in the liver, which is a main target of filovirus infection (Geisbert et al., 2003a). In rhesus macaque models, administration of recombinant human APC (rhAPC) at 30–60 min after challenge and continuing for 7 days, protected 2 of 11 (18%) monkeys against lethal EBOV infection (Hensley et al., 2007). The mean survival time in the rhAPC-treated monkeys was prolonged compared with the untreated monkeys (Hensley et al., 2007).

PHOSPHORODIAMIDATE MORPHOLINO OLIGOMER

Phosphorodiamidate morpholino oligomers (PMOs) inhibit targeted gene translation by steric blockage of ribosomal assembly. A combination of EBOV-specific PMOs targeting sequences of viral mRNAs for the VP24, VP35, and RNA polymerase L protected rodents in both pre- and post-exposure therapeutic regimens (Warfield et al., 2006). In rhesus macaque models, treatment with a combination of the PMOs of VP24, VP35, and L from 2 days prior to EBOV challenge through Day 9 of the infection protected 3 of 4 (75%) rhesus macaques against lethal infection (Warfield et al., 2006). Furthermore, it was demonstrated that the antiviral potency of PMOs could be enhanced by chemical modification, either by conjugating PMOs with peptides or by introducing positively charge to the PMOs (PMO*plus*™, Avi BioPharma, Inc.; Swenson et al., 2009). Subsequently, PMO*plus* targeting EBOV VP24 and VP35 or MARV Musoke VP24 and NP showed significant protection of mice and guinea pigs against lethal challenge with EBOV and MARV Musoke, respectively (Warren et al., 2010). AVI-6002 PMO*plus* against both EBOV VP24 and VP35, and AVI-6003 PMO*plus* against MARV VP24 and NP, were developed and tested for treatment efficacy using NHP models. These PMOs, delivered 30–60 min post-exposure, protected 62.5% of rhesus macaques against lethal EBOV infection and 100% of cynomolgus macaques against MARV Musoke infection (Warren et al., 2010). AVI-6002 and AVI-6003 are currently in phase I clinical trials.

RNA INTERFERENCE

RNA interference (RNAi) inhibits gene expression to the extent that their function is abrogated through a highly regulated enzyme-mediated process. It was demonstrated that small-interfering RNA (siRNA) down-regulated various MARV mRNA transcripts, resulting in a significant decrease in viral protein production and subsequent viral release *in vitro* (Fowler et al., 2005). Furthermore, siRNA targeting the EBOV RNA polymerase L protein formulated in stable nucleic acid-lipid particles (SNALPs) completely protected guinea pigs when administered shortly after a lethal EBOV infection (Geisbert et al., 2006). In rhesus macaques, a combination of siRNA targeting the EBOV L, VP24, and VP35 were formulated in SNALPs and administrated to the monkeys. Two of three monkeys, which were treated four times with siRNA at 30 min, 1, 3, and 5 days after challenge, survived lethal infection. Furthermore, all four monkeys treated seven times at 30 min, 1–6 days after challenge survived (Geisbert et al., 2010d).

THERAPEUTIC EFFICACY IN THE MOUSE MODEL AND *in vitro*

In the mouse model, administration of recombinant mannose-binding lectin and hexaamminecobalt (III) chloride showed efficacy in protecting against EBOV infections (Michelow et al., 2011). Mannose-binding lectin targets diverse microorganisms for phagocytosis and complement-mediated lysis by binding specific surface glycans. Hexaamminecobalt (III) chloride is a complex of a cobalt (III) ion surrounded by six ammonia ligands in a full octahedral coordination. Furthermore, by high-throughput screening, some compounds such as FGI-103, FGI-106, and NSC 62914 (a reactive oxygen species scavenger), were identified to have high antiviral activity against filoviruses (Aman et al., 2009; Warren et al., 2010). Some other substances, for example inhibitors of heat-shock protein 90 and Niemann-Pick C1, showed antiviral activity *in vitro* (Smith et al., 2010; Cote et al., 2011). As mentioned above (**Table 3**), several candidates are discussed as therapeutic agents for Ebola and Marburg HFs, but no licensed therapeutics are yet available (Friedrich et al., 2012).

CONCLUSIONS

Significant progress has been made in developing animal models, including mice, guinea pigs, hamsters and NHPs, for EHF and MHF. The NHPs are the most feasible model, because they are the only animals that are lethally infected with non-adapted virus isolates and the pathophysiology is close to that demonstrated in patients. The rodent models need serial passages of original filoviruses in rodents for acquiring lethal infection capacity and they have limited value, because the disease course in rodents differs from that demonstrated in humans and NHPs. However, the rodent models are the first choice for preliminary studies to explore vaccines and therapeutic agents, because of their ease to handling. The newly developed Golden hamster model will also be used for studies on pathogenesis and evaluation of efficacy of candidate vaccines and therapeutics because they show manifestations similar to those of patients and NHPs, including severity of coagulopathy that is lacking in mouse and guinea pig models. Among the candidate vaccines so far developed, recombinant VSV-based vaccines against EHF and MHF are confirmed to be effective in mouse, guinea pig, Golden hamster, and NHP models, and are the only platform with the potential to prevent lethal infection, especially via both vaccine and post-exposure treatment (Jones et al., 2005; Daddario-Dicaprio et al., 2006b; Feldmann et al., 2007; Geisbert et al., 2008, 2009, 2010c; Qiu et al., 2009; Tsuda et al., 2011b). Furthermore, the VSV have been used as a treatment following a recent laboratory exposure (Tuffs, 2009). Further research is needed to develop vaccines with sufficient long-term efficacy by single-dose vaccination, because expensive and time-consuming vaccinations may pose difficulties due to logistical and financial problems in developing countries, where EHF and MHF are endemic. Neither licensed vaccines nor therapeutic agents are available so far. The development of vaccines and therapeutic testing using the animal models has only recently begun to progress. We hope that further research facilitates progress toward elucidating the disease pathophysiology and developing prophylactic and therapeutic measures against EHF and MHF.

REFERENCES

Abbink, P., Lemckert, A. A., Ewald, B. A., Lynch, D. M., Denholtz, M., Smits, S., et al. (2007). Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J. Virol.* 81, 4654–4663. doi: 10.1128/JVI.02696-06

Alves, D. A., Glynn, A. R., Steele, K. E., Lackemeyer, M. G., Garza, N. L., Buck, J. G., et al. (2010). Aerosol exposure to the angola strain of marburg virus causes lethal viral hemorrhagic Fever in cynomolgus macaques. *Vet. Pathol.* 47, 831–851. doi: 10.1177/0300985810378597

Aman, M. J., Kinch, M. S., Warfield, K., Warren, T., Yunus, A., Enterlein, S., et al. (2009). Development of a broad-spectrum antiviral with activity against Ebola virus. *Antiviral Res.* 83, 245–251. doi: 10.1016/j.antiviral.2009.06.001

Arai, A., Hirano, H., Ueta, Y., Hamada, T., Mita, T., and Shirahata, A. (2000). Detection of mononuclear cells as the source of the increased tissue factor mRNA in the liver from lipopolysaccharide-treated rats. *Thromb. Res.* 97, 153–162. doi: 10.1016/S0049-3848(99)00147-4

Baize, S., Leroy, E. M., Georges, A. J., Georges-Courbot, M. C., Capron, M., Bedjabaga, I., et al. (2002). Inflammatory responses in Ebola virus-infected patients. *Clin. Exp. Immunol.* 128, 163–168. doi: 10.1046/j.1365-2249.2002.01800.x

Baize, S., Leroy, E. M., Georges-Courbot, M. C., Capron, M., Lansoud-Soukate, J., Debre, P., et al. (1999). Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nat. Med.* 5, 423–426. doi: 10.1038/7422

Baskerville, A., Bowen, E. T., Platt, G. S., McArdell, L. B., and Simpson, D. I. (1978). The pathology of experimental Ebola virus infection in monkeys. *J. Pathol.* 125, 131–138. doi: 10.1002/path.1711250303

Baskerville, A., Fisher-Hoch, S. P., Neild, G. H., and Dowsett, A. B. (1985). Ultrastructural pathology of experimental Ebola haemorrhagic fever virus infection. *J. Pathol.* 147, 199–209. doi: 10.1002/path.1711470308

Basler, C. F., Wang, X., Muhlberger, E., Volchkov, V., Paragas, J., Klenk, H. D., et al. (2000). The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12289–12294. doi: 10.1073/pnas.220398297

Bente, D., Gren, J., Strong, J. E., and Feldmann, H. (2009). Disease modeling for Ebola and Marburg viruses. *Dis. Model. Mech.* 2, 12–17. doi: 10.1242/dmm.000471

Bowen, E. T., Lloyd, G., Harris, W. J., Platt, G. S., Baskerville, A., and Vella, E. E. (1977). Viral haemorrhagic fever in southern Sudan and northern Zaire. Preliminary studies on the aetiological agent. *Lancet* 1, 571–573. doi: 10.1016/S0140-6736(77)92001-3

Bowen, E. T., Platt, G. S., Lloyd, G., Raymond, R. T., and Simpson, D. I. (1980). A comparative study of strains of Ebola virus isolated from southern Sudan and northern Zaire in 1976. *J. Med. Virol.* 6, 129–138. doi: 10.1002/jmv.1890060205

Bowen, E. T., Platt, G. S., Simpson, D. I., McArdell, L. B., and Raymond, R. T. (1978). Ebola haemorrhagic fever: experimental infection of monkeys. *Trans. R. Soc. Trop. Med. Hyg.* 72, 188–191. doi: 10.1016/0035-9203(78)90058-5

Bradfute, S. B., Braun, D. R., Shamblin, J. D., Geisbert, J. B., Paragas, J., Garrison, A., et al. (2007). Lymphocyte death in a mouse model of Ebola virus infection. *J. Infect. Dis.* 196(Suppl. 2), S296–S304. doi: 10.1086/520602

Bradfute, S. B., Warfield, K. L., and Bray, M. (2012). Mouse models for filovirus infections. *Viruses* 4, 1477–1508. doi: 10.3390/v4091477

Bray, M. (2001). The role of the Type I interferon response in the resistance of mice to filovirus infection. *J. Gen. Virol.* 82, 1365–1373.

Bray, M., Davis, K., Geisbert, T., Schmaljohn, C., and Huggins, J. (1999). A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *J. Infect. Dis.* 179(Suppl. 1), S248–S258. doi: 10.1086/514292

Bray, M., Hatfill, S., Hensley, L., and Huggins, J. W. (2001). Haematological, biochemical and coagulation changes in mice, guinea-pigs and monkeys infected with a mouse-adapted variant of Ebola Zaire virus. *J. Comp. Pathol.* 125, 243–253. doi: 10.1053/jcpa.2001.0503

Bukreyev, A., Marzi, A., Feldmann, F., Zhang, L., Yang, L., Ward, J. M., et al. (2009). Chimeric human parainfluenza virus bearing the Ebola virus glycoprotein as the sole surface protein is immunogenic and highly protective against Ebola virus challenge. *Virology* 383, 348–361. doi: 10.1016/j.virol.2008.09.030

Bukreyev, A., Rollin, P. E., Tate, M. K., Yang, L., Zaki, S. R., Shieh, W. J., et al. (2007). Successful topical respiratory tract immunization of primates against Ebola virus. *J. Virol.* 81, 6379–6388. doi: 10.1128/JVI.00105-07

Bukreyev, A., Yang, L., Zaki, S. R., Shieh, W. J., Rollin, P. E., Murphy, B. R., et al. (2006). A single intranasal inoculation with a paramyxovirus-vectored vaccine protects guinea pigs against a lethal-dose Ebola virus challenge. *J. Virol.* 80, 2267–2279. doi: 10.1128/JVI.80.5.2267-2279.2006

Bukreyev, A. A., Dinapoli, J. M., Yang, L., Murphy, B. R., and Collins, P. L. (2010). Mucosal parainfluenza virus-vectored vaccine against Ebola virus replicates in the respiratory tract of vector-immune monkeys and is immunogenic. *Virology* 399, 290–298. doi: 10.1016/j.virol.2010.01.015

Bwaka, M. A., Bonnet, M. J., Calain, P., Colebunders, R., De Roo, A., Guimard, Y., et al. (1999). Ebola hemorrhagic fever in Kikwit, Democratic Republic of the Congo: clinical observations in 103 patients. *J. Infect. Dis.* 179(Suppl. 1), S1–S7. doi: 10.1086/514308

Chupurnov, A. A., Chernukhin, I. V., Ternovoi, V. A., Kudoiarova, N. M., Makhova, N. M., Azaev, M., et al. (1995). Attempts to develop a vaccine against Ebola fever. *Vopr. Virusol.* 40, 257–260.

Connolly, B. M., Steele, K. E., Davis, K. J., Geisbert, T. W., Kell, W. M., Jaax, N. K., et al. (1999). Pathogenesis of experimental Ebola virus infection in guinea pigs. *J. Infect. Dis.* 179(Suppl. 1), S203–S217. doi: 10.1086/514305

Cote, M., Misasi, J., Ren, T., Bruchez, A., Lee, K., Filone, C. M., et al. (2011). Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature* 477, 344–348. doi: 10.1038/nature10380

Daddario-Dicaprio, K. M., Geisbert, T. W., Geisbert, J. B., Stroher, U., Hensley, L. E., Grolla, A., et al. (2006a). Cross-protection against Marburg virus strains by using a live, attenuated recombinant vaccine. *J. Virol.* 80, 9659–9666. doi: 10.1128/JVI.00959-06

Daddario-Dicaprio, K. M., Geisbert, T. W., Stroher, U., Geisbert, J. B., Grolla, A., Fritz, E. A., et al. (2006b). Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. *Lancet* 367, 1399–1404. doi: 10.1016/S0140-6736(06)68546-2

Dalgard, D. W., Hardy, R. J., Pearson, S. L., Pucak, G. J., Quander, R. V., Zack, P. M., et al. (1992). Combined simian hemorrhagic fever and Ebola virus infection in cynomolgus monkeys. *Lab. Anim. Sci.* 42, 152–157.

Davis, K. J., Anderson, A. O., Geisbert, T. W., Steele, K. E., Geisbert, J. B., Vogel, P., et al. (1997). Pathology of experimental Ebola virus infection in African green monkeys. Involvement of fibroblastic reticular cells. *Arch. Pathol. Lab. Med.* 121, 805–819.

Dietrich, M., Schumacher, H. H., Peters, D., and Knobloch, J. (1978). "Human pathology of Ebola (Maridi) virus infection in the Sudan," in *Ebola Virus Haemorrhagic Fever*, ed S. R. Pattyn (New York, NY: Elsevier, North-Holland Biomedical Press), 37–41.

Ebihara, H., Rockx, B., Marzi, A., Feldmann, F., Haddock, E., Brining, D., et al. (2011). Host response dynamics following lethal infection of rhesus macaques with Zaire ebolavirus. *J. Infect. Dis.* 204(Suppl. 3), S991–999. doi: 10.1093/infdis/jir336

Ebihara, H., Takada, A., Kobasa, D., Jones, S., Neumann, G., Theriault, S., et al. (2006). Molecular determinants of Ebola virus virulence in mice. *PLoS Pathog.* 2:e73. doi: 10.1371/journal.ppat.0020073

Ebihara, H., Zivcec, M., Gardner, D., Falzarano, D., Lacasse, R., Rosenke, R., et al. (2013). A syrian golden hamster model recapitulating ebola hemorrhagic fever. *J. Infect. Dis.* 207, 306–318. doi: 10.1093/infdis/jis626

Ellis, D. S., Bowen, E. T., Simpson, D. I., and Stamford, S. (1978). Ebola virus: a comparison, at ultrastructural level, of the behaviour of the Sudan and Zaire strains in monkeys. *Br. J. Exp. Pathol.* 59, 584–593.

Falzarano, D., Geisbert, T. W., and Feldmann, H. (2011). Progress in filovirus vaccine development: evaluating the potential for clinical use. *Expert Rev. Vaccines* 10, 63–77. doi: 10.1586/erv.10.152

Feldmann, H., Jones, S. M., Daddario-Dicaprio, K. M., Geisbert, J. B., Stroher, U., Grolla, A., et al. (2007). Effective post-exposure treatment of Ebola infection. *PLoS Pathog.* 3:e2. doi: 10.1371/journal.ppat.0030002

Fisher-Hoch, S. P., Brammer, T. L., Trappier, S. G., Hutwagner, L. C., Farrar, B. B., Ruo, S. L., et al. (1992). Pathogenic potential of filoviruses: role of geographic origin of primate host and virus strain.

J. Infect. Dis. 166, 753–763. doi: 10.1093/infdis/166.4.753

Fisher-Hoch, S. P., Platt, G. S., Neild, G. H., Southee, T., Baskerville, A., Raymond, R. T., et al. (1985). Pathophysiology of shock and hemorrhage in a fulminating viral infection (Ebola). *J. Infect. Dis.* 152, 887–894. doi: 10.1093/infdis/152.5.887

Fowler, T., Bamberg, S., Moller, P., Klenk, H. D., Meyer, T. F., Becker, S., et al. (2005). Inhibition of Marburg virus protein expression and viral release by RNA interference. *J. Gen. Virol.* 86, 1181–1188. doi: 10.1099/vir.0.80622-0

Friedrich, B. M., Trefry, J. C., Biggins, J. E., Hensley, L. E., Honko, A. N., Smith, D. R., et al. (2012). Potential vaccines and post-exposure treatments for filovirus infections. *Viruses* 4, 1619–1650. doi: 10.3390/v4091619

Geisbert, T. W., Bailey, M., Geisbert, J. B., Asiedu, C., Roederer, M., Grazia-Pau, M., et al. (2010a). Vector choice determines immunogenicity and potency of genetic vaccines against Angola Marburg virus in nonhuman primates. *J. Virol.* 84, 10386–10394. doi: 10.1128/JVI.00594-10

Geisbert, T. W., Bausch, D. G., and Feldmann, H. (2010b). Prospects for immunisation against Marburg and Ebola viruses. *Rev. Med. Virol.* 20, 344–357.

Geisbert, T. W., Hensley, L. E., Geisbert, J. B., Leung, A., Johnson, J. C., Grolla, A., et al. (2010c). Postexposure treatment of Marburg virus infection. *Emerging Infect. Dis.* 16, 1119–1122. doi: 10.3201/eid1607.100159

Geisbert, T. W., Lee, A. C., Robbins, M., Geisbert, J. B., Honko, A. N., Sood, V., et al. (2010d). Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* 375, 1896–1905. doi: 10.1016/S0140-6736(10)60357-1

Geisbert, T. W., Bailey, M., Hensley, L., Asiedu, C., Geisbert, J., Stanley, D., et al. (2011). Recombinant adenovirus serotype 26 (Ad26) and Ad35 vaccine vectors bypass immunity to Ad5 and protect nonhuman primates against ebolavirus challenge. *J. Virol.* 85, 4222–4233. doi: 10.1128/JVI.02407-10

Geisbert, T. W., Daddario-Dicaprio, K. M., Geisbert, J. B., Young, H. A., Formenty, P., Fritz, E. A., et al. (2007). Marburg virus Angola infection of rhesus macaques: pathogenesis and treatment with recombinant nematode anticoagulant protein c2. *J. Infect. Dis.* 196(Suppl. 2), S372–S381. doi: 10.1086/520608

Geisbert, T. W., Daddario-Dicaprio, K. M., Williams, K. J., Geisbert, J. B., Leung, A., Feldmann, F., et al. (2008). Recombinant vesicular stomatitis virus vector mediates postexposure protection against Sudan Ebola hemorrhagic fever in nonhuman primates. *J. Virol.* 82, 5664–5668. doi: 10.1128/JVI.00456-08

Geisbert, T. W., Geisbert, J. B., Leung, A., Daddario-Dicaprio, K. M., Hensley, L. E., Grolla, A., et al. (2009). Single-injection vaccine protects nonhuman primates against infection with marburg virus and three species of ebola virus. *J. Virol.* 83, 7296–7304. doi: 10.1128/JVI.00561-09

Geisbert, T. W., Hensley, L. E., Gibb, T. R., Steele, K. E., Jaax, N. K., and Jahrling, P. B. (2000). Apoptosis induced *in vitro* and *in vivo* during infection by Ebola and Marburg viruses. *Lab. Invest.* 80, 171–186. doi: 10.1038/labinvest.3780021

Geisbert, T. W., Hensley, L. E., Jahrling, P. B., Larsen, T., Geisbert, J. B., Paragas, J., et al. (2003a). Treatment of Ebola virus infection with a recombinant inhibitor of factor VIIa/tissue factor: a study in rhesus monkeys. *Lancet* 362, 1953–1958. doi: 10.1016/S0140-6736(03)15012-X

Geisbert, T. W., Hensley, L. E., Larsen, T., Young, H. A., Reed, D. S., Geisbert, J. B., et al. (2003b). Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. *Am. J. Pathol.* 163, 2347–2370.

Geisbert, T. W., Jahrling, P. B., Larsen, T., Davis, K. J., and Hensley, L. E. (2003c). *Filovirus Pathogenesis in Nonhuman Primates*. Wymondham, Norfolk: Taylor and Francis.

Geisbert, T. W., Young, H. A., Jahrling, P. B., Davis, K. J., Kagan, E., and Hensley, L. E. (2003d). Mechanisms underlying coagulation abnormalities in ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. *J. Infect. Dis.* 188, 1618–1629. doi: 10.1086/379724

Geisbert, T. W., Young, H. A., Jahrling, P. B., Davis, K. J., Larsen, T., Kagan, E., et al. (2003e). Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. *Am. J. Pathol.* 163, 2371–2382.

Geisbert, T. W., Hensley, L. E., Kagan, E., Yu, E. Z., Geisbert, J. B., Daddario-Dicaprio, K., et al. (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 193, 1650–1657. doi: 10.1086/504267

Geisbert, T. W., Pushko, P., Anderson, K., Smith, J., Davis, K. J., and Jahrling, P. B. (2002). Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerging Infect. Dis.* 8, 503–507. doi: 10.3201/eid0805.010284

Gibb, T. R., Norwood, D. A. Jr., Woollen, N., and Henchal, E. A. (2001). Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J. Clin. Microbiol.* 39, 4125–4130. doi: 10.1128/JCM.39.11.4125-4130.2001

Gonchar, N. I., Pshenichnov, V. A., Pokhodiaev, V. A., Lopatov, K. L., and Firsova, I. V. (1991). The sensitivity of different experimental animals to the Marburg virus. *Vopr. Virusol.* 36, 435–437.

Gupta, M., Greer, P., Mahanty, S., Shieh, W. J., Zaki, S. R., Ahmed, R., et al. (2005). CD8-mediated protection against Ebola virus infection is perforin dependent. *J. Immunol.* 174, 4198–4202.

Halfmann, P., Ebihara, H., Marzi, A., Hatta, Y., Watanabe, S., Suresh, M., et al. (2009). Replication-deficient ebolavirus as a vaccine candidate. *J. Virol.* 83, 3810–3815. doi: 10.1128/JVI.00074-09

Halfmann, P., Neumann, G., and Kawaoka, Y. (2011). The Ebolavirus VP24 protein blocks phosphorylation of p38 mitogen-activated protein kinase. *J. Infect. Dis.* 204(Suppl. 3), S953–S956. doi: 10.1093/infdis/jir325

Hall, W. C., Geisbert, T. W., Huggins, J. W., and Jahrling, P. B. (1996). Experimental infection of guinea pigs with Venezuelan hemorrhagic fever virus (Guanarito): a model of human disease. *Am. J. Trop. Med. Hyg.* 55, 81–88.

Hass, R., and Maass, G. (1971). "Experimental infection of monkeys with the marburg virus," in *Marburg Virus Disease*, eds G. A. Martini and R. Siegert (New York, NY: Springer, Berlin Heidelberg), 136–143.

Hensley, L. E., Alves, D. A., Geisbert, J. B., Fritz, E. A., Reed, C., Larsen, T., et al. (2011). Pathogenesis of Marburg hemorrhagic fever in cynomolgus macaques. *J. Infect. Dis.* 204(Suppl. 3), S1021–S1031. doi: 10.1093/infdis/jir339

Hensley, L. E., Stevens, E. L., Yan, S. B., Geisbert, J. B., Macias, W. L., Larsen, T., et al. (2007). Recombinant human activated protein C for the postexposure treatment of Ebola hemorrhagic fever. *J. Infect. Dis.* 196(Suppl. 2), S390–S399. doi: 10.1086/520598

Hevey, M., Negley, D., Pushko, P., Smith, J., and Schmaljohn, A. (1998). Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* 251, 28–37. doi: 10.1006/viro.1998.9367

Ignat'ev, G. M., Agafonov, A. P., Strel'tsova, M. A., Kuz'min, V. A., Mainagasheva, G. I., Spirin, G. V., et al. (1991). A comparative study of the immunological indices in guinea pigs administered an inactivated Marburg virus. *Vopr. Virusol.* 36, 421–423.

Ignatiev, G. M., Dadaeva, A. A., Luchko, S. V., and Chepurnov, A. A. (2000). Immune and pathophysiological processes in baboons experimentally infected with Ebola virus adapted to guinea pigs. *Immunol. Lett.* 71, 131–140. doi: 10.1016/S0165-2478(99)00169-8

Ignatyev, G. M., Agafonov, A. P., Streltsova, M. A., and Kashentseva, E. A. (1996). Inactivated Marburg virus elicits a nonprotective immune response in Rhesus monkeys. *J. Biotechnol.* 44, 111–118. doi: 10.1016/0168-1656(95)00104-2

Isaacson, M., Sureau, P., Courteille, G., and Pattyn, S. R. (1978). "Clinical aspets of Ebola virus disease at the Ngaliema hospital, Kinshasa, Zaire, 1976," in *Ebola Virus Haemorrhagic Fever*, ed S. R. Pattyn (New York, NY: Elsevier, North-Holland Biomedical Press), 15–20.

Jaax, N., Jahrling, P., Geisbert, T., Geisbert, J., Steele, K., McKee, K., et al. (1995). Transmission of Ebola virus (Zaire strain) to uninfected control monkeys in a biocontainment laboratory. *Lancet* 346, 1669–1671. doi: 10.1016/S0140-6736(95)92841-3

Jaax, N. K., Davis, K. J., Geisbert, T. J., Vogel, P., Jaax, G. P., Topper, M., et al. (1996). Lethal experimental infection of rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure. *Arch. Pathol. Lab. Med.* 120, 140–155.

Jahrling, P. B., Geisbert, T. W., Dalgard, D. W., Johnson, E. D., Ksiazek, T. G., Hall, W. C., et al. (1990). Preliminary report: isolation of Ebola virus from monkeys imported

to USA. *Lancet* 335, 502–505. doi: 10.1016/0140-6736(90)90737-P

Jahrling, P. B., Geisbert, T. W., Jaax, N. K., Hanes, M. A., Ksiazek, T. G., and Peters, C. J. (1996). Experimental infection of cynomolgus macaques with Ebola-Reston filoviruses from the 1989–1990 U.S. epizootic. *Arch. Virol. Suppl.* 11, 115–134. doi: 10.1007/978-3-7091-7482-1_11

Jahrling, P. B., Smith, S., Hesse, R. A., and Rhoderick, J. B. (1982). Pathogenesis of Lassa virus infection in guinea pigs. *Infect. Immun.* 37, 771–778.

Johnson, E., Jaax, N., White, J., and Jahrling, P. (1995). Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. *Int. J. Exp. Pathol.* 76, 227–236.

Johnson, E. D., Johnson, B. K., Silverstein, D., Tukei, P., Geisbert, T. W., Sanchez, A. N., et al. (1996). Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. *Arch. Virol. Suppl.* 11, 101–114. doi: 10.1007/978-3-7091-7482-1_10

Jones, S. M., Feldmann, H., Stroher, U., Geisbert, J. B., Fernando, L., Grolla, A., et al. (2005). Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nat. Med.* 11, 786–790. doi: 10.1038/nm1258

Kenyon, R. H., Condie, R. M., Jahrling, P. B., and Peters, C. J. (1990). Protection of guinea pigs against experimental Argentine hemorrhagic fever by purified human IgG: importance of elimination of infected cells. *Microb. Pathog.* 9, 219–226. doi: 10.1016/0882-4010(90)90010-N

Konduru, K., Bradfute, S. B., Jacques, J., Manangeeswaran, M., Nakamura, S., Morshed, S., et al. (2011). Ebola virus glycoprotein Fc fusion protein confers protection against lethal challenge in vaccinated mice. *Vaccine* 29, 2968–2977. doi: 10.1016/j.vaccine.2011.01.113

Korb, G., and Slenczka, W. (1971). "Histologic findings in livers and spleens of guinea pigs after inoculation by the marburg virus," in *Marburg Virus Disease*, eds G. A. Martini and R. Siegert (New York, NY: Springer, Berlin Heidelberg), 123–128.

Ksiazek, T. G., Rollin, P. E., Williams, A. J., Bressler, D. S., Martin, M. L., Swanepoel, R., et al. (1999). Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. *J. Infect. Dis.* 179(Suppl. 1), S177–S187. doi: 10.1086/514321

Kuhn, J. H. (2008). *Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies*. New York, NY: Springer Wien.

Kuhn, J. H., Bao, Y., Bavari, S., Becker, S., Bradfute, S., Brister, J. R., et al. (2013). Virus nomenclature below the species level: a standardized nomenclature for natural variants of viruses assigned to the family Filoviridae. *Arch. Virol.* 158, 301–311. doi: 10.1007/s00705-012-1454-0

Kuhn, J. H., Dodd, L. E., Wahl-Jensen, V., Radoshitzky, S. R., Bavari, S., and Jahrling, P. B. (2011). Evaluation of perceived threat differences posed by filovirus variants. *Biosecur. Bioterror.* 9, 361–371. doi: 10.1089/bsp.2011.0051

Leroy, E. M., Epelboin, A., Mondonge, V., Pourrut, X., Gonzalez, J. P., Muyembe-Tamfum, J. J., et al. (2009). Human Ebola outbreak resulting from direct exposure to fruit bats in Luebo, Democratic Republic of Congo, 2007. *Vector Borne Zoonotic Dis.* 9, 723–728. doi: 10.1089/vbz.2008.0167

Leroy, E. M., Rouquet, P., Formenty, P., Souquiere, S., Kilbourne, A., Froment, J. M., et al. (2004). Multiple Ebola virus transmission events and rapid decline of central African wildlife. *Science* 303, 387–390. doi: 10.1126/science.1092528

Luchko, S. V., Dadaeva, A. A., Ustinova, E. N., Sizikova, L. P., Riabchikova, E. I., and Sandakhchiev, L. S. (1995). Experimental study of Ebola hemorrhagic fever in baboon models. *Biull. Eksp. Biol. Med.* 120, 302–304. doi: 10.1007/BF02445023

Martin, J. E., Sullivan, N. J., Enama, M. E., Gordon, I. J., Roederer, M., Koup, R. A., et al. (2006). A DNA vaccine for Ebola virus is safe and immunogenic in a phase I clinical trial. *Clin. Vaccine Immunol.* 13, 1267–1277. doi: 10.1128/CVI.00162-06

Martini, G. A. (1971). "Marburg virus disease. Clinical symptome," in *Marburg Virus Disease*, eds G. A. Martini and R. Siegert (New York, NY: Springer, Berlin Heidelberg), 1–9.

Mast, T. C., Kierstead, L., Gupta, S. B., Nikas, A. A., Kallas, E. G., Novitsky, V., et al. (2010). International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine* 28, 950–957. doi: 10.1016/j.vaccine.2009.10.145

Mateo, M., Carbonnelle, C., Reynard, O., Kolesnikova, L., Nemirov, K., Page, A., et al. (2011). VP24 is a molecular determinant of Ebola virus virulence in guinea pigs. *J. Infect. Dis.* 204(Suppl. 3), S1011–S1020. doi: 10.1093/infdis/jir338

Mateo, M., Reid, S. P., Leung, L. W., Basler, C. F., and Volchkov, V. E. (2010). Ebolavirus VP24 binding to karyopherins is required for inhibition of interferon signaling. *J. Virol.* 84, 1169–1175. doi: 10.1128/JVI.01372-09

Michelow, I. C., Lear, C., Scully, C., Prugar, L. I., Longley, C. B., Yantosca, L. M., et al. (2011). High-dose mannose-binding lectin therapy for Ebola virus infection. *J. Infect. Dis.* 203, 175–179. doi: 10.1093/infdis/jiq025

Mikhailov, V. V., Borisevich, I. V., Chernikova, N. K., Potryvaeva, N. V., and Krasnianskii, V. P. (1994). The evaluation in hamadryas baboons of the possibility for the specific prevention of Ebola fever. *Vopr. Virusol.* 39, 82–84.

Miranda, M. E., Ksiazek, T. G., Retuya, T. J., Khan, A. S., Sanchez, A., Fulhorst, C. F., et al. (1999). Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. *J. Infect. Dis.* 179(Suppl. 1), S115–S119. doi: 10.1086/514314

Miranda, M. E., Yoshikawa, Y., Manalo, D. L., Calaor, A. B., Miranda, N. L., Cho, F., et al. (2002). Chronological and spatial analysis of the 1996 Ebola Reston virus outbreak in a monkey breeding facility in the Philippines. *Exp. Anim.* 51, 173–179. doi: 10.1538/expanim.51.173

Murphy, F. A., Simpson, D. I., Whitfield, S. G., Zlotnik, I., and Carter, G. B. (1971). Marburg virus infection in monkeys. Ultrastructural studies. *Lab. Invest.* 24, 279–291.

Ndambi, R., Akamituna, P., Bonnet, M. J., Tukadila, A. M., Muyembe-Tamfum, J. J., and Colebunders, R. (1999). Epidemiologic and clinical aspects of the Ebola virus epidemic in Mosango, Democratic Republic of the Congo, 1995. *J. Infect. Dis.* 179(Suppl. 1), S8–S10. doi: 10.1086/514297

Negredo, A., Palacios, G., Vazquez-Moron, S., Gonzalez, F., Dopazo, H., Molero, F., et al. (2011). Discovery of an ebolavirus-like filovirus in europe. *PLoS Pathog.* 7:e1002304. doi: 10.1371/journal.ppat.1002304

Phoolcharoen, W., Bhoo, S. H., Lai, H., Ma, J., Arntzen, C. J., Chen, Q., et al. (2011). Expression of an immunogenic Ebola immune complex in Nicotiana benthamiana. *Plant Biotechnol. J.* 9, 807–816. doi: 10.1111/j.1467-7652.2011.00593.x

Piedra, P. A., Poveda, G. A., Ramsey, B., McCoy, K., and Hiatt, P. W. (1998). Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: implication for gene therapy with adenovirus vectors. *Pediatrics* 101, 1013–1019. doi: 10.1542/peds.101.6.1013

Piot, P., Sureau, P., Breman, G., Heymann, D., Kintoki, V., Masamba, M., et al. (1978). "Clinical aspects of Ebola virus infection in Tambuku area, Zaire, 1976," in *Ebola Virus Haemorrhagic Fever*, ed S. R. Pattyn (New York, NY: Elsevier, North-Holland Biomedical Press), 7–14.

Pokhodiaev, V. A., Gonchar, N. I., and Pshenichnov, V. A. (1991). An experimental study of the contact transmission of the Marburg virus. *Vopr. Virusol.* 36, 506–508.

Pushko, P., Bray, M., Ludwig, G. V., Parker, M., Schmaljohn, A., Sanchez, A., et al. (2001). Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. *Vaccine* 19, 142–153. doi: 10.1016/S0264-410X(00)00113-4

Qiu, X., Fernando, L., Alimonti, J. B., Melito, P. L., Feldmann, F., Dick, D., et al. (2009). Mucosal immunization of cynomolgus macaques with the VSVDeltaG/ZEBOVGP vaccine stimulates strong ebola GP-specific immune responses. *PLoS ONE* 4:e5547. doi: 10.1371/journal.pone.0005547

Ramanan, P., Edwards, M. R., Shabman, R. S., Leung, D. W., Endlich-Frazier, A. C., Borek, D. M., et al. (2012). Structural basis for Marburg virus VP35-mediated immune evasion mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20661–20666. doi: 10.1073/pnas.1213559109

Ramanan, P., Shabman, R. S., Brown, C. S., Amarasinghe, G. K., Basler, C. F., and Leung, D. W. (2011). Filoviral immune evasion mechanisms. *Viruses* 3, 1634–1649. doi: 10.3390/v3091634

Reid, S. P., Valmas, C., Martinez, O., Sanchez, F. M., and Basler, C. F. (2007). Ebola virus VP24 proteins inhibit the interaction of NPI-1 subfamily karyopherin alpha

proteins with activated STAT1. *J. Virol.* 81, 13469–13477. doi: 10.1128/JVI.01097-07

Riemenschneider, J., Garrison, A., Geisbert, J., Jahrling, P., Hevey, M., Negley, D., et al. (2003). Comparison of individual and combination DNA vaccines for *B. anthracis*, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus. *Vaccine* 21, 4071–4080. doi: 10.1016/S0264-410X(03)00362-1

Robin, Y., Bres, P., and Camain, R. (1971). "Passage of marburg virus in guinea pigs," in *Marburg Virus Disease*, eds G. A. Martini and R. Siegert (New York, NY: Springer, Berlin Heidelberg), 117–122.

Ryabchikova, E., Kolesnikova, L., Smolina, M., Tkachev, V., Pereboeva, L., Baranova, S., et al. (1996). Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis. *Arch. Virol.* 141, 909–921. doi: 10.1007/BF01718165

Ryabchikova, E., Smolina, M. A. G., and Rassadkin, J. (2003). *Ebola virus infection in the guinea pig*. Wymondham, Norfolk: Taylor and Francis.

Ryabchikova, E. I., Kolesnikova, L. V., and Luchko, S. V. (1999). An analysis of features of pathogenesis in two animal models of Ebola virus infection. *J. Infect. Dis.* 179(Suppl. 1), S199–S202. doi: 10.1086/514293

Sanchez, A., Geisbert, T. W., and Felsmann, H. (2007). *Marburg and Ebola Viruses.* Philadelphia, PA: Lippincott Williams and Wilkins.

Sanchez, A., Lukwiya, M., Bausch, D., Mahanty, S., Sanchez, A. J., Wagoner, K. D., et al. (2004). Analysis of human peripheral blood samples from fatal and nonfatal cases of Ebola (Sudan) hemorrhagic fever: cellular responses, virus load, and nitric oxide levels. *J. Virol.* 78, 10370–10377. doi: 10.1128/JVI.78.19.10370-10377.2004

Schulick, A. H., Vassalli, G., Dunn, P. F., Dong, G., Rade, J. J., Zamarron, C., et al. (1997). Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity. *J. Clin. Invest.* 99, 209–219. doi: 10.1172/JCI119149

Simpson, D. I. (1969). Marburg agent disease: in monkeys. *Trans. R. Soc. Trop. Med. Hyg.* 63, 303–309. doi: 10.1016/0035-9203(69)90002-9

Simpson, D. I., Zlotnik, I., and Rutter, D. A. (1968). Vervet monkey disease. Experiment infection of guinea pigs and monkeys with the causative agent. *Br. J. Exp. Pathol.* 49, 458–464.

Smith, D. H., Francis, F., and Simpson, D. I. H. (1978). "African haemorrhagic fever in the Southern Sudan, 1976: the clinical manifestations," in *Ebola Virus Haemorrhagic Fever*, ed S. R. Pattyn (New York, NY: Elsevier, North-Holland Biomedical Press), 21–26.

Smith, D. R., McCarthy, S., Chrovian, A., Olinger, G., Stossel, A., Geisbert, T. W., et al. (2010). Inhibition of heat-shock protein 90 reduces Ebola virus replication. *Antiviral Res.* 87, 187–194. doi: 10.1016/j.antiviral.2010.04.015

Spiridonov, V. A., Bazhutin, N. B., Belanov, E. F., Voitenko, A. V., Zolin, V. V., Krivenchuk, N. A., et al. (1992). Changes in the blood serum aminotransferase activity in the experimental infection of *Cercopithecus aethiops* monkeys with the Marburg virus. *Vopr. Virusol.* 37, 156–157.

Subbotina, E., Dadaeva, A., Kachko, A., and Chepurnov, A. (2010). Genetic factors of Ebola virus virulence in guinea pigs. *Virus Res.* 153, 121–133. doi: 10.1016/j.virusres.2010.07.015

Sullivan, N. J., Geisbert, T. W., Geisbert, J. B., Xu, L., Yang, Z. Y., Roederer, M., et al. (2003). Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 424, 681–684. doi: 10.1038/nature01876

Sullivan, N. J., Sanchez, A., Rollin, P. E., Yang, Z. Y., and Nabel, G. J. (2000). Development of a preventive vaccine for Ebola virus infection in primates. *Nature* 408, 605–609. doi: 10.1038/35046108

Sun, Y., Carrion, R. Jr., Ye, L., Wen, Z., Ro, Y. T., et al. (2009). Protection against lethal challenge by Ebola virus-like particles produced in insect cells. *Virology* 383, 12–21. doi: 10.1016/j.virol.2008.09.020

Swenson, D. L., Wang, D., Luo, M., Warfield, K. L., Woraratanadharm, J., Holman, D. H., et al. (2008a). Vaccine to confer to nonhuman primates complete protection against multistrain Ebola and Marburg virus infections. *Clin. Vaccine Immunol.* 15, 460–467. doi: 10.1128/CVI.00431-07

Swenson, D. L., Warfield, K. L., Larsen, T., Alves, D. A., Coberley, S. S., and Bavari, S. (2008b). Monovalent virus-like particle vaccine protects guinea pigs and nonhuman primates against infection with multiple Marburg viruses. *Expert Rev. Vaccines* 7, 417–429.

Swenson, D. L., Warfield, K. L., Warren, T. K., Lovejoy, C., Hassinger, J. N., Ruthel, G., et al. (2009). Chemical modifications of antisense morpholino oligomers enhance their efficacy against Ebola virus infection. *Antimicrob. Agents Chemother.* 53, 2089–2099. doi: 10.1128/AAC.00936-08

Towner, J. S., Rollin, P. E., Bausch, D. G., Sanchez, A., Crary, S. M., Vincent, M., et al. (2004). Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. *J. Virol.* 78, 4330–4341. doi: 10.1128/JVI.78.8.4330-4341.2004

Tsuda, Y., Caposio, P., Parkins, C. J., Botto, S., Messaoudi, I., Cicin-Sain, L., et al. (2011a). A replicating cytomegalovirus-based vaccine encoding a single Ebola virus nucleoprotein CTL epitope confers protection against Ebola virus. *PLoS Negl. Trop. Dis.* 5:e1275. doi: 10.1371/journal.pntd.0001275

Tsuda, Y., Safronetz, D., Brown, K., Lacasse, R., Marzi, A., Ebihara, H., et al. (2011b). Protective efficacy of a bivalent recombinant vesicular stomatitis virus vaccine in the Syrian hamster model of lethal Ebola virus infection. *J. Infect. Dis.* 204(Suppl. 3), S1090–S1097. doi: 10.1093/infdis/jir379

Tuffs, A. (2009). Experimental vaccine may have saved Hamburg scientist from Ebola fever. *BMJ* 338:b1223. doi: 10.1136/bmj.b1223

van der Groen, G., Jacob, W., and Pattyn, S. R. (1979). Ebola virus virulence for newborn mice. *J. Med. Virol.* 4, 239–240. doi: 10.1002/jmv.1890040309

Vanderzanden, L., Bray, M., Fuller, D., Roberts, T., Custer, D., Spik, K., et al. (1998). DNA vaccines expressing either the GP or NP genes of Ebola virus protect mice from lethal challenge. *Virology* 246, 134–144. doi: 10.1006/viro.1998.9176

Vogels, R., Zuijdgeest, D., van Rijnsoever, R., Hartkoorn, E., Damen, I., de Bethune, M. P., et al. (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of pre-existing adenovirus immunity. *J. Virol.* 77, 8263–8271. doi: 10.1128/JVI.77.15.8263-8271.2003

Volchkov, V. E., Chepurnov, A. A., Volchkova, V. A., Ternovoj, V. A., and Klenk, H. D. (2000). Molecular characterization of guinea pig-adapted variants of Ebola virus. *Virology* 277, 147–155. doi: 10.1006/viro.2000.0572

Wahl-Jensen, V., Bollinger, L., Safronetz, D., de Kok-Mercado, F., Scott, D. P., and Ebihara, H. (2012). Use of the Syrian hamster as a new model of ebola virus disease and other viral hemorrhagic fevers. *Viruses* 4, 3754–3784. doi: 10.3390/v4123754

Wang, D., Hevey, M., Juompan, L. Y., Trubey, C. M., Raja, N. U., Deitz, S. B., et al. (2006a). Complex adenovirus-vectored vaccine protects guinea pigs from three strains of Marburg virus challenges. *Virology* 353, 324–332. doi: 10.1016/j.virol.2006.05.033

Wang, D., Raja, N. U., Trubey, C. M., Juompan, L. Y., Luo, M., Woraratanadharm, J., et al. (2006b). Development of a cAdVax-based bivalent ebola virus vaccine that induces immune responses against both the Sudan and Zaire species of Ebola virus. *J. Virol.* 80, 2738–2746. doi: 10.1128/JVI.80.6.2738-2746.2006

Warfield, K. L., Bosio, C. M., Welcher, B. C., Deal, E. M., Mohamadzadeh, M., Schmaljohn, A., et al. (2003). Ebola virus-like particles protect from lethal Ebola virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15889–15894. doi: 10.1073/pnas.2237038100

Warfield, K. L., Bradfute, S. B., Wells, J., Lofts, L., Cooper, M. T., Alves, D. A., et al. (2009). Development and characterization of a mouse model for Marburg hemorrhagic fever. *J. Virol.* 83, 6404–6415. doi: 10.1128/JVI.00126-09

Warfield, K. L., Posten, N. A., Swenson, D. L., Olinger, G. G., Esposito, D., Gillette, W. K., et al. (2007a). Filovirus-like particles produced in insect cells: immunogenicity and protection in rodents. *J. Infect. Dis.* 196(Suppl. 2), S421–429.

Warfield, K. L., Swenson, D. L., Olinger, G. G., Kalina, W. V., Aman, M. J., and Bavari, S. (2007b). Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. *J. Infect. Dis.* 196(Suppl. 2), S430–S437.

Warfield, K. L., Swenson, D. L., Olinger, G. G., Nichols, D. K., Pratt, W. D., Blouch, R., et al. (2006). Gene-specific countermeasures against Ebola virus based on antisense phosphorodiamidate morpholino oligomers. *PLoS Pathog.* 2:e1. doi: 10.1371/journal.ppat.0020001

Warren, T. K., Warfield, K. L., Wells, J., Swenson, D. L., Donner, K. S.,

van Tongeren, S. A., et al. (2010). Advanced antisense therapies for postexposure protection against lethal filovirus infections. *Nat. Med.* 16, 991–994. doi: 10.1038/nm.2202

Xu, L., Sanchez, A., Yang, Z., Zaki, S. R., Nabel, E. G., Nichol, S. T., et al. (1998). Immunization for Ebola virus infection. *Nat. Med.* 4, 37–42. doi: 10.1038/nm0198-037

Zaki, S. R., and Goldsmith, C. S. (1999). Pathologic features of filovirus infections in humans. *Curr. Top. Microbiol. Immunol.* 235, 97–116. doi: 10.1007/978-3-642-59949-1_7

Zhang, A. P., Bornholdt, Z. A., Liu, T., Abelson, D. M., Lee, D. E., Li, S., et al. (2012). The ebola virus interferon antagonist VP24 directly binds STAT1 and has a novel, pyramidal fold. *PLoS Pathog.* 8:e1002550. doi: 10.1371/journal.ppat.1002550

Zivcec, M., Safronetz, D., Haddock, E., Feldmann, H., and Ebihara, H. (2011). Validation of assays to monitor immune responses in the Syrian golden hamster (Mesocricetus auratus). *J. Immunol. Methods* 368, 24–35. doi: 10.1016/j.jim.2011.02.004

Zlotnik, I. (1971). "Marburg disease" The pathology of experimentally infected hamsters," in *Marburg Virus Disease*, eds G. A. Martini and R. Siegert (New York, NY: Springer, Berlin Heidelberg), 129–135.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 June 2013; paper pending published: 03 July 2013; accepted: 19 August 2013; published online: 05 September 2013.

Citation: Nakayama E and Saijo M (2013) Animal models for Ebola and Marburg virus infections. Front. Microbiol. ***4****:267. doi: 10.3389/fmicb.2013.00267*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 26 July 2013
doi: 10.3389/fmicb.2013.00212

Hepatitis C virus infection and related liver disease: the quest for the best animal model

Laurent Mailly[1], Eric Robinet[1,2], Philip Meuleman[3], Thomas F. Baumert[1,4]* and Mirjam B. Zeisel[1]*

[1] Inserm U1110, Université de Strasbourg, Strasbourg, France
[2] Institut Hospitalo-Universitaire de Strasbourg, Strasbourg, France
[3] Center for Vaccinology, Ghent University and Hospital, Ghent, Belgium
[4] Pôle Hépato-digestif, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Ikuo Shoji, Kobe University Graduate School of Medicine, Japan
Eric J. Gowans, The University of Adelaide, Australia

***Correspondence:**
Thomas F. Baumert and Mirjam B. Zeisel, Inserm U1110, Université de Strasbourg, 3 rue Kœberlé, 67000 Strasbourg, France
e-mail: thomas.baumert@unistra.fr; mirjam.zeisel@unistra.fr

Hepatitis C virus (HCV) is a major cause of cirrhosis and hepatocellular carcinoma (HCC) making the virus the most common cause of liver failure and transplantation. HCV is estimated to chronically affect 130 million individuals and to lead to more than 350,000 deaths per year worldwide. A vaccine is currently not available. The recently developed direct acting antivirals (DAAs) have markedly increased the efficacy of the standard of care but are not efficient enough to completely cure all chronically infected patients and their toxicity limits their use in patients with advanced liver disease, co-morbidity or transplant recipients. Because of the host restriction, which is limited to humans and non-human primates, *in vivo* study of HCV infection has been hampered since its discovery more than 20 years ago. The chimpanzee remains the most physiological model to study the innate and adaptive immune responses, but its use is ethically difficult and is now very restricted and regulated. The development of a small animal model that allows robust HCV infection has been achieved using chimeric liver immunodeficient mice, which are therefore not suitable for studying the adaptive immune responses. Nevertheless, these models allowed to go deeply in the comprehension of virus-host interactions and to assess different therapeutic approaches. The immunocompetent mouse models that were recently established by genetic humanization have shown an interesting improvement concerning the study of the immune responses but are still limited by the absence of the complete robust life cycle of the virus. In this review, we will focus on the relevant available animal models of HCV infection and their usefulness for deciphering the HCV life cycle and virus-induced liver disease, as well as for the development and evaluation of new therapeutics. We will also discuss the perspectives on future immunocompetent mouse models and the hurdles to their development.

Keywords: hepatitis C virus, liver disease, hepatocellular carcinoma, animal models, antivirals, immunocompetent mouse model

INTRODUCTION

Hepatitis C virus (HCV) is a small enveloped positive sense single-stranded RNA virus from the Flaviviridae family (Lindenbach et al., 2007). HCV is one of the main causative agents of liver disease worldwide and a major problem of public health with approximately 130 million chronically infected people and more than 350,000 deaths each year. Moreover, the World Health Organization estimates that three to four million people are newly infected every year. No vaccine is available so far, and the current standard of care combination treatment of interferon-alfa (IFNα) and ribavirin is not effective against all HCV genotypes and often not well tolerated by the patients (Poordad and Dieterich, 2012). The recently developed direct-acting antivirals (DAAs) have significantly improved the treatment of chronic HCV infection (Poordad and Dieterich, 2012) but can lead to the selection of DAA-resistant viral variants (Pawlotsky, 2011; Aloia et al., 2012). Thus, despite the advances made in recent years, HCV infection remains a global health problem. Furthermore, a vaccine is not available. Research efforts are thus still to be continued in order to decipher the details of the viral life cycle within its host and to propose new therapeutic alternatives to improve patient care.

Improved understanding of the phenomena related to the interaction of the virus with its host, in its entirety, relies on the use of a model allowing the study of the whole viral life cycle as well as the host responses against the virus, especially the immune responses. Only the use of a living organism allows to achieve this goal. Chimpanzees are the only animals reliably supporting HCV infection and allowing the study of anti-viral immune responses even though they do not develop fibrosis and cirrhosis and very rarely hepatocellular carcinoma (HCC; Muchmore et al., 1988; Lanford et al., 2001). However, the use of chimpanzees in medical research is ethically very controversial and increasingly limited (Harrington, 2012). To overcome this hurdle, small animal models have been developed during the last decade. Mouse

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma

models are of particular interest since mice are easy to breed and handle and can be genetically modified.

In this review, we will discuss the advantages and limitations of the different animal models regarding their potential in leading to a better comprehension of virus-host interactions and HCV pathogenesis, as well as their utility for preclinical evaluations prior translation to clinical trials in human.

NATURALLY HCV PERMISSIVE ANIMAL MODELS

Highly genetically HCV-related viruses from the hepacivirus gender can infect new world primates (Muerhoff et al., 1995; Simons et al., 1995), dogs (Kapoor et al., 2011), horses (Burbelo et al., 2012; Lyons et al., 2012) and bats (Quan et al., 2013). But so far, HCV was only found to infect few species other than human (reviewed in Simmonds, 2013), chimpanzees and non-rodent small mammal northern treeshrew (*Tupaia belangeri*).

CHIMPANZEES

Chimpanzees are tightly linked to the history of HCV discovery (Houghton, 2009). These animals can be chronically infected with the virus, using various sources as inoculum. Although the clinical course of HCV infection in chimps and humans is not identical (Bukh, 2004), chimp studies have greatly contributed to our understanding of innate and adaptive immune responses in the course of HCV infection (Bowen and Walker, 2005; Rehermann, 2009). A large body of evidence indicates that T cell responses play a major role in viral clearance as well as protection from HCV infection (Neumann-Haefelin and Thimme, 2013). Indeed, memory T cells as well as the activation of intra-hepatic natural killer (NK) cells and type I/II interferon production were demonstrated to prevent HCV re-infection in chimpanzees (Nascimbeni et al., 2003; Barth et al., 2011). Moreover, neutralizing antibodies may also contribute to protection from HCV infection in these animals.

Chimpanzees have also been very valuable for the evaluation of various antivirals and to date, chimpanzees are the only animals permitting extensive evaluation of the efficacy of potential vaccines against HCV (Houghton, 2011). It has especially been shown that therapeutic vaccines including structural proteins are better T cell stimulators than vaccines where only non-structural proteins are present (Dahari et al., 2010). But so far, it was not possible to clearly identify a safe and effective vaccine for humans (Bailey, 2010).

Despite the fact that studies of HCV infection in chimpanzees have greatly advanced our understanding of the immune responses that are required to efficiently clear viral infection, several limitations of this model have to be pointed out (**Table 1**). Indeed, data from chimpanzee studies are highly variable and difficult to interpret mainly because of the biological variability between individual animals and the small animal cohorts. Moreover, chronic infection appears in only 30–40% of infected animals while it can reach 85% in humans (Lanford et al., 2001). Furthermore, chronically infected chimpanzees do not readily develop cirrhosis or fibrosis and have much milder symptoms (Bukh et al., 2001). It is worth noting that HCC is only rarely observed in chimpanzees (Muchmore et al., 1988; Lanford et al., 2001). Finally, the limited availability, the cost for acquiring and maintaining animals as well as ethic considerations have also been major drawbacks to the use of this animal model. Indeed, the use of chimpanzees for biomedical and behavioural research is now legally forbidden in Europe since the new European Directive 2010/63 and has been recently banned by the NIH (Harrington, 2012) following the recommendations of the Institute of Medicine (Altevogt et al., 2011).

Tupaia belangeri

Tupaia belangeri, or Northern treeshrew, is a non-rodent small mammal susceptible to HCV infection (Xie et al., 1998; Xu et al.,

Table 1 | Current animal models for the study of HCV infection.

	Chimpanzee 	Tree shrew 	uPA-SCID 	FRG 	AFC8-huHSC/Hep 	Rosa26-FLuc 	Rat 
HCV entry	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HCV production	Yes	Yes	Yes	Yes	No	No	Yes
Viremia	Weaker than in humans	Low	High	High	No	No	Low
HCV pathogenesis	Milder than in humans, HCC?	Hepatitis, fibrosis, cirrhosis	No	No	Fibrosis	N/A	Hepatitis
Immune response	Yes	Yes	No	No	Yes	Yes	No
MHC match Immune system/ hepatocytes	Yes	Yes	N/A	N/A	Yes	Yes	No
MHC match Immune system/host	Yes	Yes	N/A	N/A	No	Yes	Yes
Vaccine development	Yes	Possible	No	No	Yes	No	No

HCC, hepatocellular carcinoma; N/A, not applicable.

2007; Amako et al., 2010). HCV entry factors CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN) from tupaia origin have been shown to promote entry of HCV pseudoparticles or cell culture-derived HCV (HCVcc) into human or mouse cells engineered to express these host factors and primary tupaia hepatocytes are able to support HCV infection (Zhao et al., 2002; Barth et al., 2005; Tong et al., 2011). This model has recently been used to perform a metabolomic analysis upon HCV infection (Sun et al., 2013). In this study, the authors performed comprehensive metabolomics profiling and pathway analysis of large biological data sets in order to identify signaling pathways associated with HCV infection, but the HCV infection level of the animals was not clearly stated. Another study assessed the protective effect of xanthohumol, a main prenylated chalcone from hops, on HCV-induced hepatitis (Yang et al., 2013). Even though the authors were able to detect liver injury and HCV Core protein expression in the liver of HCV inoculated animals, neither serum HCV RNA nor anti-HCV antibodies could be detected. Indeed, despite the development of chronic liver disease in some animals, the infection rate of tupaia is weak and viremia appears to be low and rarely sustained (Xu et al., 2007; Amako et al., 2010; **Table 1**). Moreover, the limited availability of these animals, their cost of housing and the absence of tupaia-specific reagents to assess HCV-host interactions in this model still limit the use of tupaia for the study of HCV pathogenesis and vaccine design. The development of tupaia-adapted viruses may be one strategy to make this model more efficient and robust.

RODENT MODELS OF HCV INFECTION

Mice and rats are naturally resistant to HCV infection as rodent hepatocytes do not support HCV entry and replication (Ploss et al., 2009; Dorner et al., 2011). It is worth noting that in contrast to the early steps of the viral life cycle, HCV assembly and release do not appear to be restricted in mouse cells (Long et al., 2011; Vogt et al., 2013). With the advent of transgenic technology different mouse models for the study of HCV infection could be engineered. Several transgenic mice carrying different parts of the HCV genome were the first available mouse models to study HCV-host interactions (reviewed in Lerat et al., 2011; Billerbeck et al., 2013). These mice display liver pathologies mimicking human disease, principally steatosis and primary liver cancer. However, in contrast to the human setting, as the mouse immune system tolerates the transgenically expressed viral proteins, liver pathogenesis establishes in the absence of local inflammation. Moreover, the absence of active HCV RNA replication in these mice precludes the study of HCV infection. Research efforts thus further focused on developing rodent models supporting productive HCV infection mainly by humanizing mice or rats to render them permissive to HCV. Humanization can be achieved by two different strategies: (i) xenografting human cells or (ii) transgenesis.

THE uPA-SCID MOUSE MODEL

These immunodeficient (SCID) mice with hepatocyte-lethal phenotype due to the overexpression of the urokinase-type plasminogen activator (uPA) transgene in their liver can be efficiently engrafted with primary human hepatocytes in order to initiate infection with HCV (**Table 1**). The uPA-SCID mouse model was for the first time described more than 10 years ago (Mercer et al., 2001). It has been shown that the liver of these chimeric mice can be nearly completely repopulated by the transplanted human hepatocytes (Tateno et al., 2004). Such human liver chimeric mice then become susceptible to HCV infection. Following viral inoculation, HCV titers of more than 10^7 IU/mL can been observed (Hiraga et al., 2007; Vanwolleghem et al., 2010) and viral infection can be sustained up to 10 months (Mercer et al., 2001). As for chimpanzees, in addition to HCV-positive patient-derived serum of different genotypes (Mercer et al., 2001; Hsu et al., 2003; Bukh et al., 2010), HCVcc may also be used to inoculate these mice (Lindenbach et al., 2006; Akazawa et al., 2013) and this thus enables researchers to study a wide variety of different inocula.

The uPA-SCID model has been extensively used to evaluate different strategies to prevent or treat HCV infection. Targeting cell entry of the virus is one of these approaches. HCV entry is a crucial step to establish infection and can be blocked by using neutralizing antibodies binding to the virions or by monoclonal antibodies (mAbs) targeting host entry factors. Studies using neutralizing antibodies purified from blood of a genotype 1a infected patient demonstrated the efficacy of this approach to inhibit viral infection with homologous and heterologous HCV strains (Vanwolleghem et al., 2008; Meuleman et al., 2011b). Another study assessed the potential of neutralizing mAbs from a phage display library constructed from the bone marrow mononuclear cell RNA of a chronically infected patient (Law et al., 2008). These antibodies have been shown to bind to the HCV envelope glycoprotein E2 and to exhibit broadly cross-neutralizing activity against heterologous HCV quasispecies in the uPA-SCID model. In addition to antibodies targeting the virus, HCV entry can also been inhibited by targeting host factors essential for this process (Zeisel et al., 2013). MAbs against the HCV entry factors CD81 (Meuleman et al., 2008) and SR-BI (Lacek et al., 2012; Meuleman et al., 2012) have been successfully tested in the chimeric uPA-SCID mouse model and were proven to be efficient at inhibiting HCV infection in challenge studies with different genotypes. Moreover, chimeric-liver mice that were already infected for 3 days could still be efficiently treated with five injections of 400 μg of anti-SR-BI mAb (Lacek et al., 2012; Meuleman et al., 2012). The uPA-SCID model has also been successfully used to assess the efficacy of small molecule drugs (Lupberger et al., 2011; Sainz et al., 2012) and various other molecules targeting host entry factors (Matsumura et al., 2009; Meuleman et al., 2011a). Indeed, administration of the clinically approved drug erlotinib, which specifically targets the tyrosine kinase activity of epidermal growth factor receptor (EGFR), significantly delayed the kinetics of HCV infection with a genotype 2a infectious serum (Lupberger et al., 2011). Another study showed that pretreatment with ezetimibe, an antagonist of the HCV co-entry factor Niemann-Pick C1-like1 (NPC1L1), could prevent infection of some chimeric mice infected with a genotype 1b virus (Sainz et al., 2012).

Moreover, the uPA-SCID mouse model has also been efficiently used to assess the efficacy of recently developed DAAs specifically targeting HCV encoded proteins required for viral replication. One of the first HCV protease inhibitor, BILN 2061, has been evaluated by Vanwolleghem et al. (2007) in this mouse model.

Although the authors could see a very rapid viral load decline of about 2.5 $\log_{10}$ after a 4-day treatment, they also observed a deteriorating effect of this compound on the mouse cardiomyocytes. This latter observation confirmed results already seen in rhesus monkeys where BILN 2061 also induced cardiotoxicity (Reiser et al., 2005). Of note, the clinical development of BILN 2061 was halted due to these toxic effects. More recently, the clinically licensed NS3-4A protease inhibitor telaprevir has been evaluated in this model, alone (Kamiya et al., 2010) or in combination with the NS5B inhibitor MK-0608 (Ohara et al., 2011). Furthermore, a study by Shi et al. (2013) evaluated the antiviral activity of different combinations of DAAs against genotype 1b, 2a, and 2b in these mice. The authors assessed the effect of the NS3 protease inhibitor BMS-605339, the NS5A inhibitor BMS-788329 and the NS5B non-nucleoside analog inhibitor BMS-821095 and showed that different combination therapies were very efficient against genotype 1b virus but not against genotype 2a or 2b strains. Quasispecies population before and after treatment as well as selection of mutations leading to the appearance of resistant variants were also analyzed by ultra deep sequencing in these animals (Shi et al., 2013). Furthermore, several other compounds with an effect on viral replication have also been tested with success as monotherapy against HCV infection (Nakagawa et al., 2007; Kneteman et al., 2009; Narjes et al., 2011).

The uPA-SCID mouse model has further been used to study the relevance of the genetic polymorphism near the interleukin-28B (IL-28B) region described to be associated with a better response to IFNα and ribavirin in patients (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009). In this study, Watanabe et al. (2012) showed that there was no significant difference in response to IFNα treatment in mice with human hepatocytes with different types of polymorphism indicating that the effect observed in patients was attributable to their immune responses, since these mice do not have an adaptive immune system.

Although the uPA-SCID mouse model has proven its usefulness and has become relevant for the preclinical evaluation of novel antiviral compounds, this model has several limitations. Indeed, these mice are very fragile and have to be engrafted within the first weeks of life given that these animals are born with a hepatocyte-lethal phenotype (Meuleman et al., 2005; Vanwolleghem et al., 2010). Moreover, the uPA transgene can be deleted in some mice leading to the restoration of a wild-type phenotype and thus loss of the human hepatocyte graft (Sandgren et al., 1991). Another drawback of this model is the absence of a functional adaptive immune system. Due to the severe combined immunodeficiency background, these mice are deficient in functional mature T and B cells. Therefore, they cannot be used for the study of adaptive immune responses or for the evaluation of vaccines. Nevertheless, this model is not devoid of innate immunity and has been used to decipher HCV specific innate immune responses (Walters et al., 2006).

The Fah$^{-/-}$ Rag2$^{-/-}$ γ-c$^{-/-}$ (FRG) MOUSE MODEL

In order to overcome some of the limitations of the uPA-SCID mouse model, another model has been developed using immunodeficient mice with genetic alterations leading to a hepatocyte-lethal phenotype. These "FRG" mice are deficient for the Rag2 recombinase and the common γ-chain of the interleukin receptors, leading to a more profound immunodeficiency (Goldman et al., 1998; Mazurier et al., 1999). Moreover, they are deficient for the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah), which leads to liver degeneration (Grompe et al., 1993, 1995; Overturf et al., 1996). In this model, liver degeneration can be prevented as long as the 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) drug is provided to the mice. Thus, the time of transplantation of human hepatocytes is easier to control than in the uPA-SCID mouse model since it can be done at anytime in adult mice upon drug withdrawal. Moreover, in contrast to the uPA-SCID mouse model, spontaneous reversion of the hepatocyte-lethal phenotype does not occur since there is a full deletion within the Fah encoding gene (Grompe et al., 1993).

Upon NTBC withdrawal, FRG mice can be efficiently transplanted with human hepatocytes to obtain liver repopulation (Azuma et al., 2007; Bissig et al., 2007). Moreover, Bissig et al. (2010) successfully infected these transplanted mice with a genotype 2a HCV JFH-1 strain, a clinical isolate of HCV genotype 1a and chimeric genotype 1a/2a and 1b/2a viruses. Up to now, this study is the only one reporting HCV infection in the FRG model. Although this chimeric liver mouse model has so far been less extensively used than the uPA-SCID model, it should be as efficient to allow preclinical evaluation of antiviral compounds (**Table 1**) and several reports using this model are expected within the next years.

Like the uPA-SCID mouse model, the FRG model suffers from a lack of an immune system. Due to the Rag2 and γ-c deficiencies, these mice do not harbor T-, B- and NK-cells. These animals are therefore not more suitable than the uPA-SCID mice for the study of HCV immunopathogenesis and for the development and evaluation of vaccines.

AFC8-huHSC/Hep MICE

Recently, another model of HCV permissive mice has been developed to overcome the lack of immunity intrinsic to the uPA-SCID and FRG models. This model is based on immunodeficient Balb/c Rag2$^{-/-}$ γ-c$^{-/-}$ (BRG) mice that have been genetically modified to overexpress in the mouse liver a fusion protein of the FK506 binding protein (FKPB) and caspase-8 under the albumin promoter (AFC8; Washburn et al., 2011). Injection of AP20187 induces the homodimerization of the caspase-8 active domain leading to a suicidal activity of this enzyme and thus death of mouse hepatocytes (Pajvani et al., 2005). This induced hepatodeficiency improves the engraftment of human hepatocytes. These transgenic mice can therefore be transplanted, within the 5 first days of life, with human hepatocyte progenitor cells and CD34^{+} hematopoietic stem cells derived from the same fetal liver allowing human leukocyte antigen (HLA) matching between these cells. Following injection of AP20187, the authors demonstrated development of human immune cells and human hepatocytes in these mice. About 50% of these AFC8-huHSC/Hep mice were subsequently effectively infected with patient serum-derived HCV of genotype 1a but HCV RNA could only be demonstrated in the liver of these animals. No HCV RNA was detected in the blood of infected mice. Despite this absence of viremia, the authors observed human immune cell infiltration in the liver of HCV^{+}

mice as well as HCV-specific CD4 and CD8 T cell responses. Specific B cell responses could not be observed in these animals. Interestingly, half of these HCV^+ mice developed severe portal fibrosis with numerous septa. This was the first report on a small animal model of HCV infection exhibiting development of HCV-specific adaptive immune responses and virally induced immunopathogenesis (**Table 1**).

Although these mice represent a first breakthrough for the development of a fully immunocompetent mouse model for the study of HCV infection, the absence of serum HCV particles and the lack of fully functional B cells are important issues for the evaluation of potential antiviral drugs and vaccines. Furthermore, the education of the immune system from human HSC is done on the murine major histocompatibility complex (MHC). It is not yet clear whether the selection of human immune cells on mouse MHC will allow proper recognition of HCV infected HLA expressing human hepatocytes and be comparable with the human setting.

Rosa26-Fluc HCV ENTRY FACTOR HUMANIZED MICE

Another model of immunocompetent mice permissive to HCV infection has been developed shortly after the AFC8-huHSC/Hep. In contrast to the previous model, these mice have a fully functional murine immune system and liver. To overcome the species-specific restriction of viral entry and to achieve HCV susceptibility of the mouse liver, the authors targeted the mouse hepatocytes *in vivo* with adenoviral vectors to express human HCV entry factors CD81, SR-BI, CLDN1, and OCLN (Dorner et al., 2011; **Figure 1**). About 5% of the murine hepatocytes expressed all four human entry factors upon adenoviral transduction. The Rosa26-Fluc background of these mice allows to detect viral entry by *in vivo* bioluminescence. Indeed, as HCV does not efficiently replicate in mouse cells (Zhu et al., 2003; Uprichard et al., 2006; Lin et al., 2010), Dorner et al. (2011) engineered the virus to express the Cre recombinase (HCV-CRE). This latter, once expressed in the mouse liver, leads to the activation of a loxP-flanked luciferase reporter in the genome of the Rosa26-Fluc mice (Safran et al., 2003). The emitted photons then reflect viral entry into hepatocytes. This model was successfully used to study for the first time entry of HCVcc chimeras of different genotypes into mouse hepatocytes *in vivo*.

The authors also assessed the efficacy of anti-CD81 and anti-E2 antibodies on HCV entry as well as a prophylactic vaccine based on recombinant vaccinia virus encoding HCV proteins from Core to NS2 of genotype 1a. With this vaccine approach, robust titers of anti-E2 antibodies and a decreased susceptibility to heterologous challenge with HCV-CRE harboring structural proteins of genotypes 1b, 2a, or 4a was observed. This is the first report of the usage of a fully immunocompetent small animal model for the study of HCV infection and, so far, this model is the only available mouse

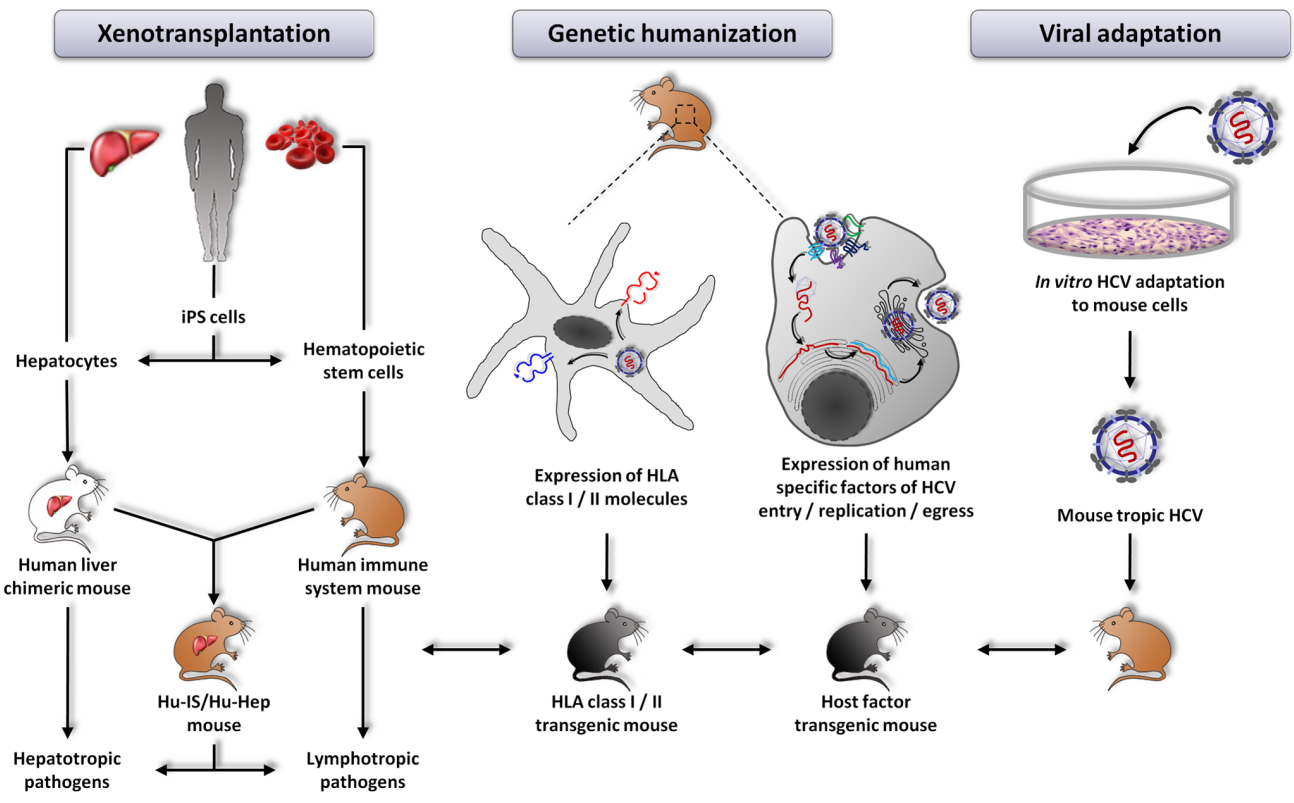


FIGURE 1 | Currently explored approaches aiming at developing new animal models for the study of hepatitis C virus infection. From left to right: xenotransplantation of human cells, genetic humanization by expression of defined human specific factors, viral adaptation of HCV to mouse cells. HLA transgenic mice can be crossed with the different models in order to improve immune responses.

model for combined immunization and challenge studies. This is also the only mouse model with a perfect MHC matching between the immune system and infected hepatocytes, which would allow a better understanding of the entire immune responses induced by the virus (**Table 1**).

Although conceptually markedly advancing the field, the humanized Rosa26-Fluc model still has its limitations. First, there is no virus production by the infected mouse hepatocytes due to inefficient viral replication in these cells. This makes this model unsuitable for the evaluation of DAAs or antivirals targeting the assembly and egress steps of the viral life cycle. Moreover, the usage of adenoviral vectors to introduce the human entry factors into mouse hepatocytes leads to the induction of an immune response against the vector. This renders the study of HCV-specific immune responses difficult since the induction of interferon stimulated genes (ISGs) and a rapid NK cell-mediated innate immunity lead to the loss of adenovirus-transduced hepatocytes and therefore to the loss of HCV infected cells. Thus, it is not possible to study HCV-induced immunopathogenesis in this model. This model is currently being extended by developing transgenic mice stably expressing the human HCV entry factors and ultimately further engineering the mouse hepatocytes to support HCV replication. A first transgenic mouse expressing human CD81, SR-BI, CLDN1, and OCLN did not appear to be susceptible to HCV entry (Hikosaka et al., 2011) but technical limitations may have precluded detection of viral entry in this study. Follow-up studies using human entry factor transgenic mice are thus awaited to assess the potential of such mice for further studying HCV pathogenesis.

IMMUNOCOMPETENT RAT MODEL

Another immunocompetent small animal model susceptible to HCV infection was generated using rats tolerant to human hepatocytes (Ouyang et al., 2001). Fetal rats were intraperitoneally injected with human hepatoma Huh7 cells between 15 and 17 days of gestation in order to induce a specific tolerance of the rat immune system toward these cells. This tolerance allows the transplantation of Huh7 cells via an intrasplenic injection in newborn rats, within 24 h of birth. This results in survival of these cells without rejection by the rat immune system as demonstrated by human albumin detection in the liver as well as in the serum of the tolerized transplanted rats (Ouyang et al., 2001). These rats may then be inoculated with serum-derived HCV of genotype 1 one week after transplantation and are able to develop transient viremia as shown by an HCV viral load of 2×10^4 copies/mL by week 12 (Wu et al., 2005). Infected animals where characterized by inflammation of the liver as demonstrated by elevated serum alanine aminotransferase with a peak at week 13 as well as by mononuclear cell infiltration in the liver (**Table 1**).

Unfortunately, the set-up of this model is difficult since the intraperitoneal injection in fetal rats is challenging. Moreover, this model is limited by the fact that the transplanted cells are not primary human hepatocytes but a human hepatoma cell line. Furthermore, the number of transplanted cells is low and viremia remains weak, around 2×10^4 copies/mL, in comparison to viral loads observed in HCV-infected patients or human liver chimeric mice. Despite the fact that this animal model is immunocompetent and can be infected with HCV, the mismatch between human HLA and rat MHC prevents the study of adaptive immune responses against infected hepatoma cells. It is worth noting that this model has not been extensively used so far and only one publication reported its usage (Wu et al., 2005). Further reports would thus be useful in order to compare the potential of this model to the potential of the different mouse models to study HCV pathogenesis and assess antiviral strategies.

FUTURE DIRECTIONS

In the past years, much progress has been made in developing novel animal models for the study of HCV-host interactions. The major drawbacks of the different small animal models described so far are (i) absent or not fully functional adaptive immunity, (ii) low-level viral replication and absent viremia and consequently (iii) absent or low fibrosis and no cirrhosis. The uPA-SCID and FRG models both lack T and B cells. The AFC8-huHSC/Hep model is characterized by a human immune system educated on murine MHC molecules and absent viremia. The Rosa26-Fluc model has matched murine immune cells and hepatocytes but these latter are unable to efficiently replicate the viral genome and to egress the virus. Finally, the immune system of the immunocompetent rat model does not match the HLA molecules at the surface of the transplanted human hepatoma cells. Thus, additional immunocompetent mouse models need to be developed to study different aspects of HCV pathogenesis in the context of robust viral replication. So, ultimately, what would be the best model to study HCV infection? Different lines of development are currently pursued: (i) adapting the virus to mice to allow the virus to accomplish its entire life cycle in mouse hepatocytes, (ii) further humanizing mice to render these animals permissive to HCV and (iii) combining these two approaches (**Figure 1**).

HCV-ADAPTED IMMUNOCOMPETENT MOUSE MODEL

Given that mouse hepatocytes are resistant to HCV infection, a possibility to overcome this species-specific restriction of viral infection without genetically engineering the host is to adapt the virus to mouse cells, i.e., develop a virus able to complete its whole life cycle - entry, translation, replication, egress – in normal mouse hepatocytes (**Figure 1**). Bitzegeio et al. (2010) have explored this trail by the selection of a HCV Jc1-derived mutant (genotype 2a) adapted to the murine entry factor CD81. In contrast to wild-type Jc1, this HCV mutant is able to enter cells expressing mouse CD81. However, despite efficiently entering mouse cells, this selected HCV strain is unable to replicate in mouse hepatocytes, indicating that host factors limit productive infection downstream of virus cell entry (Bitzegeio et al., 2010). Several lines of evidence indicate that innate immune responses interfere with HCV replication and although HCV has evolved strategies to evade innate immunity in human cells, this may be less efficient in mouse cells (Lin et al., 2010; Billerbeck et al., 2013; Schoggins and Rice, 2013; Vogt et al., 2013). So potentially the selection of a mouse innate immune response-insensitive HCV strain might overcome the limitations imposed by mouse hepatocytes and lead to the development of viremia in immunocompetent mice. However, even though such a model might be achievable, it will remain to be determined to which extent it would be comparable to HCV infection in humans.

In order to mimic more precisely the human immune response, it might be of interest to use HLA expressing mice (Pajot et al., 2004), in line with a recent study where hepatitis B virus genome was introduced into the liver of HLA-A2/DR1 mice (Dion et al., 2013). However, the main limitation of such an approach is the fact that it will be very difficult to mimic the variability of HLA combinations present in humans. Thus, efforts should focus on defined HLA transgene combinations. Nevertheless, there is no certainty that the mouse immune responses developed against the virus would be comparable to those observed in human (Mestas and Hughes, 2004).

Therefore, another strategy to generate a fully immunocompetent mouse model for the study of HCV infection is to focus on the development of mice with humanized immune system and humanized liver (hu-IS/hu-Hep mice).

HUMAN IMMUNE SYSTEM – HUMAN HEPATOCYTE CHIMERIC MOUSE MODEL

The optimal way to assess the role of the immune system in response to HCV infection and to explore virus-induced immunopathogenesis in a setting comparable to humans would be to use mice harboring both human immune cells and human hepatocytes (**Figure 1**). The study by Washburn et al. (2011) described above paved the road to the development of such an animal model. However, the selection of human immune cells on murine MHC instead of HLA molecules may have precluded the development of very efficient T and B cell responses. An alternative approach would thus be to use hepatodeficient and immunodeficient HLA-expressing mice in order to allow the engraftment of HLA matched human hematopoietic stem cells (hHSC) and human hepatocytes. The selection of immune cells on HLA molecules and the recognition of the hepatocytes as being from self might give a better view of the immune response against HCV and allow the design of efficient vaccines and new therapeutic products.

Unfortunately, the development of all hematopoietic compartments from hHSC in mice is not efficient because of the inability of several mouse cytokines to stimulate human cells (Manz, 2007; Legrand et al., 2009). In order to ameliorate the immune reconstitution from hHSC, several approaches have been attempted using injection of exogenous recombinant cytokines or by creating transgenic or knock-in mice (reviewed in Willinger et al., 2011). However, to achieve a complete human immune reconstitution, several different human cytokines and growth factors would be necessary. Moreover, several mouse strains with different genetic backgrounds reject transplanted human cells because of the inefficient interaction between human CD47 and the mouse signal-regulatory protein alpha (SIRPα) expressed on macrophages (Takenaka et al., 2007). This inability of human CD47 to interact with mouse SIRPα leads to activation of the phagocytic activity of macrophages (Takizawa and Manz, 2007). In line with this observation, it has been shown that human progenitor cells expressing mouse CD47 can efficiently be engrafted in BRG mice leading to a better homeostasis of T- and NK-cells in lymphoid organs (Legrand et al., 2011). Similarly, the same BRG background, which is one of the most efficient to allow hHSC transplantation, has been used to create human SIRPα transgenic mice in order to improve engraftment of hHSC (Strowig et al., 2011). In order to avoid genetic manipulations and selection procedures of the cells to be transplanted, which can be in limited number at the time of the graft, other genetic backgrounds have been investigated for their efficiency to accept xenogenic transplantations. This lead to the selection of the non-obese diabetic (NOD) background which appears to be the best recipient for hHSC transplantation. Indeed, this strain exhibits a polymorphism of the gene encoding SIRPα, allowing a more efficient binding to human CD47 (Takenaka et al., 2007; Takizawa and Manz, 2007; Yamauchi et al., 2013). Therefore, the NOD background may be more suitable to introduce xenogenic cells without the need of genetic modification in order to achieve high degree of chimerism (reviewed in Ito et al., 2008). The recent advances in the field of induced pluripotent stem cells (iPS) may allow in the future to develop a mouse model engrafted with iPS-derived hepatocytes and HSC from the same donor (Espejel et al., 2010; Huang et al., 2011; Liu et al., 2011; Sekiya and Suzuki, 2011; Schwartz et al., 2012; Wu et al., 2012). This might overcome the issues of HLA-matching between the immune system and hepatocytes but will not resolve HLA matching with the host. However, this strategy using iPS cells, which could be available in large amount, would be easier to implement than the use of hHSC and fetal hepatocytes which are more complicated to access due to ethical reasons.

The hu-IS/hu-Hep mouse model would certainly be the best model to assess the immune responses against HCV, to decipher more deeply the immunopathogenesis developed during chronic infection, to explore HCV-host interactions during acute infection and to unravel the mechanisms leading to virus eradication as well as to develop vaccines and new therapeutic approaches.

GENETICALLY HUMANIZED MOUSE MODEL

Another explored approach in order to develop an immunocompetent mouse model of HCV infection relies on genetic modifications of mice by introducing essential human specific factors for the viral life cycle (**Figure 1**). It has previously been shown that viral entry, the first step of HCV infection, requires the presence of at least two human cell surface factors, CD81 and OCLN (Ploss et al., 2009). The study by Dorner et al. (2011) described above has shown that adenoviral expression of human entry factors in mouse liver enables viral entry into murine hepatocytes *in vivo*. HCV RNA replication thus appears to be the next and last essential step to overcome in mouse cells in order to reconstitute the entire viral life cycle since mouse cells are able to support viral assembly and egress (Long et al., 2011). Indeed, it has been shown that viral RNA is translated in mouse cells but is unable to replicate efficiently (McCaffrey et al., 2002; Dorner et al., 2011). It is worth noting that several studies have shown that HCV replicons can replicate in murine cell lines (Zhu et al., 2003; Uprichard et al., 2006; Frentzen et al., 2011), indicating that there are no dominant murine inhibitory factors implied in the low replication of HCV in mouse cells and that murine orthologs of host factor required for viral replication are able to participate in the full life cycle of HCV. The activation of mouse innate immune responses may thus most likely be responsible for the limited HCV replication in mouse cells (Schoggins and Rice, 2013). Indeed, it has been shown that inactivation of several antiviral cellular molecules involved

in innate immunity enhances HCV replication and allows HCV production by mouse cells (Chang et al., 2006; Lin et al., 2010; Aly et al., 2011; Vogt et al., 2013). It might thus be wise to ascertain *in vivo* the relevance of these findings and to generate a mouse model devoid of some of these innate immune pathways or knock-in mice expressing human orthologs of these innate immune mediators.

CONCLUSIONS AND PERSPECTIVES

Since the development of the first small animal model of HCV infection – the uPA-SCID mouse model (Mercer et al., 2001), other increasingly sophisticated models emerged (Wu et al., 2005; Bissig et al., 2010; Dorner et al., 2011; Washburn et al., 2011). Each of these models allowed to significantly advance our understanding of defined aspects of HCV infection and HCV-host interactions and to pave the way for future animal models combining different characteristics and advantages of each model. Although the uPA-SCID mouse model has been most intensively used as a preclinical model in order to assess different classes of antivirals, none of the current models prevails over the others with respect to analysis of all aspects of viral infection (virus life cycle, immune response, pathogenesis, vaccine development. . .). The combination of different technologies and efforts will ultimately lead to the development of additional models better suited for the study of HCV immunopathogenesis and vaccine development. Given the natural history of HCV infection, requiring decades to evolve toward an HCC, one may consider that obtaining HCV-induced cirrhosis and HCC will be highly challenging in rodents, whose life expectancy is around 2 years. However, numerous models of HCC based on the transgenic expression of HCV proteins have been published (reviewed in Billerbeck et al., 2013), suggesting that HCV infection-induced HCC may be achievable, provided that sufficient host-responses are generated. As the chimpanzee model has to be abandoned in favor of small rodent models, the well known genetics of the mouse and the ease of modification of its genome should put this animal first in line to become the next gold standard for HCV research. Fully mouse or half human, the possibilities remain open. The complementarities of both approaches will raise new perspectives in the field of animal research for HCV and for the development of new therapeutic alternatives. The quest for the "holy Grail" is on, but the road is still long and full of pitfalls.

ACKNOWLEDGMENTS

The authors acknowledge financial support of their work by the European Union (ERC-2008-AdG-233130-HEPCENT and INTERREG-IV-FEDER-Hepato-Regio-Net 2009 and 2012), Laboratoire d'Excellence HepSYS (Investissement d'Avenir; ANR-10-LAB-28), Institut Hospitalo-Universitaire Mix-Surg, ANRS (2012/239, 2012/318, 2013/108), Inserm, University of Strasbourg and the Strasbourg University Hospitals, France, the Research Foundation Flanders (FWO project #3G052112), the Ghent University (GOA #01G01712), the Belgian state (IUAP P7/47-HEPRO2) and the European Union (FP7-HepaMab).

REFERENCES

Akazawa, D., Moriyama, M., Yokokawa, H., Omi, N., Watanabe, N., Date, T., et al. (2013). Neutralizing antibodies induced by cell culture-derived hepatitis C virus protect against infection in mice. *Gastroenterology* doi: 10.1053/j.gastro.2013.05.007 [Epub ahead of print].

Aloia, A. L., Locarnini, S., and Beard, M. R. (2012). Antiviral resistance and direct-acting antiviral agents for HCV. *Antivir. Ther.* 17, 1147–1162. doi: 10.3851/IMP2426

Altevogt, B. M., Pankevich, D. E., Shelton-Davenport, M. K., and Kahn, J. P.. (2011). *Chimpanzees in Biomedical and Behavioral Research: Assessing the Necessity*. Washington, DC: The National Academies Press.

Aly, H. H., Oshiumi, H., Shime, H., Matsumoto, M., Wakita, T., Shimotohno, K., et al. (2011). Development of mouse hepatocyte lines permissive for hepatitis C virus (HCV). *PLoS ONE* 6:e21284. doi: 10.1371/journal.pone.0021284

Amako, Y., Tsukiyama-Kohara, K., Katsume, A., Hirata, Y., Sekiguchi, S., Tobita, Y., et al. (2010). Pathogenesis of hepatitis C virus infection in *Tupaia belangeri*. *J. Virol.* 84, 303–311. doi: 10.1128/JVI.01448-09

Azuma, H., Paulk, N., Ranade, A., Dorrell, C., Al-Dhalimy, M., Ellis, E., et al. (2007). Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/- mice. *Nat. Biotechnol.* 25, 903–910. doi: 10.1038/nbt1326

Bailey, J. (2010). An assessment of the use of chimpanzees in hepatitis C research past, present and future: 1. Validity of the chimpanzee model. *Altern. Lab. Anim.* 38, 387–418.

Barth, H., Cerino, R., Arcuri, M., Hoffmann, M., Schurmann, P., Adah, M. I., et al. (2005). Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. *J. Virol.* 79, 5774–5785. doi: 10.1128/JVI.79.9.5774-5785.2005

Barth, H., Rybczynska, J., Patient, R., Choi, Y., Sapp, R. K., Baumert, T. F., et al. (2011). Both innate and adaptive immunity mediate protective immunity against hepatitis C virus infection in chimpanzees. *Hepatology* 54, 1135–1148. doi: 10.1002/hep.24489

Billerbeck, E., De Jong, Y., Dorner, M., De La Fuente, C., and Ploss, A. (2013). Animal models for hepatitis C. *Curr. Top. Microbiol. Immunol.* 369, 49–86. doi: 10.1007/978-3-642-27340-7_3

Bissig, K. D., Le, T. T., Woods, N. B., and Verma, I. M. (2007). Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20507–20511. doi: 10.1073/pnas.0710528105

Bissig, K. D., Wieland, S. F., Tran, P., Isogawa, M., Le, T. T., Chisari, F. V., et al. (2010). Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *J. Clin. Invest.* 120, 924–930. doi: 10.1172/JCI40094

Bitzegeio, J., Bankwitz, D., Hueging, K., Haid, S., Brohm, C., Zeisel, M. B., et al. (2010). Adaptation of hepatitis C virus to mouse CD81 permits infection of mouse cells in the absence of human entry factors. *PLoS Pathog.* 6:e1000978. doi: 10.1371/journal.ppat.1000978

Bowen, D. G., and Walker, C. M. (2005). Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436, 946–952. doi: 10.1038/nature04079

Bukh, J. (2004). A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology* 39, 1469–1475. doi: 10.1002/hep.20268

Bukh, J., Forns, X., Emerson, S. U., and Purcell, R. H. (2001). Studies of hepatitis C virus in chimpanzees and their importance for vaccine development. *Intervirology* 44, 132–142. doi: 10.1159/000050040

Bukh, J., Meuleman, P., Tellier, R., Engle, R. E., Feinstone, S. M., Eder, G., et al. (2010). Challenge pools of hepatitis C virus genotypes 1-6 prototype strains: replication fitness and pathogenicity in chimpanzees and human liver-chimeric mouse models. *J. Infect. Dis.* 201, 1381–1389. doi: 10.1086/651579

Burbelo, P. D., Dubovi, E. J., Simmonds, P., Medina, J. L., Henriquez, J. A., Mishra, N., et al. (2012). Serology-enabled discovery of genetically diverse hepaciviruses in a new host. *J. Virol.* 86, 6171–6178. doi: 10.1128/JVI.00250-12

Chang, K. S., Cai, Z., Zhang, C., Sen, G. C., Williams, B. R., and Luo, G. (2006). Replication of hepatitis C virus (HCV) RNA in mouse embryonic fibroblasts: protein kinase R (PKR)-dependent and PKR-independent mechanisms for controlling HCV RNA replication and mediating interferon activities. *J. Virol.* 80, 7364–7374. doi: 10.1128/JVI.00586-06

Dahari, H., Feinstone, S. M., and Major, M. E. (2010). Meta-analysis of hepatitis C virus vaccine efficacy in chimpanzees indicates an importance for structural proteins. *Gastroenterology* 139, 965–974. doi: 10.1053/j.gastro.2010.05.077

Dion, S., Bourgine, M., Godon, O., Levillayer, F., and Michel, M. L. (2013). Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2

and HLA-DR1 molecules. *J. Virol.* 87, 5554–5563. doi: 10.1128/JVI.03134-12

Dorner, M., Horwitz, J. A., Robbins, J. B., Barry, W. T., Feng, Q., Mu, K., et al. (2011). A genetically humanized mouse model for hepatitis C virus infection. *Nature* 474, 208–211. doi: 10.1038/nature10168

Espejel, S., Roll, G. R., Mclaughlin, K. J., Lee, A. Y., Zhang, J. Y., Laird, D. J., et al. (2010). Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. *J. Clin. Invest.* 120, 3120–3126. doi: 10.1172/JCI43267

Frentzen, A., Huging, K., Bitzegeio, J., Friesland, M., Haid, S., Gentzsch, J., et al. (2011). Completion of hepatitis C virus replication cycle in heterokaryons excludes dominant restrictions in human non-liver and mouse liver cell lines. *PLoS Pathog.* 7:e1002029. doi: 10.1371/journal.ppat.1002029

Ge, D., Fellay, J., Thompson, A. J., Simon, J. S., Shianna, K. V., Urban, T. J., et al. (2009). Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461, 399–401. doi: 10.1038/nature08309

Goldman, J. P., Blundell, M. P., Lopes, L., Kinnon, C., Di Santo, J. P., and Thrasher, A. J. (1998). Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *Br. J. Haematol.* 103, 335–342. doi: 10.1046/j.1365-2141.1998.00980.x

Grompe, M., Al-Dhalimy, M., Finegold, M., Ou, C. N., Burlingame, T., Kennaway, N. G., et al. (1993). Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes Dev.* 7, 2298–2307. doi: 10.1101/gad.7.12a.2298

Grompe, M., Lindstedt, S., Al-Dhalimy, M., Kennaway, N. G., Papaconstantinou, J., Torres-Ramos, C. A., et al. (1995). Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. *Nat. Genet.* 10, 453–460. doi: 10.1038/ng0895-453

Harrington, M. (2012). State of the (research) chimp. *Lab Anim. (NY)* 41, 31. doi: 10.1038/laban0212-31a

Hikosaka, K., Noritake, H., Kimura, W., Sultana, N., Sharkar, M. T., Tagawa, Y., et al. (2011). Expression of human factors CD81, claudin-1, scavenger receptor, and occludin in mouse hepatocytes does not confer susceptibility to HCV entry. *Biomed. Res.* 32, 143–150. doi: 10.2220/biomedres.32.143

Hiraga, N., Imamura, M., Tsuge, M., Noguchi, C., Takahashi, S., Iwao, E., et al. (2007). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett.* 581, 1983–1987. doi: 10.1016/j.febslet.2007.04.021

Houghton, M. (2009). The long and winding road leading to the identification of the hepatitis C virus. *J. Hepatol.* 51, 939–948. doi: 10.1016/j.jhep.2009.08.004

Houghton, M. (2011). Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunol. Rev.* 239, 99–108. doi: 10.1111/j.1600-065X.2010.00977.x

Hsu, E. C., Hsi, B., Hirota-Tsuchihara, M., Ruland, J., Iorio, C., Sarangi, F., et al. (2003). Modified apoptotic molecule (BID) reduces hepatitis C virus infection in mice with chimeric human livers. *Nat. Biotechnol.* 21, 519–525. doi: 10.1038/nbt817

Huang, P., He, Z., Ji, S., Sun, H., Xiang, D., Liu, C., et al. (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature.* 475, 386–389. doi: 10.1038/nature10116

Ito, M., Kobayashi, K., and Nakahata, T. (2008). NOD/Shi-scid IL2rgamma(null) (NOG) mice more appropriate for humanized mouse models. *Curr. Top. Microbiol. Immunol.* 324, 53–76. doi: 10.1007/978-3-540-75647-7_3

Kamiya, N., Iwao, E., Hiraga, N., Tsuge, M., Imamura, M., Takahashi, S., et al. (2010). Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor. *J. Gen. Virol.* 91, 1668–1677. doi: 10.1099/vir.0.019315-0

Kapoor, A., Simmonds, P., Gerold, G., Qaisar, N., Jain, K., Henriquez, J. A., et al. (2011). Characterization of a canine homolog of hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11608–11613. doi: 10.1073/pnas.1101794108

Kneteman, N. M., Howe, A. Y., Gao, T., Lewis, J., Pevear, D., Lund, G., et al. (2009). HCV796: a selective nonstructural protein 5B polymerase inhibitor with potent anti-hepatitis C virus activity in vitro, in mice with chimeric human livers, and in humans infected with hepatitis C virus. *Hepatology* 49, 745–752. doi: 10.1002/hep.22717

Lacek, K., Vercauteren, K., Grzyb, K., Naddeo, M., Verhoye, L., Slowikowski, M. P., et al. (2012). Novel human SR-BI antibodies prevent infection and dissemination of HCV in vitro and in humanized mice. *J. Hepatol.* 57, 17–23. doi: 10.1016/j.jhep.2012.02.018

Lanford, R. E., Bigger, C., Bassett, S., and Klimpel, G. (2001). The chimpanzee model of hepatitis C virus infections. *ILAR J.* 42, 117–126. doi: 10.1093/ilar.42.2.117

Law, M., Maruyama, T., Lewis, J., Giang, E., Tarr, A. W., Stamataki, Z., et al. (2008). Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat. Med.* 14, 25–27. doi: 10.1038/nm1698

Legrand, N., Huntington, N. D., Nagasawa, M., Bakker, A. Q., Schotte, R., Strick-Marchand, H., et al. (2011). Functional CD47/signal regulatory protein alpha (SIRP(alpha)) interaction is required for optimal human T- and natural killer- (NK) cell homeostasis in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13224–13229. doi: 10.1073/pnas.1101398108

Legrand, N., Ploss, A., Balling, R., Becker, P. D., Borsotti, C., Brezillon, N., et al. (2009). Humanized mice for modeling human infectious disease: challenges, progress, and outlook. *Cell Host Microbe* 6, 5–9. doi: 10.1016/j.chom.2009.06.006

Lerat, H., Higgs, M., and Pawlotsky, J. M. (2011). Animal models in the study of hepatitis C virus-associated liver pathologies. *Expert Rev. Gastroenterol. Hepatol.* 5, 341–352. doi: 10.1586/egh.11.14

Lin, L. T., Noyce, R. S., Pham, T. N., Wilson, J. A., Sisson, G. R., Michalak, T. I., et al. (2010). Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. *J. Virol.* 84, 9170–9180. doi: 10.1128/JVI.00559-10

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., Mckeating, J. A., et al. (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3805–3809. doi: 10.1073/pnas.0511218103

Lindenbach, B. D., Thiel, H. J., and Rice, C. M. (2007). "Flaviviridae: the viruses and their replication," *Fields Virology*, 5th Edition, eds D. M. Knipe and P. M. Howley (Philadelphia: Lippincott-Raven Publishers), 1101–1152.

Liu, H., Kim, Y., Sharkis, S., Marchionni, L., and Jang, Y. Y. (2011). In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Sci. Transl. Med.* 3, 82ra39. doi: 10.1126/scitranslmed.3002376

Long, G., Hiet, M. S., Windisch, M. P., Lee, J. Y., Lohmann, V., and Bartenschlager, R. (2011). Mouse hepatic cells support assembly of infectious hepatitis C virus particles. *Gastroenterology* 141, 1057–1066. doi: 10.1053/j.gastro.2011.06.010

Lupberger, J., Zeisel, M. B., Xiao, F., Thumann, C., Fofana, I., Zona, L., et al. (2011). EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat. Med.* 17, 589–595. doi: 10.1038/nm.2341

Lyons, S., Kapoor, A., Sharp, C., Schneider, B. S., Wolfe, N. D., Culshaw, G., et al. (2012). Nonprimate hepaciviruses in domestic horses, United kingdom. *Emerg. Infect. Dis.* 18, 1976–1982. doi: 10.3201/eid1812.120498

Manz, M. G. (2007). Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity* 26, 537–541. doi: 10.1016/j.immuni.2007.05.001

Matsumura, T., Hu, Z., Kato, T., Dreux, M., Zhang, Y. Y., Imamura, M., et al. (2009). Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. *Gastroenterology* 137, 673–681. doi: 10.1053/j.gastro.2009.04.048

Mazurier, F., Fontanellas, A., Salesse, S., Taine, L., Landriau, S., Moreau-Gaudry, F., et al. (1999). A novel immunodeficient mouse model – RAG2 x common cytokine receptor gamma chain double mutants – requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J. Interferon Cytokine Res.* 19, 533–541. doi: 10.1089/107999099313983

McCaffrey, A. P., Ohashi, K., Meuse, L., Shen, S., Lancaster, A. M., Lukavsky, P. J., et al. (2002). Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice. *Mol. Ther.* 5, 676–684. doi: 10.1006/mthe.2002.0600

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., et al. (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* 7, 927–933. doi: 10.1038/90968

Mestas, J., and Hughes, C. C. (2004). Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172, 2731–2738.

Meuleman, P., Albecka, A., Belouzard, S., Vercauteren, K., Verhoye, L., Wychowski, C., et al. (2011a). Griffithsin has antiviral activity against hepatitis C virus. *Antimicrob. Agents Chemother.* 55, 5159–5167. doi: 10.1128/AAC.00633-11

Meuleman, P., Bukh, J., Verhoye, L., Farhoudi, A., Vanwolleghem, T., Wang, R. Y., et al. (2011b). In vivo evaluation of the cross-genotype neutralizing activity of polyclonal antibodies against hepatitis C virus. *Hepatology* 53, 755–762. doi: 10.1002/hep.24171

Meuleman, P., Catanese, M. T., Verhoye, L., Desombere, I., Farhoudi, A., Jones, C. T., et al. (2012). A human monoclonal antibody targeting scavenger receptor class B type I precludes hepatitis C virus infection and viral spread in vitro and in vivo. *Hepatology* 55, 364–372. doi: 10.1002/hep.24692

Meuleman, P., Hesselgesser, J., Paulson, M., Vanwolleghem, T., Desombere, I., Reiser, H., et al. (2008). Anti-CD81 antibodies can prevent a hepatitis C virus infectionin vivo. *Hepatology* 48, 1761–1768. doi: 10.1002/hep.22547

Meuleman, P., Libbrecht, L., De Vos, R., De Hemptinne, B., Gevaert, K., Vandekerckhove, J., et al. (2005). Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* 41, 847–856. doi: 10.1002/hep.20657

Muchmore, E., Popper, H., Peterson, D. A., Miller, M. F., and Lieberman, H. M. (1988). Non-A, non-B hepatitis-related hepatocellular carcinoma in a chimpanzee. *J. Med. Primatol.* 17, 235–246.

Muerhoff, A. S., Leary, T. P., Simons, J. N., Pilot-Matias, T. J., Dawson, G. J., Erker, J. C., et al. (1995). Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. *J. Virol.* 69, 5621–5630.

Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., and Kohara, M. (2007). Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. *Biochem. Biophys. Res. Commun.* 353, 882–888. doi: 10.1016/j.bbrc.2006.12.117

Narjes, F., Crescenzi, B., Ferrara, M., Habermann, J., Colarusso, S., Ferreira Mdel, R., et al. (2011). Discovery of (7R)-14-cyclohexyl-7-{[2-(dimethylamino)ethyl](methyl)amino}-7,8-dihydro-6H-indolo[1,2-e][1,5]benzoxazocine-11-carboxylic acid (MK-3281), a potent and orally bioavailable finger-loop inhibitor of the hepatitis C virus NS5B polymerase. *J. Med. Chem.* 54, 289–301. doi: 10.1021/jm1013105

Nascimbeni, M., Mizukoshi, E., Bosmann, M., Major, M. E., Mihalik, K., Rice, C. M., et al. (2003). Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. *J. Virol.* 77, 4781–4793. doi: 10.1128/JVI.77.8.4781-4793.2003

Neumann-Haefelin, C., and Thimme, R. (2013). Adaptive immune responses in hepatitis C virus infection. *Curr. Top. Microbiol. Immunol.* 369, 243–262. doi: 10.1007/978-3-642-27340-7_10

Ohara, E., Hiraga, N., Imamura, M., Iwao, E., Kamiya, N., Yamada, I., et al. (2011). Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice. *J. Hepatol.* 54, 872–878. doi: 10.1016/j.jhep.2010.08.033

Ouyang, E. C., Wu, C. H., Walton, C., Promrat, K., and Wu, G. Y. (2001). Transplantation of human hepatocytes into tolerized genetically immunocompetent rats. *World J. Gastroenterol.* 7, 324–330.

Overturf, K., Al-Dhalimy, M., Tanguay, R., Brantly, M., Ou, C. N., Finegold, M., et al. (1996). Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. *Nat. Genet.* 12, 266–273. doi: 10.1038/ng0396-266

Pajot, A., Michel, M.-L., Fazilleau, N., Pancré, V., Auriault, C., Ojcius, D. M., et al. (2004). A mouse model of human adaptive immune functions:HLA-A2.1-/HLA-DR1-transgenic H-2 classI-/classII-knockout mice. *Eur. J. Immunol.* 34, 3060–3069.

Pajvani, U. B., Trujillo, M. E., Combs, T. P., Iyengar, P., Jelicks, L., Roth, K. A., et al. (2005). Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. *Nat. Med.* 11, 797–803. doi: 10.1038/nm1262

Pawlotsky, J.-M. (2011). Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology* 53, 1742–1751. doi: 10.1002/hep.24262

Ploss, A., Evans, M. J., Gaysinskaya, V. A., Panis, M., You, H., De Jong, Y. P., et al. (2009). Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457, 882–886. doi: 10.1038/nature07684

Poordad, F., and Dieterich, D. (2012). Treating hepatitis C: current standard of care and emerging direct-acting antiviral agents. *J. Viral Hepat.* 19, 449–464. doi: 10.1111/j.1365-2893.2012.01617.x

Quan, P. L., Firth, C., Conte, J. M., Williams, S. H., Zambrana-Torrelio, C. M., Anthony, S. J., et al. (2013). Bats are a major natural reservoir for hepaciviruses and pegiviruses. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8194–8199. doi: 10.1073/pnas.1303037110

Rehermann, B. (2009). Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J. Clin. Invest.* 119, 1745–1754. doi: 10.1172/JCI39133

Reiser, M., Hinrichsen, H., Benhamou, Y., Reesink, H. W., Wedemeyer, H., Avendano, C., et al. (2005). Antiviral efficacy of NS3-serine protease inhibitor BILN-2061 in patients with chronic genotype 2 and 3 hepatitis C. *Hepatology* 41, 832–835. doi: 10.1002/hep.20612

Safran, M., Kim, W. Y., Kung, A. L., Horner, J. W., Depinho, R. A., and Kaelin, W. G. Jr. (2003). Mouse reporter strain for noninvasive bioluminescent imaging of cells that have undergone Cre-mediated recombination. *Mol. Imaging* 2, 297–302. doi: 10.1162/153535003322750637

Sainz, B., Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., et al. (2012). Identification of the Niemann-Pick C1–like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat. Med.* 18, 281–285. doi: 10.1038/nm.2581

Sandgren, E. P., Palmiter, R. D., Heckel, J. L., Daugherty, C. C., Brinster, R. L., and Degen, J. L. (1991). Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. *Cell* 66, 245–256. doi: 10.1016/0092-8674(91)90615-6

Schoggins, J. W., and Rice, C. M. (2013). Innate immune responses to hepatitis C virus. *Curr. Top. Microbiol. Immunol.* 369, 219–242. doi: 10.1007/978-3-642-27340-7_9

Schwartz, R. E., Trehan, K., Andrus, L., Sheahan, T. P., Ploss, A., Duncan, S. A., et al. (2012). Modeling hepatitis C virus infection using human induced pluripotent stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2544–2548. doi: 10.1073/pnas.1121400109

Sekiya, S., and Suzuki, A. (2011). Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature* 475, 390–393. doi: 10.1038/nature10263

Shi, N., Hiraga, N., Imamura, M., Hayes, C. N., Zhang, Y., Kosaka, K., et al. (2013). Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice. *Gut* 62, 1055–1061. doi: 10.1136/gutjnl-2012-302600

Simmonds, P. (2013). The origin of hepatitis C virus. *Curr. Top. Microbiol. Immunol.* 369, 1–15. doi: 10.1007/978-3-642-27340-7_1

Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., et al. (1995). Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. U.S.A.* 92, 3401–3405. doi: 10.1073/pnas.92.8.3401

Strowig, T., Rongvaux, A., Rathinam, C., Takizawa, H., Borsotti, C., Philbrick, W., et al. (2011). Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13218–13223. doi: 10.1073/pnas.1109769108

Sun, H., Zhang, A., Yan, G., Piao, C., Li, W., Sun, C., et al. (2013). Metabolomic analysis of key regulatory metabolites in hepatitis C virus-infected tree shrews. *Mol. Cell. Proteomics* 12, 710–719. doi: 10.1074/mcp.M112.019141

Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M. L., et al. (2009). IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* 41, 1100–1104. doi: 10.1038/ng.447

Takenaka, K., Prasolava, T. K., Wang, J. C., Mortin-Toth, S. M., Khalouei, S., Gan, O. I., et al. (2007). Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nat. Immunol.* 8, 1313–1323. doi: 10.1038/ni1527

Takizawa, H., and Manz, M. G. (2007). Macrophage tolerance: CD47-SIRP-alpha-mediated signals matter. *Nat. Immunol.* 8, 1287–1289. doi: 10.1038/ni1207-1287

Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., et al. (2009). Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* 41, 1105–1109. doi: 10.1038/ng.449

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., et al. (2004). Near completely humanized liver in mice shows human-type metabolic responses to

drugs. *Am. J. Pathol.* 165, 901–912. doi: 10.1016/S0002-9440(10)63352-4

Tong, Y., Zhu, Y., Xia, X., Liu, Y., Feng, Y., Hua, X., et al. (2011). Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. *J. Virol.* 85, 2793–2802. doi: 10.1128/JVI.01818-10

Uprichard, S. L., Chung, J., Chisari, F. V., and Wakita, T. (2006). Replication of a hepatitis C virus replicon clone in mouse cells. *Virol. J.* 3, 89. doi: 10.1186/1743-422X-3-89

Vanwolleghem, T., Bukh, J., Meuleman, P., Desombere, I., Meunier, J. C., Alter, H., et al. (2008). Polyclonal immunoglobulins from a chronic hepatitis C virus patient protect human liver-chimeric mice from infection with a homologous hepatitis C virus strain. *Hepatology* 47, 1846–1855. doi: 10.1002/hep.22244

Vanwolleghem, T., Libbrecht, L., Hansen, B. E., Desombere, I., Roskams, T., Meuleman, P., et al. (2010). Factors determining successful engraftment of hepatocytes and susceptibility to hepatitis B and C virus infection in uPA-SCID mice. *J. Hepatol.* 53, 468–476. doi: 10.1016/j.jhep.2010.03.024

Vanwolleghem, T., Meuleman, P., Libbrecht, L., Roskams, T., De Vos, R., and Leroux-Roels, G. (2007). Ultra-rapid cardiotoxicity of the hepatitis C virus protease inhibitor BILN 2061 in the urokinase-type plasminogen activator mouse. *Gastroenterology* 133, 1144–1155. doi: 10.1053/j.gastro.2007.07.007

Vogt, A., Scull, M. A., Friling, T., Horwitz, J. A., Donovan, B. M., Dorner, M., et al. (2013). Recapitulation of the hepatitis C virus life-cycle in engineered murine cell lines. *Virology* doi: 10.1016/j.virol.2013.05.036 [Epub ahead of print].

Walters, K. A., Joyce, M. A., Thompson, J. C., Smith, M. W., Yeh, M. M., Proll, S., et al. (2006). Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: role of the innate antiviral immune response. *PLoS Pathog.* 2:e59. doi: 10.1371/journal.ppat.0020059

Washburn, M. L., Bility, M. T., Zhang, L., Kovalev, G. I., Buntzman, A., Frelinger, J. A., et al. (2011). A humanized mouse model to study hepatitis C virus infection, immune response, and liver disease. *Gastroenterology* 140, 1334–1344. doi: 10.1053/j.gastro.2011.01.001

Watanabe, T., Sugauchi, F., Tanaka, Y., Matsuura, K., Yatsuhashi, H., Murakami, S., et al. (2012). Hepatitis C virus kinetics by administration of pegylated interferon-alpha in human and chimeric mice carrying human hepatocytes with variants of the IL28B gene. *Gut* doi: 10.1136/gutjnl-2012-302553 [Epub ahead of print].

Willinger, T., Rongvaux, A., Strowig, T., Manz, M. G., and Flavell, R. A. (2011). Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. *Trends Immunol.* 32, 321–327. doi: 10.1016/j.it.2011.04.005

Wu, G. Y., Konishi, M., Walton, C. M., Olive, D., Hayashi, K., and Wu, C. H. (2005). A novel immunocompetent rat model of HCV infection and hepatitis. *Gastroenterology* 128, 1416–1423. doi: 10.1053/j.gastro.2005.03.015

Wu, X., Robotham, J. M., Lee, E., Dalton, S., Kneteman, N. M., Gilbert, D. M., et al. (2012). Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. *PLoS Pathog.* 8:e1002617. doi: 10.1371/journal.ppat.1002617

Xie, Z. C., Riezu-Boj, J. I., Lasarte, J. J., Guillen, J., Su, J. H., Civeira, M. P., et al. (1998). Transmission of hepatitis C virus infection to tree shrews. *Virology* 244, 513–520. doi: 10.1006/viro.1998.9127

Xu, X., Chen, H., Cao, X., and Ben, K. (2007). Efficient infection of tree shrew (*Tupaia belangeri*) with hepatitis C virus grown in cell culture or from patient plasma. *J. Gen. Virol.* 88, 2504–2512. doi: 10.1099/vir.0.82878-0

Yamauchi, T., Takenaka, K., Urata, S., Shima, T., Kikushige, Y., Tokuyama, T., et al. (2013). Polymorphic Sirpa is the genetic determinant for NOD-based mouse lines to achieve efficient human cell engraftment. *Blood* 121, 1316–1325. doi: 10.1182/blood-2012-06-440354

Yang, M., Li, N., Li, F., Zhu, Q., Liu, X., Han, Q., et al. (2013). Xanthohumol, a main prenylated chalcone from hops, reduces liver damage and modulates oxidative reaction and apoptosis in hepatitis C virus infected *Tupaia belangeri*. *Int. Immunopharmacol.* 16, 466–474. doi: 10.1016/j.intimp.2013.04.029

Zeisel, M. B., Lupberger, J., Fofana, I., and Baumert, T. F. (2013). Host-targeting agents for prevention and treatment of chronic hepatitis C – perspectives and challenges. *J. Hepatol.* 58, 375–384. doi: 10.1016/j.jhep.2012.09.022

Zhao, X., Tang, Z. Y., Klumpp, B., Wolff-Vorbeck, G., Barth, H., Levy, S., et al. (2002). Primary hepatocytes of Tupaia belangeri as a potential model for hepatitis C virus infection. *J. Clin. Invest.* 109, 221–232.

Zhu, Q., Guo, J. T., and Seeger, C. (2003). Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *J. Virol.* 77, 9204–9210. doi: 10.1128/JVI.77.17.9204-9210.2003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 June 2013; accepted: 08 July 2013; published online: 26 July 2013.

Citation: Mailly L, Robinet E, Meuleman P, Baumert TF and Zeisel MB (2013) Hepatitis C virus infection and related liver disease: the quest for the best animal model. Front. Microbiol. ***4****:212. doi: 10.3389/fmicb.2013.00212*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 11 October 2013
doi: 10.3389/fmicb.2013.00305

Can non-human primates serve as models for investigating dengue disease pathogenesis?

Kristina B. Clark[1], Nattawat Onlamoon[2], Hui-Mien Hsiao[1], Guey C. Perng[1,3,4] and Francois Villinger[1]*

[1] *Department of Pathology and Laboratory Medicine, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA, USA*
[2] *Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[3] *Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan, Taiwan*
[4] *Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan*

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Youichi Suzuki, National University of Singapore, Singapore
Hirofumi Akari, Kyoto University, Japan

***Correspondence:**
Francois Villinger, Yerkes National Primate Research Center, Emory University, 954 Gatewood Rd, Atlanta, GA 30329, USA
e-mail: fvillin@emory.edu

Dengue Virus (DV) infects between 50 and 100 million people globally, with public health costs totaling in the billions. It is the causative agent of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), vector-borne diseases that initially predominated in the tropics. Due to the expansion of its mosquito vector, *Aedes* spp., DV is increasingly becoming a global problem. Infected individuals may present with a wide spectrum of symptoms, spanning from a mild febrile to a life-threatening illness, which may include thrombocytopenia, leucopenia, hepatomegaly, hemorrhaging, plasma leakage and shock. Deciphering the underlining mechanisms responsible for these symptoms has been hindered by the limited availability of animal models that can induce classic human pathology. Currently, several permissive non-human primate (NHP) species and mouse breeds susceptible to adapted DV strains are available. Though virus replication occurs in these animals, none of them recapitulate the cardinal features of human symptomatology, with disease only occasionally observed in NHPs. Recently our group established a DV serotype 2 intravenous infection model with the Indian rhesus macaque, which reliably produced cutaneous hemorrhages after primary virus exposure. Further manipulation of experimental parameters (virus strain, immune cell expansion, depletion, etc.) can refine this model and expand its relevance to human DF. Future goals include applying this model to elucidate the role of pre-existing immunity upon secondary infection and immunopathogenesis. Of note, virus titers in primates *in vivo* and *in vitro*, even with our model, have been consistently 1000-fold lower than those found in humans. We submit that an improved model, capable of demonstrating severe pathogenesis may only be achieved with higher virus loads. Nonetheless, our DV coagulopathy disease model is valuable for the study of select pathomechanisms and testing DV drug and vaccine candidates.

Keywords: dengue virus, disease pathogenesis, non-human primate, hemorrhage, platelet, bone marrow, platelet-lymphocyte aggregate, rhesus macaque

INTRODUCTION

Dengue Virus (DV), the causative agent of dengue fever (DF), is the most important vector-borne human pathogen, infecting between 50 and 100 million people annually (Who, 2012). Moreover, DF is an escalating human problem that is increasingly spreading across the globe and extending in seasonality. This recent growth is attributed to the expansion in the niche of the virus-transmitting vectors, primarily *Aedes albopictus* and *Aedes aegypti* (Who, 2011). Thanks to the lack of vector control, increased human travel and global warming, DF, once considered a tropical disease, may reach a worldwide distribution.

The majority of DV infections are asymptomatic or mild, but for about a quarter of infected people, disease may present as an illness that is indistinguishable from other febrile diseases or as DF with minor hemorrhagic abnormalities, bone pain, decreases in platelet counts and leucopenia, the most common form of disease. Rarely, people present with the severe forms—dengue hemorrhagic fever (DHF) in which patients display hematomas with a marked thrombocytopenia or extremely low platelet counts and dengue shock syndrome (DSS), a disease similar to DHF but including plasma leakage/heme concentration, pleural effusion and the increased risk of multi-organ failure (Who and Tdr, 2009). Other symptoms (abnormal bleeding, melena, hepatomegaly, vomiting, etc.) have also been reported (Cobra et al., 1995). The majority of severe DHF/DSS cases in endemic countries occur in healthy adolescents 10–24 years of age (Tsai et al., 2012). Early identification of the causative agent and immediate hydration therapy with extensive monitoring of symptoms is important for resolving symptoms and preventing fatal outcomes (Who and Tdr, 2009). There is currently no targeted therapy to modulate disease severity of those most vulnerable.

It has been surmised that factors such as genetic susceptibility, developmental stage, environmental exposures and immune system programming induced by previous infections may predispose young adults to more severe disease (Halstead et al., 2007). Epidemiological data obtained from endemic countries reveal

that DHF/DSS most often occurs in people with a secondary antibody response, which has led many to champion the antibody-dependent enhancement (ADE) of infection hypothesis (Endy et al., 2004; Fox et al., 2011). ADE proponents believe that weakly specific, cross-reacting antibodies facilitate virus entry into permissive cells, increasing titers and thus, disease. Though some ADE proponents suggest that dengue-specific antibody increases immunopathology without necessarily enhancing virus replication (Markoff et al., 1991; Lei et al., 2001; Oishi et al., 2003). On the contrary, many reports have failed to demonstrate an association of DHF/DSS with secondary infection (Murgue et al., 1999, 2000; Cordeiro et al., 2007; Guilarde et al., 2008; Libraty et al., 2009; Meltzer et al., 2012). A better association may exist between virus titers and disease severity (Murgue et al., 2000; Libraty et al., 2002). Despite the uncertainty over ADE, it is required that this potential risk factor be considered during the formulation of all vaccines under development (Who, 2011). Standard preventative modalities incorporate representative antigens of each serotype in effort to simultaneously induce protection to all four DV strains.

In the past, vaccines were designed without an exact understanding of the mechanism(s) responsible for disease pathogenesis; this was done by selecting for candidates that reduced viremia and elicited strong antibody responses (Cox, 1953; Togo, 1964). Unfortunately this approach has failed with DV, a pathogen that does not elicit strong humoral immunity in natural infections. Neutralizing antibody to DV can be elicited in a variety of primates (chimpanzees, cynomolgus macaques, African green monkeys, etc.) after primary infection, but they are often weak and short-lived (Scherer et al., 1978; Bernardo et al., 2008; Martin et al., 2009). In addition, protection from viremia has been reported in rhesus macaques that develop poor neutralizing antibody titers (Scott et al., 1980; Putnak et al., 1996) and after the response waned (Raviprakash et al., 2000). Interestingly, some evidence suggests that humans may also be protected from disease during high viremia without ever developing specific antibodies (Stramer et al., 2012; Perng and Chokephaibulkit, 2013); these observations raise concern that neutralizing antibody quantification is not the best approach to evaluate vaccine efficacy.

A more thorough understanding of the mechanisms contributing to disease and protection in humans is clearly needed to accelerate progress toward better drug and vaccine candidates. Severe disease is known to arise after the clearance of viremia, suggesting that DHF/DSS and lethality are more likely immune than viral-mediated (Who and Tdr, 2009). In fact, immune activities elicited via antibodies (Saito et al., 2004), complement (Avirutnan et al., 2006) and T cells (Green et al., 1999) have been associated with disease in human studies. Importantly, the delay in severe disease presentation until late after infection limits our ability to interrogate early events that set the stage for immunopathogenesis. Thrombocytopenia, plasma leakage, and coagulation abnormalities appear to be the critical phenomena to prevent in patients, but the events preceding these phenomena have been incompletely elucidated. Carefully controlled experiments performed in relevant animal models are needed to explore the dynamics of hematological dysfunction and other factors potentially involved in dengue disease. Unfortunately an adequate animal model that is capable of recapitulating human disease is largely unavailable.

DEVELOPMENT OF DV INFECTION ANIMAL MODEL SYSTEMS

The search for animal model systems began in the early 1900s, far before the availability of cell culture techniques to propagate or quantify virus stocks. Pathogens had to be amplified in animals that were permissive and quantified by mortality studies. Unfortunately none of the animals tested (hamster, mouse, rat, lizard, etc.) ever displayed signs of disease, limiting the progress in studying DV (Simmons et al., 1931). The research that was conducted often involved virus propagation in human volunteers, who suffered from typical DF (Simmons et al., 1931). Eventually, a young suckling mouse model inoculated intracranially with DV that displayed mild disease was developed (Sabin and Schlesinger, 1945). This model was quite limited, with paralysis observed only after 3–4 weeks in 10–20% of the mice, but this provided a starting point for virus adaptation and lead to the first small animal infection model.

MOUSE MODEL

There are a number of mouse breeds that have been employed in DV investigations–wildtype, engrafted-SCID, AG129, RAG-hu, and the NOD/SCID/IL-2Rγ/human CD34 transplant or humanized mouse (Lin et al., 1998; Kuruvilla et al., 2007; Zhang et al., 2007; Mota and Rico-Hesse, 2011; Zompi et al., 2011). AG129 mice have been the most commonly utilized strain; they are highly susceptible to dengue, replicate virus to high titers and display vascular leakage (Shresta et al., 2006; Zompi and Harris, 2012). The NOD/SCID/IL-2Rγ mice reconstituted with human CD34+ cells are infrequently used but have the greatest potential as future mouse models. These animals demonstrate several symptoms of human disease (fever, erythema, thrombocytopenia) (Mota and Rico-Hesse, 2011; Cox et al., 2012).

However, the symptomatology observed with inbred, immune-compromised mice differs from that seen in humans, likely because of the susceptibility of various cell lineages and the extensive differences in immune system dynamics (Nussenblatt et al., 2010). AG129 mice predominantly display neurological symptoms and splenomegaly (Schul et al., 2007; Zompi and Harris, 2012) and engrafted-SCID mice present with paralysis (Zompi and Harris, 2012). While the humanized mouse may be the closest to replicating patient pathology, there still remain a few caveats to using this model. Challenges involved in humanized mouse preparation and data interpretation are compounded by the considerable mouse-to-mouse variation observed (Akkina et al., 2011). Additionally this mouse model, with murine stroma and endothelium, cannot completely mimic the immune response of humans. A number of mechanisms suspected to play critical roles in dengue pathology are differentially regulated in these mice. Processes that are dependent on stromal cell interactions, such as B lymphocyte maturation and specific antibody production (Akkina, 2013), and involve endothelial microparticle signaling, such as the coagulation cascade (Mairuhu et al., 2003; Lynch, 2007), may unfold differently in these mice and lead

to alternative outcomes. The human CD34+ engrafted mouse model system can provide a great starting point in interpreting important biological processes involved in human DV disease but results will still need to be confirmed in non-human primate (NHP) species.

NON-HUMAN PRIMATE (NHP) MODELS

It has been hypothesized that the close genetic relationship between primates and humans and the presence of a comparable immune responses make NHPs the best models for studying DV. While this may be, NHPs have been particularly unreliable at modeling DV pathology, producing mild symptoms at best (Scherer et al., 1972; Halstead et al., 1973b). Monkeys thus far appear to be incapable of succumbing to life-threatening DV disease. However, several Old and New World primate species are in fact permissive to experimental DV infection (Scherer et al., 1978; Schiavetta et al., 2003; Onlamoon et al., 2010; Yoshida et al., 2012). A recently published review detailed the characteristics of viremia in many of these species (Hanley et al., 2013). **Table 1** summarizes the pathology and immunopathology observed thus far in ∼20 NHP species from 15 different genera.

The most consistent pathological finding in these animals have been lymphadenopathy of the inguinal and auxiliary lymph nodes (Halstead et al., 1973a; Marchette et al., 1973; Scherer et al., 1978; Schiavetta et al., 2003). In one species, *Chlorocebus aethiops sabaeus*, the absence of lymphomegaly (Martin et al., 2009) and in a few reports, splenomegaly (a rare symptom in humans) were noted (Scherer et al., 1978; Schiavetta et al., 2003). Fever is a valid parameter to assess, but its recording in DV-infected primates is logistically difficult, and is therefore rarely reported (Scherer et al., 1972). NHPs in general have higher body temperatures and greater variability than human bodies (Scherer et al., 1972; Fuller et al., 1985), so unless readings are measured on awake animals by telemetry, the anesthesia used profoundly alters the body's temperature, making accurate readings impossible (Baker et al., 1976). Another human dengue symptom, cutaneous rashes, are not commonly observed in primates but may be underreported; also tourniquet tests are never performed on primates to assess capillary fragility. Behavioral changes, like lethargy, have been documented in only a few studies (Chandler and Rice, 1923; Scherer et al., 1978; Schiavetta et al., 2003). In general, primates kept and bred in captivity rarely display overt disease.

Despite the low incidence of pathology observed in these studies, dengue infections in primates share many characteristics with human disease. The onset and duration of viremia is similar to humans, or about 3–6 days starting from the second day after inoculation (Freire et al., 2007; Koraka et al., 2007). Leucopenia has been observed (Onlamoon et al., 2010). Thrombocytopenia has never been captured in NHPs, likely because of their naturally high platelet counts, but moderate platelet decreases have been document in *M. mulatta* (Halstead et al., 1973a; Onlamoon et al., 2010). A DV-induced reduction of dengue-specific antibodies during the early phases of secondary homologous infection, a phenomenon observed in viremic patients, has been seen in marmosets (Omatsu et al., 2011). The anti-dengue antibodies that are elicited in primates are highly cross-reactive against other closely related flaviviruses (Scherer et al., 1978). DV infection of monkeys elicits a vigorous innate response (Sariol et al., 2007) leading to activation and marked shifts in circulating subsets of T, NK, and NK-T cells in the marmoset model (Yoshida et al., 2013). The role of DV specific cell-mediated responses in NHP models has received relatively less attention, although some studies reported recognition of non-structural proteins in addition to viral components by both CD4+ and CD8+ T cells (Koraka et al., 2007; Mladinich et al., 2012). However, such responses have been difficult to detect in immunized monkeys, even in those that show protection from challenge (Chen et al., 2007; Porter et al., 2012).

The similarities observed in these studies imply that primates may present with more suitable symptoms than mouse models upon further manipulation. A comparison of the benefits to using the NHP and murine animal models is given (**Table 2**). Several strategies to improve the NHP model may be explored—for instance increasing the number of permissive cells or altering the immune environment. Here we discuss boosting viremia with different virus delivery strategies.

VIRUS DELIVERY

Only a limited number of studies have attempted determining the infectious dose delivered during natural dengue infection. One study suggests the amount of DV transmitted by *A. aegypti* ranges from 1×10^4 to 1×10^5 (Gubler and Rosen, 1976). However, there are disagreements over the best methods to conduct such studies; the controversial points include mosquito species, generation number, feeding strategy, infection method, incubation temperature and length, virus strain and technique used to quantify transmitted virus. All these variables have the potential to affect the infection dynamics and alter the conclusions of the study (Chamberlain et al., 1954; Grimstad et al., 1980; Mellink, 1982; Watts et al., 1987; Colton et al., 2005; Smith et al., 2005). Some studies have suggested levels as high as $1 \times 10^{8.7}$ genome equivalents or almost 1×10^7 PFUs can be transmitted, though rarely (Colton et al., 2005; Styer et al., 2007). Currently we know as few as 1000 PFUs can cause viremia and disease symptoms in humans (Sun et al., 2013). Ultimately the natural inoculum dose is more suggestive of the amount of virus needed for continual DV transmission *in vivo* and does not necessarily reflect the quantity required for disease induction. Viremia levels and disease may be less dependent on inoculum size and more contingent on host-pathogen interactions. These matters should be considered when modeling DV infection in animals.

Virus delivery to the proper tissues is important for inducing the appropriate interactions with the host and promoting disease presentation. DV deposition is believed to occur exclusively by direct inoculation into the subcutaneous layer by mosquitoes. However, the subcutaneous infection route does not promote adequate virus dissemination (Marchette et al., 1973; Pamungkas et al., 2011). Potentially the virus is restricted by less frequent encounters with migrating cells and immobilization by attachment to extracellular matrix proteins (Anez et al., 2009). Consider that mosquito feeding involves the probing of all layers of skin, including the cutaneous layer and capillaries, to find a blood meal. These tissues are an integral part of the arbovirus-vector lifecycle and are frequently evaluated in transmission studies (Chamberlain et al., 1954; Styer et al., 2007). Virus injected

Table 1 | Summary of *in vivo* DV studies.

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Macaca mulatta	iv, sc	ND	ND	Humans	ND	ND	ND	No disease, leucopenia (Lavinder and Francis, 1914)
Macaca cyclopis	sc, iv, ip	ND	ND	Humans	ND	ND	ND	No disease (Koizumi and Tonomura, 1917)
Macaca mulatta	NI	ND	ND	Humans	ND	Yes	ND	Animal chilly and morose, rash on chin, and throat (Chandler and Rice, 1923)
Macaca fascicularis	sc	ND	ND	Humans	ND	Yes	ND	First to demonstrate unquestionably that some primates were permissive to DV
Cercopithecus callitrichus		ND	ND	Humans	ND	Yes	ND	infection but that they are asymptomatic
Papio spp.		ND	ND	Humans	ND	No	ND	
Cercocebus spp.		ND	ND	Humans	ND	No	ND	(Blanc et al., 1929)
Macaca mulatta	sc, mi	ND	ND	Humans, mosquitoes	ND	No	ND	
Macaca fascicularis philippinensis	sc, mi, ic	ND	ND	Humans, mosquitoes	ND	Yes	ND	No fever, some leukopenia and lymphocytosis, demonstrated transmission of DV from primates to humans through mosquitoes
*Macaca fascicularis fusca**	sc, mi	ND	ND	Humans, mosquitoes	ND	Yes	ND	(Simmons et al., 1931)
*Pan troglodytes**	sc, id	Hawaiian	NI	Human	ND	Yes	ND	Mild fever (101°F) (Paul et al., 1948)
Homo sapiens	id	NI	NI	Human	1[d]	Yes	+	Low dose gave multiple patterns of disease: (1) unmodified attack, (2) short febrile illness without rash or 3) no illness but partial immunity
	id				10[d]	Yes	+	Progression of symptoms:(1) edema and erythema, (2) fever, (3) maculopapular eruptions with sparing at the site of the original skin lesion
	into scars				Conc. human serum	Yes	+	Unmodified dengue
	eye				2E5[d]	Yes	+	Typical dengue
	eye				1E4[d]	No	−	No disease or immunity
	in				1E6[d]	Yes	+	Unmodified dengue or mild rash
	in				1E4[d]	No	−	No disease or immunity (Sabin, 1952)

(Continued)

Table 1 | Continued

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Cebus capucinus	sc or ip	Hawaiian, NGC	DV1, DV2	Human	ND	Yes	+	No overt signs of illness
Ateles geoffroyi						Yes	+	
Ateles fusciceps						Yes	+	
Alouatta palliata						Yes	+	
*Callithrix geoffroyi**						Yes	ND	
Saimiri oerstedii						Yes	ND	
Aotus trivirgatus						Yes	ND	(Rosen, 1958)
Hylobates lar	sc	BKM725-67	DV1	LLC-MK2	800	Yes	+	Fever and hemorrhagic manifestations occurred but were associated with acute
		BKM1179-67	DV1		800			Lymphomatous leukemia, no correlation between antibody titers to
		BKM1749	DV2		1.6E3			DV and protection from viremia
		24969	DV3		6.6E2			
		KS168-68	DV4		5E3			(Whitehead et al., 1970)
Saimiri sciureus	sc	Hawaii	DV1	Mice	1E6.4[e]	Yes	+	Some fever in DV1 infection,
		16007	DV1	LLC-MK2	1E5.7	Yes	–	No platelet, hematocrit or leukocyte count changes
		NGC	DV2	Mice	1E6.7[e]	Yes	+	
		NGC	DV2	mosquitoes	1E2.5	Yes	+	
		16681	DV2	LLC-MK2	1E5.5	Yes	–	
		Pak-20	DV3	LLC-MK2	1E3.4	Yes	50	
		16562	DV3	LLC-MK2	1E5.7	Yes	–	
		4328S	DV4	LLC-MK2	1E3.9	No	–	
Saguinus oedipus	sc	Hawaii	DV1	Mice	1E6.4[e]	Yes	+	Brief fever in DV1 infection
		NGC	DV2	Mice	1E6.7[e]	Yes	+	
		NGC	DV2	Mosquitoes	1E2.5	Yes	+	
		H87	DV3	Mice	1E5.8	Yes	–	
		Pak-20	DV3	LLC-MK2	1E3.4	Yes	–	
		H241	DV4	Mice	1E6.6[e]	No	–	
Saimiri sciureus	in	Hawaii	DV1	Mice	1E6.4[e]	Yes	ND	No disease reported
		NGC	DV2	Mice	1E5[e]	No	ND	
		NGC	DV2	mosquitoes	1E2.5	No	ND	
		Pak-20	DV3	LLC-MK2	1E2.1	No	ND	
		H-241	DV4	Mice	1E6.6[e]	No	ND	

(Continued)

Table 1 | Continued

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Saguinus oedipus	in	NGC	DV1	Mice	1E5.3[e]	Yes	+	
		H87	DV3	Mice	1E6.2[e]	Yes	ND	
		Pak-20	DV3	LLC-MK2	1E2.7	No	ND	
		H241	DV4	Mice	1E5.6[e]	No	ND	
Aotus trivirgatus	in	Hawaii	DV1	Mice	1E5.7[e]	No	ND	
		NGC	DV2	Mice	1E6.6[e]	Yes	ND	
		Pak-20	DV3	LLC-MK2	1E2.1	Yes	ND	(Scherer et al., 1972)
Macaca mulatta (Indian)	sc	16007	DV1	LLC-MK2	5E5	Yes	1.7E3	Lymphadenopathy in DV1, 2, & 4, rare hemorrhaging in DV1& 4, leucopenia
		16681	DV2	LLC-MK2	5E5	Yes	4.8E2	In DV2 & 4, lymphocytosis was common
		16562	DV3	LLC-MK2	5E5	Yes	+	Thrombocytopenia in 21–33% of animals with all serotypes
		4328S	DV4	LLC-MK2	5E5	Yes	2.8E2	Complement decreases in secondary DV2, no change in behavior, eating or prothrombin
Macaca	sc, id	16007	DV1	LLC-MK2	NI	Yes	–	No disease
fascicularis		16681	DV2		NI	Yes	–	
*fascicularis**		16562	DV3		NI	Yes	–	
		4328S	DV4		NI	Yes	–	
Chlorocebus	sc, id	16007	DV1	LLC-MK2	1E5	Yes	+	No disease
*aethiops**		16681	DV2		1E5	Yes	+	
		16562	DV3		1E4.5	Yes	+	
Erythrocebus	sc, id	16007	DV1	LLC-MK2	NI	Yes	+	No disease
patas		16681	DV2		1E5	Yes	+	
		16562	DV3		1E4.5	Yes	–	
		4328S	DV4		1E3.3	Yes	–	(Halstead et al., 1973a,b)
Macaca mulatta	sc	16007	DV1	LLC-MK2	1.2E5	Yes	350	Lymphadenopathy, virus distribution after sc injection indicated that most virus did not move far from the inoculation site, day after viremia virus was distributed widely throughout skin (Marchette et al., 1973)
		16681	DV2		2E6	Yes	443	
		16562	DV3		1E5	Yes	40	
		4328S	DV4		1E6	Yes	1085	
Pan troglodytes	id, sc	49313	DV1	Mosquitoes	1E3.1	Yes	1E6.6[g]	Nasal discharges and lymphadenopathy
		NC38	DV2	Humans	1E3.6	Yes	1E5.6[g]	Symptoms found in individual animals
		49080	DV3	Mosquitoes	1E2.7	Yes	1E5.2[g]	Splenomegaly, leucopenia
		17111	DV4	Mosquitoes	1E2.8	Yes	1E6[g]	Hemorrhage, shaking chill, lethargy (Scherer et al., 1978)

(Continued)

Table 1 | Continued

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Macaca mulatta	sc	16681	DV2	LLC-MK2	1E5	Yes	1E5.7	Cyclophosphamide treatment caused chronic infection, 3/9 died, internal hemorrhaging, enlarged kidney, severe acute proliferative glomerulonephritis, pleural effusion, passively transferred antibody aided viral clearance (Marchette et al., 1980)
Macaca mulatta	sc	PR-159	DV2	FRhL	5.6	Yes	ND	No disease
		H-241	DV4		1.44			(Kraiselburd et al., 1985)
Macaca mulatta & Macaca fascicularis	is, im, it	16007	DV1	PDK	2.5E5	Yes	ND	Mild neurovirulence (Angsubhakorn et al., 1987)
Aotus nancymae	sc	Western Pacific 74	DV1	NI	2E4	Yes	+	Pathology more pronounced in DV1, mild leucopenia, changes in attitude and appetite
		S16803	DV2					Changes in fecal consistency, 2/20 became lethargic
		CH53489	DV3					Common symptoms: lymphadenopathy, nasal discharges and splenomegaly (Schiavetta et al., 2003)
		341750	DV4					
Aotus	sc	IQT6152	DV1	NI	1E4	Yes	+	No disease
nancymae		IQT2124	DV2				−	
		OBS8041	DV2				+	(Kochel et al., 2005)
Macaca	sc	60305	DV1	Vero	1E5	Yes	1E1.6	No disease
mulatta		16007	DV1	Vero	1E5	Yes	1E2.4	
		16007	DV1	C6/36	1E5	Yes	1E1.9	
		40247	DV2	C6/36	1E5	Yes	1E3.6	
		44/2	DV2	Vero	1E5	Yes	1E2.9	
		H87	DV3	Vero	1E5	Yes	1E2.7	
		16562	DV3	Vero	1E5	No	−	
		74886	DV3	C6/36	1E5[f]	Yes	1E2.2	(Freire et al., 2007)
Macaca fascicularis	sc	40514	DV1	NI	1E6.4[f]	Yes	400[f]	No disease, characterized T-cell and neut antibody cross-reactivity, no changes in
		28128	DV4		1E6.2[f]		20[f]	IFN-γ, TNFα, IL4, IL8, IL10 transcription during infection (Koraka et al., 2007)
Macaca mulatta	sc	Western Pacific 74	DV1	NI	1E4	Yes	ND	No disease, increases in AST, transcriptional upregulation of

(Continued)

Table 1 | Continued

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
								ISGs, OASs, Mxs, etc., no increases in cytokine gene expression (Sariol et al., 2007)
Chlorocebus aethiops sabaeus	sc	SB8553	DV2	NI	1E6	Yes	+	No fever or lymphomegaly, no changes in behavior or weight, no respiratory, digestive or nervous system disturbances, lower inoculum titers gave prolonged viremia and better neut antibody responses (Martin et al., 2009)
Macaca mulatta (Indian)	iv	16681	DV2	Vero	1E7	Yes	~8E3	Consistent hemorrhaging in 9/9 animals, decline in platelet count and leucopenia, elevated thrombin-antithrombin, D-dimers, ALT, and CK, no increases in hematocrit, prothrombin or activated PTT (Onlamoon et al., 2010)
Callithrix jacchus	sc	02–17/1	DV1	C6/36	3.5E7	Yes	5E5[h]	No disease
		DHF0663	DV2		6.7E7		1.6E7[h]	Found differing NK, NKT, and niave effector memory and central T-cell kinetics during DV infection with different strains
		DSS1403	DV3		4.5E6		5.5E4[h]	
		05-40/1	DV4		1.5E6		2.5E4[h]	
		Jam/77/07	DV2		1.2E5		2.8E6[h]	
		Mal/77/08	DV2		1.9E5		9.6E6[h]	(Omatsu et al., 2011; Yoshida et al., 2013)
Homo sapiens	sc	45AZ5	DV1	FRhL	2E3	Yes	+	CD8+T-cell-dervied IFN-γ associated with protection from fever and viremia, sIL-R2α correlated with disease onset and severity, PBMC-derived TNF-α, IL-2, 4, 5, 10 did not correlate with protection or disease (Gunther et al., 2011; Sun et al., 2013)
		CH53489	DV3	FRhL	1E5			
Macaca nemestrina	sc	98900645	DV3	C6/36	1E7-1E8	Yes	62.94	Inoculation route influenced virus-tissue distribution
	id						47.98	Minimal hepatitis
	iv						58.62	(Pamungkas et al., 2011)
Saguinus midas and Saguinus labiatus	sc	DHF0663	DV2	C6/36	6.7E7	Yes	2.7E6[h]	No disease, CD16+ NK cell depletion did not alter virus replication or pathogenesis
	iv						2E7[h]	(Yoshida et al., 2012)

(Continued)

Table 1 | Continued

Primate	Route	Strain	Type	Virus stock[a]	Dose[b]	Infected	Viremia[c]	Findings (source)
Macaca mulatta (Indian)	sc	NGC	DV2	NI	1E5	Yes	257	Day 14 PI showed the highest levels in T-cell activation, Anti-NS1, 3, & 5 T-cell responses were characterized (Mladinich et al., 2012)
Macaca mulatta (Chinese)	iv, sc	16681	DV2	Vero	1E7	Yes	+	Hemorrhaging in 50% of iv inoculated primates (unpublished)

*[a] Cell type or organism in which DV stock was propagated; [b] Highest inoculum dose is given when there were variable doses; [c] Titers given when available; [d] HID; [e] MLD50 or MLD50/ml; [f] TCID50 or TCID50/ml; [g] MID50/ml; [h] RNA/ml; +/−, indicates presence or absence of viremia, ic, intracardial; mi, mosquito inoculation; iv, intravenous; sc, subcutaneous; id, intradermal, ip, intraperitoneal; in, intranasal; im, intramuscular; is, intraspinal; it, intrathalmic; NI, not indicated; ND, not determined; MID50, mosquito infectious dose 50; TCID50, tissue culture infectious dose 50; MLD50, suckling mouse intracranial lethal dose 50; HID, human minimal infectious dose; *indicates species name change.*

Table 2 | Relative advantages in using primate and murine model systems to study DV disease.

	Primate models	Murine models
Ease of use/cost	−	+
Susceptibility to human DV strains	+	−
Mimic human viremia	(+) reduced	+
Mimic human immune responses	+	−
MODEL HUMAN DISEASE		
Fever	−	CD34-engrafted humanized mouse
Hemorrhages	Indian rhesus monkey	CD34-engrafted humanized mouse, C57BL/6
Platelet count reduction	Indian rhesus monkey	CD34-engrafted humanized mouse
Hepatomegaly	−	Balb/c
Pleural effusion	−	−
CNS disease*	−	+
DHF/DSS	−	−
Lethality	−	+

*+, commonly present; −, absent; *Rarely observed in human dengue infections.*

directly into these tissues have better access to and faster dissemination throughout the body, affording the virus more opportunities to rapidly reach distant target cells (Pamungkas et al., 2011). Additionally, pathology induction is likely promoted by rapid viral dissemination and replication in distant cells and organs. This assumption led us to hypothesize that an intravenous infection strategy would favor wide dissemination and allow for rapid simultaneous replication of virus in various tissues, invoking a more pronounced innate immune response, potentially reflective of the human immune environment during high viremia. Although the kinetics of viremia did not markedly differ between subcutaneous and intravenous DV2 infection (Onlamoon et al., 2010; Omatsu et al., 2011), it will be critical to delineate the overall kinetics of DV dissemination to and replication in various tissues and how this relates to the induction of symptoms.

RHESUS MACAQUE MODEL OF COAGULOPATHY

Only a few NHP dengue investigations have reported rashes post-infection (PI) (Lavinder and Francis, 1914; Halstead et al., 1973b; Onlamoon et al., 2010). In most of these studies, hemorrhaging was a rare event. However, our group reported a reproducible coagulopathy disease model in the Indian rhesus macaque when 9 out of 9 monkeys inoculated intravenously with 1×10^7 PFUs of DV2 (16681) displayed evidence of subcutaneous hemorrhage (Onlamoon et al., 2010). The viremia noted in these animals remained at the high end of the range typically reported in other NHP studies and were reached relatively consistently at early time points PI.

The most prominent symptoms observed in our studies with the Indian rhesus macaque were cutaneous hemorrhages, starting at Days 3 and 4 PI and lasting as long as 10 days (**Figure 1A**) (Onlamoon et al., 2010). In a pilot study using Chinese rhesus macaques, disease presentation with the same virus was more modest, suggesting that these NHPs may be less susceptible to disease. Large hematomas developed in only one of the two primates infected intravenously with DV2 (**Figure 1B**).

The dynamics of various leukocyte subsets were followed longitudinally PI. Similar to human dengue, these animals experienced the typical leucopenia or a modest but consistent decrease in white blood cells that reached a nadir at Day 7 PI, but returned to normal levels by Day 10 (Onlamoon et al., 2010). Platelets also modestly decreased until Day 3, corresponding to the time of peak DV RNA load (Noisakran et al., 2012). While these leukocyte values did not fall out-of-range for macaques the changes were clearly noticeable and consistent. There was also a modest decrease in hematocrit, which resolved with the clearance of viremia at Day 7, in spite of continuous blood and bone marrow (BM) draws (Onlamoon et al., 2010).

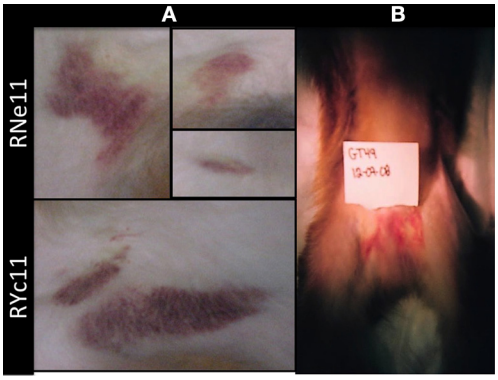


FIGURE 1 | Hematomas are seen in intravenously inoculated rhesus macaques. (A) Indian rhesus macaques were injected intravenously with 1×10^7 PFUs of DV2 16681 as previously reported (Onlamoon et al., 2010). Hematomas of various degrees of severity were present on Days 3 till 14 PI. Prominent ecchymoses were visible in two young male animals, RNell and RYc11, on Day 7. **(B)** Four Chinese rhesus macaques were injected intravenously ($n = 2$) or subcutaneously ($n = 2$) with 1×10^7 PFUs of DV2 16681 strain. Hemorrhaging was only observed in 1 of 2 IV-injected monkeys (GT49), depicted in the picture above on Day 6 PI. No hematomas were observed in subcutaneously inoculated macaques.

A longitudinal monitoring of coagulatory parameters hinted that a number of features may be important for hemorrhage formation (Onlamoon et al., 2010). Increased time to clotting was noted during blood collection of some Indian rhesus macaques, indicating an increased susceptibility toward bleeding. However, thromboplastin and prothrombin times did not indicate abnormal clotting. Protein C and anti-thrombin III levels did not vary from pre-inoculation values, but they were predominantly in the high end of the reference range. Marked elevations were noted for D-dimers, TAT complexes and protein S, with peaks most consistently present on Days 5–10 PI, corresponding to the resolution of viremia. This data requires further confirmation with additional time points, more animals spanning various ages and other DV isolates. However, we submit that we might for the first time have a model to investigate coagulopathy similar to DHF, which can allow for better evaluation of preventative and therapeutic strategies to prevent pathogenesis, not just infection.

Interestingly, analysis of serum chemistry parameters indicated relatively modest changes for all parameters except creatine phosphokinase (CK), which was markedly elevated on Day 7 (Onlamoon et al., 2010). CK is a component in energy metabolism (with multiple isoenzymatic forms: MM, MB, and BB) that are altered in individuals with a number of different illnesses (Roberts and Sobel, 1973; Saks et al., 1978). Heightened levels of CK have been noted in Crimean Congo and Influenza patients (Middleton et al., 1970; Ergonul et al., 2004). Additionally, a recent report confirms elevation of this enzyme in dengue patients and suggests it is linked to muscle weakness/dysfunction during malaise (Misra et al., 2011). However, CK is a non-specific biomarker that is elevated in various conditions, and thus its diagnostic value is limited. Since these enzymes are quite highly elevated during DV infection, there could be a meaningful relationship between CK and disease. CK and creatine phosphates in combination are known as ADP scavengers and participate in modulating platelet activities, such as aggregation (Chignard et al., 1979; Chesney et al., 1982; Krishnamurthi et al., 1984; Jennings, 2009), which may consequently modulate immune cell activation/function and by extension, pathogenesis (Wong et al., 2013).

BONE MARROW (BM) TARGETING

The BM can be involved in hemodynamic defects; alterations in the BM environment may result in altered leukocyte function and contribute to pathogenesis (Wilson and Trumpp, 2006; Duffy et al., 2012). DV has long been known to alter hematopoiesis in human BM (Bierman and Nelson, 1965; La Russa and Innis, 1995). However, collecting BM aspirates from DV patients is contraindicated. Additionally, infections in patients can be misleading due to the variability in disease onset and the uncertainty of sample time points. Experimentation in animal models in which the induction of infection is known allows for better analysis in real time. Our rhesus monkeys were sampled for BM repeatedly on a rotating basis resulting in the collection of at least 3 samples at each time point spanning Days 1–14 PI. This has allowed for us to confirm that BM cellularity is indeed depressed during early acute DV infection (Noisakran et al., 2012). Aspirates were also monitored for the presence of DV in attempts to identify the initial cellular reservoirs of infection. While the general consensus is that DV targets phagocytes, such acquisition could be secondary to amplification in other cell types. *In vitro* both human and monkey BMs are permissive for DV replication, and similar to *in vivo*, peak titers differ by 1000-fold (**Figure 2**) (Clark et al., 2012). Characteristics of the early host cells were also evaluated in our model both *in vivo* and *in vitro* (Clark et al., 2012; Noisakran et al., 2012). Of interest DV antigen was primarily detected in CD41+ CD61+ cells during the first 3 days, followed by a gradual shift toward CD14+ phagocytes at later time points, coinciding with viral clearance (Clark et al., 2012). The results suggest that megakaryocytes represent the initial target of DV in BM, rather than a member of the monocytic lineage. Direct infection of these cells may account for the altered megakaryocyte composition (Nelson et al., 1964), impaired platelet function (Srichaikul and Nimmannitya, 2000; Cheng et al., 2009) and the incidence of platelet phagocytosis observed in previous studies (Nelson et al., 1966; Honda et al., 2009; Onlamoon et al., 2010). Platelet activation and function during the course of infection has been under-investigated but may be critical for unraveling the mechanisms responsible for dengue pathology.

PLATELET ACTIVITIES

The role of platelets in the crafting of the immune response is imperfectly defined and only recently becoming recognized (Klinger and Jelkmann, 2002; Ombrello et al., 2010). These anucleated cells are able to associate with and deliver signals to other lineages and shape immune responses. Abnormal platelet behavior during dengue infection may play a significant role in modifying lymphocyte, monocyte and granulocyte function. When platelet-leukocyte interactions were quantified *in vivo*, macrophages/monocytes appeared to be the most commonly

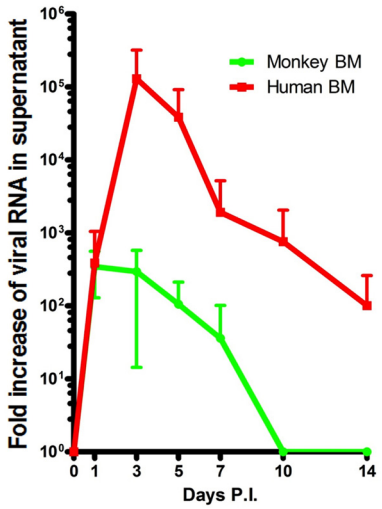


FIGURE 2 | Peak DV titers in rhesus macaque BMs is markedly lower than that of humans. BMs were acquired and infected as previously described (Clark et al., 2012). Samples from Days 1 through 14 were quantified by realtime PCR. Human (red) and monkey (green) titers are depicted in RNA copy numbers per ml. The *in vitro* experimentation of whole BM indicates that human BM is able to produce far more virus than monkey BM. Titers appear to max out on average closer to Day 1 in monkey BM but reach their peak (∼1000-fold higher) on Day 3 PI in humans.

associated cell lineage with platelets (Onlamoon et al., 2010), with a majority of these monocyte-platelet aggregates expressing activation marker CD62P (Onlamoon et al., 2010). This data is reminiscent of other reports linking activated monocytes to disease pathology in humans (Mustafa et al., 2001; Bozza et al., 2008; Durbin et al., 2008).

Platelets binding to neutrophils and lymphocytes were less frequent (**Figures 3A–C**) (Onlamoon et al., 2010). Only about 20–40% of neutrophils were bound with platelets, with 30–60% expressing CD62P. However, the extend of neutrophil-platelet aggregatation may be underestimated, since these cells are short-lived and other markers for neutrophil (CD11b and CD66b) and platelet (CD154, cleaved PAR1, CD63) activation were not tested (Heijnen et al., 1999; Claytor et al., 2003; Kinhult et al., 2003; Sprague et al., 2008). Lymphocyte-platelet aggregation occurred the least (**Figures 3B,C**). This was examined with Indian and Chinese rhesus macaques during primary DV2 (16681) infection and in Chinese macaques during secondary DV3 (Hawaii) infection (**Figures 3B,C** respectively). Since the dominant phenotype of the lymphocyte-platelet aggregate (LymPA) population was CD62P negative, this was the only population evaluated. Chinese and Indian macaques have different baseline levels of CD41+CD61+CD62P− lymphocytes, approximately 2% and 12%, respectively (**Figures 3B,C**). The average response from 5 Indian macaques suggests that the LymPA population is down-regulated (to about 7%) during infection but returns to normal levels after viral clearance (**Figure 3B**). In Chinese macaques, there appeared to be higher LymPA frequencies with the IV-inoculated monkeys, ranging up to 8% but only as high as 4% in SC-inoculated primates (**Figure 3C**). There was a late phase expansion of this population after primary but not after secondary infection. The functional significance of such changes is unclear at the present, but it would be interesting to compare these findings with other viral infections, like influenza, which produce robust long-lived B cell memory responses (Ikonen et al., 2010; Li et al., 2012). It remains to be seen whether this observation represents a common immune phenomenon or a DV specific response, which would potentially open a new line of investigation.

POTENTIAL REFINEMENTS TO THE COAGULOPATHY MONKEY MODEL

VIRUS SELECTION

While the data obtained with our rhesus macaque model appears promising, many parameters remain to be examined and refined. Arguably, the most important factor to evaluate is different strains. The viruses we used had been propagated extensively in cell culture, and thus the next step will be to evaluate primary DV strains, which are considered more capable of inducing pathology. Interestingly, the earliest DV studies (pre-1940s) in primates were conducted with human-derived virus that had never been propagated through cell culture (Lavinder and Francis, 1914; Chandler and Rice, 1923; Blanc et al., 1929; Simmons et al., 1931), yet these investigations induced minimal overt disease. The human-derived Hawaiian and New Guinea strains from Sabin's work were pathogenic in humans (when inoculated intradermally) but demonstrated no pathology in Rosen's study when inoculated into various primate species via a subcutaneous or intraperitoneal route (Sabin, 1952; Rosen, 1958). In recent studies, a large number of the strains employed were recent clinical isolates minimally passaged *in vitro* (Freire et al., 2007; Omatsu et al., 2011; Pamungkas et al., 2011; Yoshida et al., 2012). While these viruses are often close in sequence to the original isolate, these strains are not necessarily the most virulent or capable of achieving the targeted pathology in primates (Omatsu et al., 2011) and may require further evaluation before use *in vivo*.

The major drawbacks of primate models are the logistics and cost. Ideally one would perform preliminary experiments and evaluate strain virulence through a screening tool before *in vivo* studies with NHPs. Virulence could be assessed by testing the induction of disease in the humanized mouse or potentially by growth characteristics in monkey whole BM. Alternatively, passage of dengue in organisms (humanized mice or rhesus macaques) may ensure that the strain is more fit for these studies. It has been suggested that mouse-passaged viruses are more capable at causing viremia in NHPs than *in vitro*-passaged strains (Scherer et al., 1972).

Considering the viruses that have already been tested in NHPs, a select few appear promising for future studies. WP-74 (DV1) and S16803 (DV2) caused extreme lethargy in owl monkeys (Schiavetta et al., 2003) but not in cynomolgus (Koraka et al., 2007) or rhesus macaques (Ajariyakhajorn et al., 2005; Robert Putnak et al., 2005). Besides the 16681 DV2 virus, strains 49313 (DV1), 16007 (DV1), and 43283 (DV4) were associated with hemorrhage in previous studies (Halstead et al., 1973b; Scherer et al., 1978). Testing these strains in our Indian macaque model could lead to a more frequent presentation of coagulopathy and

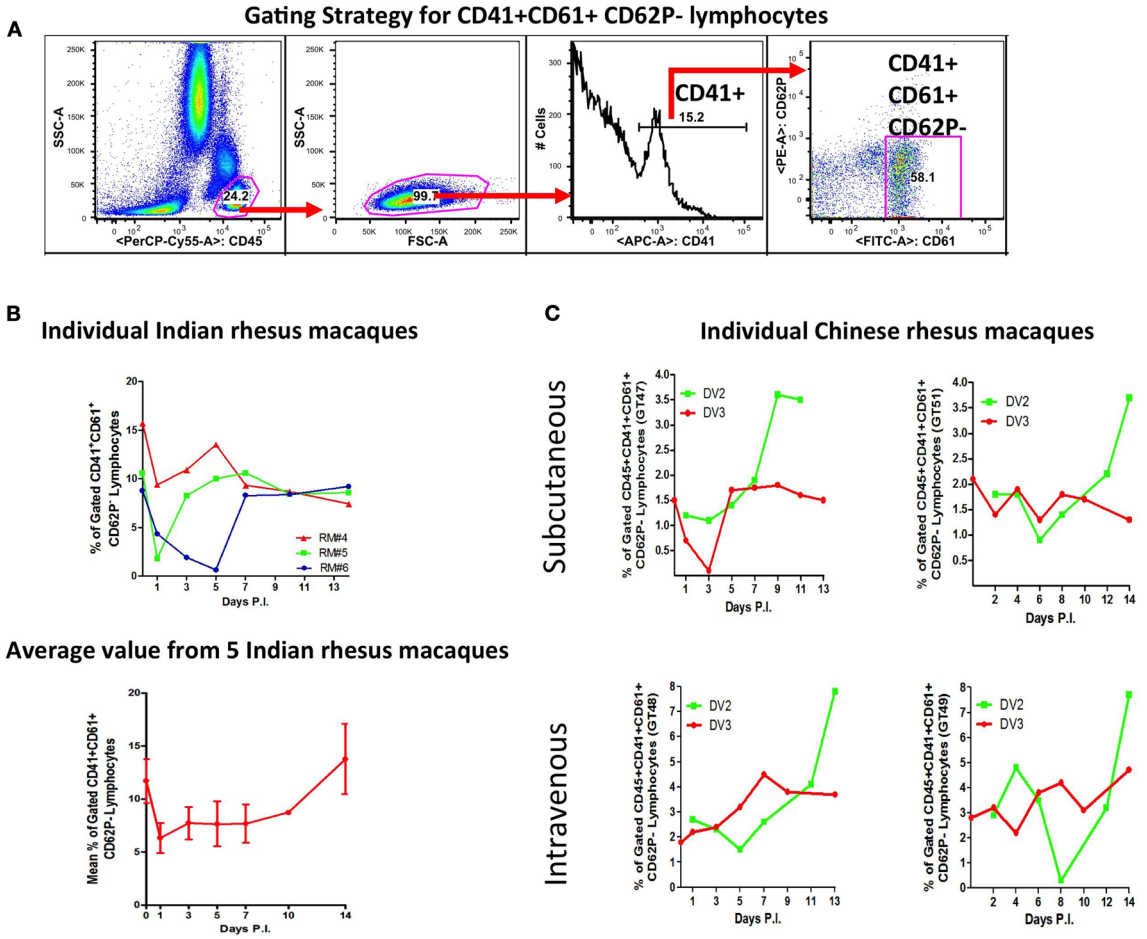


FIGURE 3 | Dynamics of lymphocyte-platelet aggregates (LymPA) during DV infection. Indian and Chinese rhesus macaques were infected as detailed in **Figure 1**. In addition, the Chinese macaques were challenged 2 months later with DV3 strain Hawaii. Peripheral blood samples obtained on Days 1 through 14 were subjected to flow cytometric analysis with CD45, CD41, CD61, and CD62P fluorescent antibodies. The frequencies of $CD45^{+}CD41^{+}CD61^{+}CD62P^{-}$ cells over time is graphed. **(A)** Panels to illustrate the gating strategy employed to analyze lymphocyte-platelet aggregates (LymPA). **(B)** The kinetics of LymPA in Indian rhesus macaques. The top graph displays LymPA frequencies from 3 individual macaques and the bottom graph, the average population frequency from 5 primates. The LymPA population is down-regulated during DV infection in Indian rhesus macaques. **(C)** LymPA kinetics in subcutaneously and intravenously infected Chinese rhesus macaques during primary DV2 (green line) and secondary DV3 infection (red line). The frequency of LymPA increases late after primary but not after secondary infection.

models for 3 of the 4 dengue serotypes. For future preclinical vaccine and drug studies, one strain of each serotype that can induce easily quantifiable disease will be needed for better vaccine evaluation.

OTHER PARAMETERS

A number of additional parameters may be manipulated in rhesus macaques that could amplify disease severity. Factors from infected mosquito saliva may potentiate the virus in down-modulating immune responses during the initiation of infection and help raise peak titer levels (Cox et al., 2012; Reagan et al., 2012; Surasombatpattana et al., 2012; Le Coupanec et al., 2013). Mosquito inoculation of DV into NHPs was modeled long ago without inducing much disease (Simmons et al., 1931). However, a number of confounding factors (preexisting immunity, inoculum quality, etc.) were not accounted for in these studies, indicating that this approach is worth revisiting.

Modulation of *in vivo* cell populations with drug treatments has rarely been attempted (Marchette et al., 1980; Yoshida et al., 2012). Potential treatment of macaques with megakaryocytic growth factors, like thrombopoetin, could increase the number of early permissive targets and enhance peak viral load if indeed megakaryocytes are the primary replication site for DV (Nakorn et al., 2003). General immunosuppression has been attempted but led to chronic viremia, which does not mimic human DV disease (Marchette et al., 1980). Depletion of macrophages, neutrophils or other innate immune responders may enhance titers by altering the dynamics of viral clearance. One previous attempt at CD16+ natural killer cell depletion did not modulate virus titers (Yoshida et al., 2012), although such depletions are generally partial at best. Additionally, various inoculum sizes and alternative inoculation routes may be tested. The intradermal inoculation route was suggested to lead to better virus tissue distribution, but did not result in better dissemination to the BM (Pamungkas et al., 2011).

Characterization of these parameters are necessary for the further refinement of the coagulopathy disease animal model.

HOST CHARACTERISTICS OR GENETIC FACTORS THAT INCREASE SUSCEPTIBILITY TO COAGULOPATHY

Epidemiological studies of dengue patient characteristics, including age, sex and genetic polymorphisms have been frequently studied, but none of these findings have been validated in animal models (Loke et al., 2001; Stephens et al., 2002; Cordeiro et al., 2007; Kalayanarooj et al., 2007; Soundravally and Hoti, 2007; Stephens, 2010). In humans, the age of greatest susceptibility to disease is seen in young adults (Tsai et al., 2012). In our Indian rhesus macaques, we have evaluated age as a contributing factor to viremia by comparing the titers of DV when propagated in whole BM *in vitro* (unpublished data). However, no difference was noted in virus growth kinetics or magnitude related to age of BM donors ($n = 11$), which spanned 2–15 years of age. *In vivo*, anecdotal observations suggested that coagulopathy appeared to be more extensive in older female macaques when compared to young males, which were the populations included in the study, although sample size was too low to be conclusive. This nevertheless raises an interesting question about the potential for host factors contributing to the severity of symptoms.

Various MHC alleles, blood group and platelet antigens have been found to be associated with dengue disease and protection (Kalayanarooj et al., 2007; Soundravally and Hoti, 2007; Alagarasu et al., 2013; Weiskopf et al., 2013). Although in general these associations are weak as biomarkers of disease. One of our goals is to assess gene alleles involved with regulating platelet activation and the coagulatory cascade e.g., *HPA1*, *HPA2* for association with disease presentation. Available techniques, such as *Macaca mulatta* typing and gene expression analyses, will need to be an integral part of future experiments with the rhesus monkey model to facilitate identification of genetic factors involved with dengue-induced abnormal coagulation.

CONCLUSION

The induction of disease symptoms upon the inoculation of DV in primates has been an elusive objective. Recently a coagulopathy disease model was developed using the serotype 2 strain 16681 injected intravenously into Indian rhesus macaques. We submit that this approach provides a strategy for detailed investigation of the mechanisms potentially involved in DHF. Moreover, the model provides an attractive algorithm for testing the efficacy of preventative vaccines and therapeutics that not only limit virus replication but also prevent disease development *in vivo*. Various host and viral parameters can begin to be evaluated *in vivo* to help us gain a better understanding of dengue biology and disease pathogenesis. Can pathology be induced in other NHPs by switching to the intravenous route? Will different virus strains promote coagulopathy, or other symptoms? Can we alter other parameters and achieve a more severe disease model? The establishment of this new rhesus macaque infection model has proved insightful on ways to improve disease presentation in primates.

HUMAN SUBJECT AND ANIMAL RESEARCH

Use of deidentified human BM was provided by Emory Hospital and approved by the Emory University Internal Review Board. Investigations with rhesus macaques were approved by Yerkes and Tulane IACUCs and conducted at either the Yerkes or Tulane National Primate Research Centers. Research was performed in accordance with institutional and national guidelines and regulations.

ACKNOWLEDGMENTS

Thanks should be given to the staffs of Yerkes and Tulane National Primate Research Centers for the handling of monkeys and facilitating these investigations. Dr. John Roback aided with acquiring human BM samples to perform *in vitro* assays. Funding was acquired from U19 Pilot Project Funds (RFA-AI-02-042), National Institutes of Health/SERCEB, Emory URC grant, and P510D11123 in support to the Yerkes National Primate Research Center.

REFERENCES

Ajariyakhajorn, C., Mammen, M. P. Jr., Endy, T. P., Gettayacamin, M., Nisalak, A., Nimmannitiya, S. et al. (2005). Randomized, placebo-controlled trial of nonpegylated and pegylated forms of recombinant human alpha interferon 2a for suppression of dengue virus viremia in rhesus monkeys. *Antimicrobial Agents Chemother.* 49, 4508–4514. doi: 10.1128/AAC.49.11.4508-4514.2005

Akkina, R. (2013). New generation humanized mice for virus research: comparative aspects and future prospects. *Virology* 435, 14–28. doi: 10.1016/j.virol.2012.10.007

Akkina, R., Berges, B. K., Palmer, B. E., Remling, L., Neff, C. P., Kuruvilla, J., et al. (2011). Humanized Rag1−/− gammac−/− mice support multilineage hematopoiesis and are susceptible to HIV-1 infection via systemic and vaginal routes. *PLoS ONE* 6:e20169. doi: 10.1371/journal.pone.0020169

Alagarasu, K., Mulay, A. P., Singh, R., Gavade, V. B., Shah, P. S., and Cecilia, D. (2013). Association of HLA-DRB1 and TNF genotypes with dengue hemorrhagic fever. *Hum. Immunol.* 74, 610–617. doi: 10.1016/j.humimm.2013.01.027

Anez, G., Men, R., Eckels, K. H., and Lai, C. J. (2009). Passage of dengue virus type 4 vaccine candidates in fetal rhesus lung cells selects heparin-sensitive variants that result in loss of infectivity and immunogenicity in rhesus macaques. *J. Virol.* 83, 10384–10394. doi: 10.1128/JVI.01083-09

Angsubhakorn, S., Moe, J. B., Marchette, N. J., Latendresse, J. R., Palumbo, N. E., Yoksan, S., et al. (1987). Neurovirulence detection of dengue virus using rhesus and cynomolgus monkeys. *J. Virol. Methods* 18, 13–24. doi: 10.1016/0166-0934(87)90106-6

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., et al. (2006). Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. *J. Infect. Dis.* 193, 1078–1088. doi: 10.1086/500949

Baker, M. A., Cronin, M. J., and Mountjoy, D. G. (1976). Variability of skin temperature in the waking monkey. *Am. J. Physiol.* 230, 449–455.

Bernardo, L., Izquierdo, A., Prado, I., Rosario, D., Alvarez, M., Santana, E., et al. (2008). Primary and secondary infections of Macaca fascicularis monkeys with Asian and American genotypes of dengue virus 2. *Clin. Vaccine Immunol.* 15, 439–446. doi: 10.1128/CVI.00208-07

Bierman, H. R., and Nelson, E. R. (1965). Hematodepressive virus diseases of Thailand. *Ann. Intern. Med.* 62, 867–884. doi: 10.7326/0003-4819-62-5-867

Blanc, G., Caminopetros, J., Dumas, J., and Saenz, A. (1929). Recherches experimentales sur la sensibilite des singes inferieurs au virus de la dengue. *Comptes rendus hebdomadaires des seances de*

l'academie des sciences T188, 468–470.

Bozza, F. A., Cruz, O. G., Zagne, S. M., Azeredo, E. L., Nogueira, R. M., Assis, E. F., et al. (2008). Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. *BMC Infect. Dis.* 8:86. doi: 10.1186/1471-2334-8-86

Chamberlain, R. W., Kissling, R. E., and Sikes, R. K. (1954). Studies on the North American arthropod-borne encephalitides. VII. Estimation of amount of eastern equine encephalitis virus inoculated by infected Aedes aegypti. *Am. J. Hyg.* 60, 286–291.

Chandler, A. S., Rice, L. (1923). Observations on the etiology of dengue fever. *Am. J. Trop. Med. Hyg.* s1–s3, 233–262.

Chen, L., Ewing, D., Subramanian, H., Block, K., Rayner, J., Alterson, K. D., et al. (2007). A heterologous DNA prime-Venezuelan equine encephalitis virus replicon particle boost dengue vaccine regimen affords complete protection from virus challenge in cynomolgus macaques. *J. Virol.* 81, 11634–11639. doi: 10.1128/JVI.00996-07

Cheng, H. J., Lei, H. Y., Lin, C. F., Luo, Y. H., Wan, S. W., Liu, H. S., et al. (2009). Anti-dengue virus nonstructural protein 1 antibodies recognize protein disulfide isomerase on platelets and inhibit platelet aggregation. *Mol. Immunol.* 47, 398–406. doi: 10.1016/j.molimm.2009.08.033

Chesney, C. M., Pifer, D. D., Byers, L. W., and Muirhead, E. E. (1982). Effect of platelet-activating factor (PAF) on human platelets. *Blood* 59, 582–585.

Chignard, M., Le Couedic, J. P., Tence, M., Vargaftig, B. B., and Benveniste, J. (1979). The role of platelet-activating factor in platelet aggregation. *Nature* 279, 799–800. doi: 10.1038/279799a0

Clark, K. B., Noisakran, S., Onlamoon, N., Hsiao, H. M., Roback, J., Villinger, F., et al. (2012). Multiploid CD61+ cells are the pre-dominant cell lineage infected during acute dengue virus infection in bone marrow. *PLoS ONE* 7:e52902. doi: 10.1371/journal.pone.0052902

Claytor, R. B., Michelson, A. D., Li, J. M., Frelinger, A. L. 3rd., Rohrer, M. J., Garnette,C. S., et al. (2003). The cleaved peptide of PAR1 is a more potent stimulant of platelet-endothelial cell adhesion than is thrombin. *J. Vasc. Surg.* 37, 440–445. doi: 10.1067/mva.2003.129

Cobra, C., Rigau-Perez, J. G., Kuno, G., and Vorndam, V. (1995). Symptoms of dengue fever in relation to host immunologic response and virus serotype, Puerto Rico, 1990–1991. *Am. J. Epidemiol.* 142, 1204–1211.

Colton, L., Biggerstaff, B. J., Johnson, A., and Nasci, R. S. (2005). Quantification of West Nile virus in vector mosquito saliva. *J. Am. Mosq. Control Assoc.* 21, 49–53. doi: 10.2987/8756-971X (2005)21[49:QOWNVI]2.0.CO;2

Cordeiro, M. T., Silva, A. M., Brito, C. A., Nascimento, E. J., Magalhaes, M. C., Guimaraes, G. F., et al. (2007). Characterization of a dengue patient cohort in Recife, Brazil. *Am. J. Trop. Med. Hyg.* 77, 1128–1134.

Cox, H. R. (1953). Active immunization against poliomyelitis. *Bull. N.Y. Acad. Med.* 29, 943–960.

Cox, J., Mota, J., Sukupolvi-Petty, S., Diamond, M. S., and Rico-Hesse, R. (2012). Mosquito bite delivery of dengue virus enhances immunogenicity and pathogenesis in humanized mice. *J. Virol.* 86, 7637–7649. doi: 10.1128/JVI.00534-12

Duffy, D., Perrin, H., Abadie, V., Benhabiles, N., Boissonnas, A., Liard, C., et al. (2012). Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. *Immunity* 37, 917–929. doi: 10.1016/j.immuni.2012.07.015

Durbin, A. P., Vargas, M. J., Wanionek, K., Hammond, S. N., Gordon, A., Rocha, C., et al. (2008). Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. *Virology* 376, 429–435. doi: 10.1016/j.virol.2008.03.028

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. *J. Infect. Dis.* 189, 990–1000. doi: 10.1086/382280

Ergonul, O., Celikbas, A., Dokuzoguz, B., Eren, S., Baykam, N., and Esener, H. (2004). Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy. *Clin. Infect. Dis.* 39, 284–287. doi: 10.1086/422000

Fox, A., Le, N. M., Simmons, C. P., Wolbers, M., Wertheim, H. F., Pham, T. K., et al. (2011). Immunological and viral determinants of dengue severity in hospitalized adults in Ha Noi, Viet Nam. *PLoS Negl. Trop. Dis.* 5:e967. doi: 10.1371/journal.pntd.0000967

Freire, M., Marchevsky, R., Almeida, L., Yamamura, A., Caride, E., Brindeiro, P., et al. (2007). Wild dengue virus types 1, 2 and 3 viremia in rhesus monkeys. *Mem. Inst. Oswaldo Cruz* 102, 203–208. doi: 10.1590/S0074-02762007005000011

Fuller, C. A., Sulzman, F. M., and Moore-Ede, M. C. (1985). Role of heat loss and heat production in generation of the circadian temperature rhythm of the squirrel monkey. *Physiol. Behav.* 34, 543–546. doi: 10.1016/0031-9384(85)90046-0

Green, S., Pichyangkul, S., Vaughn, D. W., Kalayanarooj, S., Nimmannitya, S., Nisalak, A., et al. (1999). Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. *J. Infect. Dis.* 180, 1429–1435. doi: 10.1086/315072

Grimstad, P. R., Ross, Q. E., and Craig, G. B. Jr. (1980). Aedes triseriatus (Diptera: Culicidae) and La Crosse virus. II. Modification of mosquito feeding behavior by virus infection. *J. Med. Entomol.* 17, 1–7.

Gubler, D. J., and Rosen, L. (1976). A simple technique for demonstrating transmission of dengue virus by mosquitoes without the use of vertebrate hosts. *Am. J. Trop. Med. Hyg.* 25, 146–150.

Guilarde, A. O., Turchi, M. D., Siqueira, J. B. Jr., Feres, V. C., Rocha, B., Levi, J. E., et al. (2008). Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. *J. Infect. Dis.* 197, 817–824. doi: 10.1086/528805

Gunther, V. J., Putnak, R., Eckels, K. H., Mammen, M. P., Scherer, J. M., Lyons, A., et al. (2011). A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. *Vaccine* 29, 3895–3904. doi: 10.1016/j.vaccine.2011.03.038

Halstead, S. B., Shotwell, H., and Casals, J. (1973a). Studies on the pathogenesis of dengue infection in monkeys. I. Clinical laboratory responses to primary infection. *J. Infect. Dis.* 128, 7–14. doi: 10.1093/infdis/128.1.7

Halstead, S. B., Shotwell, H., and Casals, J. (1973b). Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *J. Infect. Dis.* 128, 15–22. doi: 10.1093/infdis/128.1.15

Halstead, S. B., Suaya, J. A., and Shepard, D. S. (2007). The burden of dengue infection. *Lancet* 369, 1410–1411. doi: 10.1016/S0140-6736(07)60645-X

Hanley, K. A., Monath, T. P., Weaver, S. C., Rossi, S. L., Richman, R. L., and Vasilakis, N. (2013). Fever versus fever: the role of host and vector susceptibility and interspecific competition in shaping the current and future distributions of the sylvatic cycles of dengue virus and yellow fever virus. *Infect. Genet. Evol.* doi: 10.1016/j.meegid.2013.03.008. [Epub ahead of print].

Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J., and Sixma, J. J. (1999). Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 94, 3791–3799.

Honda, S., Saito, M., Dimaano, E. M., Morales, P. A., Alonzo, M. T., Suarez, L. A., et al. (2009). Increased phagocytosis of platelets from patients with secondary dengue virus infection by human macrophages. *Am. J. Trop. Med. Hyg.* 80, 841–845.

Ikonen, N., Strengell, M., Kinnunen, L., Osterlund, P., Pirhonen, J., Broman, M., et al. (2010). High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland. *Euro Surveill.* 15, 1–8.

Jennings, L. K. (2009). Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. *Thromb. Haemost.* 102, 248–257. doi: 10.1160/TH09-03-0192

Kalayanarooj, S., Gibbons, R. V., Vaughn, D., Green, S., Nisalak, A., Jarman, R. G., et al. (2007). Blood group AB is associated with increased risk for severe dengue disease in secondary infections. *J. Infect. Dis.* 195, 1014–1017. doi: 10.1086/512244

Kinhult, J., Egesten, A., Benson, M., Uddman, R., and Cardell, L. O. (2003). Increased expression of surface activation markers on neutrophils following migration into the nasal lumen. *Clin. Exp. Allergy* 33, 1141–1146. doi: 10.1046/j.1365-2222.2003.01682.x

Klinger, M. H., and Jelkmann, W. (2002). Role of blood platelets in infection and inflammation. *J. Interferon*

Cytokine Res. 22, 913–922. doi: 10.1089/10799900260286623

Kochel, T. J., Watts, D. M., Gozalo, A. S., Ewing, D. F., Porter, K. R., and Russell, K. L. (2005). Cross-serotype neutralization of dengue virus in Aotus nancymae monkeys. *J. Infect. Dis.* 191, 1000–1004. doi: 10.1086/427511

Koizumi, T. K. Y., and Tonomura, K. (1917). The research of dengue disease. *Taiwan Igakukai Zasshi* 177, 432–463.

Koraka, P., Benton, S., van Amerongen, G., Stittelaar, K. J., and Osterhaus, A. D. (2007). Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses. *Microbes Infect.* 9, 940–946. doi: 10.1016/j.micinf.2007.03.012

Kraiselburd, E., Gubler, D. J., and Kessler, M. J. (1985). Quantity of dengue virus required to infect rhesus monkeys. *Trans. R. Soc. Trop. Med. Hyg.* 79, 248–251. doi: 10.1016/0035-9203(85)90348-7

Krishnamurthi, S., Westwick, J., and Kakkar, V. V. (1984). Regulation of human platelet activation—analysis of cyclooxygenase and cyclic AMP-dependent pathways. *Biochem. Pharmacol.* 33, 3025–3035. doi: 10.1016/0006-2952(84)90604-X

Kuruvilla, J. G., Troyer, R. M., Devi, S., and Akkina, R. (2007). Dengue virus infection and immune response in humanized RAG2(−/−)gamma(c)(−/−) (RAG-hu) mice. *Virology* 369, 143–152. doi: 10.1016/j.virol.2007.06.005

La Russa, V. F., and Innis, B. L. (1995). Mechanisms of dengue virus-induced bone marrow suppression. *Baillieres Clin. Haematol.* 8, 249–270. doi: 10.1016/S0950-3536(05)80240-9

Lavinder, C. H., and Francis, E. (1914). The Etiology of Dengue. An attempt to produce the disease in the rhesus monkey by the inoculation of defibrated blood. *J. Infect. Dis.* 15, 341–346. doi: 10.1093/infdis/15.2.341

Le Coupanec, A., Babin, D., Fiette, L., Jouvion, G., Ave, P., Misse, D., et al. (2013). Aedes mosquito saliva modulates Rift Valley fever virus pathogenicity. *PLoS Negl. Trop. Dis.* 7:e2237. doi: 10.1371/journal.pntd.0002237

Lei, H. Y., Yeh, T. M., Liu, H. S., Lin, Y. S., Chen, S. H., and Liu, C. C. (2001). Immunopathogenesis of dengue virus infection. *J. Biomed. Sci.* 8, 377–388. doi: 10.1007/BF02255946

Li, G. M., Chiu, C., Wrammert, J., McCausland, M., Andrews, S. F., Zheng, N. Y., et al. (2012). Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9047–9052. doi: 10.1073/pnas.1118979109

Libraty, D. H., Acosta, L. P., Tallo, V., Segubre-Mercado, E., Bautista, A., Potts, J. A., et al. (2009). A prospective nested case-control study of Dengue in infants: rethinking and refining the antibody-dependent enhancement dengue hemorrhagic fever model. *PLoS Med.* 6:e1000171. doi: 10.1371/journal.pmed.1000171

Libraty, D. H., Young, P. R., Pickering, D., Endy, T. P., Kalayanarooj, S., Green, S., et al. (2002). High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J. Infect. Dis.* 186, 1165–1168. doi: 10.1086/343813

Lin, Y. L., Liao, C. L., Chen, L. K., Yeh, C. T., Liu, C. I., Ma, S. H., et al. (1998). Study of Dengue virus infection in SCID mice engrafted with human K562 cells. *J. Virol.* 72, 9729–9737.

Loke, H., Bethell, D. B., Phuong, C. X., Dung, M., Schneider, J., White, N. J., et al. (2001). Strong HLA class I—restricted T cell responses in dengue hemorrhagic fever: a double-edged sword? *J. Infect. Dis.* 184, 1369–1373. doi: 10.1086/324320

Lynch, S. L. C. (2007). Plasma microparticles and vascular disorders. *Br. J. Haematol.* 137, 36–48.

Mairuhu, A. T., Mac Gillavry, M. R., Setiati, T. E., Soemantri, A., Ten Cate, H., Brandjes, D. P., et al. (2003). Is clinical outcome of dengue-virus infections influenced by coagulation and fibrinolysis? A critical review of the evidence. *Lancet Infect. Dis.* 3, 33–41. doi: 10.1016/S1473-3099(03)00487-0

Marchette, N. J., Halstead, S. B., Falkler, W. A. Jr., Stenhouse, A., and Nash, D. (1973). Studies on the pathogenesis of dengue infection in monkeys. 3. Sequential distribution of virus in primary and heterologous infections. *J. Infect. Dis.* 128, 23–30. doi: 10.1093/infdis/128.1.23

Marchette, N. J., O'Rourke, T., and Halstead, S. B. (1980). Studies on dengue 2 virus infection in cyclophosphamide-treated rhesus monkeys. *Med. Microbiol. Immunol.* 168, 35–47. doi: 10.1007/BF02121650

Markoff, L. J., Innis, B. L., Houghten, R., and Henchal, L. S. (1991). Development of cross-reactive antibodies to plasminogen during the immune response to dengue virus infection. *J. Infect. Dis.* 164, 294–301. doi: 10.1093/infdis/164.2.294

Martin, J., Hermida, L., Castro, J., Lazo, L., Martinez, R., Gil, L., et al. (2009). Viremia and antibody response in green monkeys (Chlorocebus aethiops sabaeus) infected with dengue virus type 2: a potential model for vaccine testing. *Microbiol. Immunol.* 53, 216–223. doi: 10.1111/j.1348-0421.2009.00112.x

Mellink, J. J. (1982). Estimation of the amount of Venezuelan equine encephalomyelitis virus transmitted by a single infected Aedes aegypti (Diptera: Culicidae). *J. Med. Entomol.* 19, 275–280.

Meltzer, E., Heyman, Z., Bin, H., and Schwartz, E. (2012). Capillary leakage in travelers with dengue infection: implications for pathogenesis. *Am. J. Trop. Med. Hyg.* 86, 536–539. doi: 10.4269/ajtmh.2012.10-0670

Middleton, P. J., Alexander, R. M., and Szymanski, M. T. (1970). Severe myositis during recovery from influenza. *Lancet* 2, 533–535. doi: 10.1016/S0140-6736(70)91343-7

Misra, U. K., Kalita, J., Maurya, P. K., Kumar, P., Shankar, S. K., and Mahadevan, A. (2011). Dengue-associated transient muscle dysfunction: clinical, electromyography and histopathological changes. *Infection* 40, 125–130. doi: 10.1007/s15010-011-0203-8

Mladinich, K. M., Piaskowski, S. M., Rudersdorf, R., Eernisse, C. M., Weisgrau, K. L., Martins, M. A., et al. (2012). Dengue virus-specific CD4(+) and CD8(+) T lymphocytes target NS1, NS3 and NS5 in infected Indian rhesus macaques. *Immunogenetics* 64, 111–121. doi: 10.1007/s00251-011-0566-0

Mota, J., and Rico-Hesse, R. (2011). Dengue virus tropism in humanized mice recapitulates human dengue fever. *PLoS ONE* 6:e20762. doi: 10.1371/journal.pone.0020762

Murgue, B., Deparis, X., Chungue, E., Cassar, O., and Roche, C. (1999). Dengue: an evaluation of dengue severity in French Polynesia based on an analysis of 403 laboratory-confirmed cases. *Trop. Med. Int. Health* 4, 765–773. doi: 10.1046/j.1365-3156.1999.00478.x

Murgue, B., Roche, C., Chungue, E., and Deparis, X. (2000). Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996-1997 dengue-2 outbreak in French Polynesia. *J. Med. Virol.* 60, 432–438.

Mustafa, A. S., Elbishbishi, E. A., Agarwal, R., and Chaturvedi, U. C. (2001). Elevated levels of interleukin-13 and IL-18 in patients with dengue hemorrhagic fever. *FEMS Immunol. Med. Microbiol.* 30, 229–233. doi: 10.1111/j.1574-695X.2001.tb01575.x

Nakorn, T. N., Miyamoto, T., and Weissman, I. L. (2003). Characterization of mouse clonogenic megakaryocyte progenitors. *Proc. Natl. Acad. Sci. U.S.A.* 100, 205–210. doi: 10.1073/pnas.262655099

Nelson, E. R., Bierman, H. R., and Chulajata, R. (1964). Hematologic Findings in the 1960 Hemorrhagic Fever Epidemic (Dengue) in Thailand. *Am. J. Trop. Med. Hyg.* 13, 642–649.

Nelson, E. R., Bierman, H. R., and Chulajata, R. (1966). Hematologic phagocytosis in postmortem bone marrows of dengue hemorrhagic fever. (Hematologic phagocytosis in Thai hemorrhagic fever). *Am. J. Med. Sci.* 252, 68–74. doi: 10.1097/00000441-196607000-00010

Noisakran, S., Onlamoon, N., Hsiao, H. M., Clark, K. B., Villinger, F., Ansari, A. A., and Perng, G. C. (2012). Infection of bone marrow cells by dengue virus *in vivo*. *Exp. Hematol.* 40, 250–259 e254. doi: 10.1016/j.exphem.2011.11.011

Nussenblatt, R. B., Bielekova, B., Childs, R., Krensky, A., Strober, W., and Trinchieri, G. (2010). National Institutes of Health Center for Human Immunology Conference. *Ann. N. Y. Acad. Sci.* E1–E23. doi: 10.1111/j.1749-6632.2010.05682.x

Oishi, K., Inoue, S., Cinco, M. T., Dimaano, E. M., Alera, M. T., Alfon, J. A., et al. (2003). Correlation between increased platelet-associated IgG and thrombocytopenia in secondary dengue virus infections. *J. Med. Virol.* 71, 259–264. doi: 10.1002/jmv.10478

Omatsu, T., Moi, M. L., Hirayama, T., Takasaki, T., Nakamura, S., Tajima, S., et al. (2011). Common marmoset (Callithrix jacchus) as a primate model of dengue virus infection: development of high levels of viraemia and demonstration of protective immunity. *J. Gen. Virol.* 92, 2272–2280. doi: 10.1099/vir.0.031229-0

Ombrello, C., Block, R. C., and Morrell, C. N. (2010). Our expanding view of platelet functions and its

clinical implications. *J. Cardiovasc. Transl. Res.* 3, 538–546. doi: 10.1007/s12265-010-9213-7

Onlamoon, N., Noisakran, S., Hsiao, H. M., Duncan, A., Villinger, F., Ansari, A. A., et al. (2010). Dengue virus-induced hemorrhage in a nonhuman primate model. *Blood* 115, 1823–1834. doi: 10.1182/blood-2009-09-242990

Pamungkas, J., Iskandriati, D., Saepuloh, U., Affandi, M., Arifin, E., Paramastri, Y., et al. (2011). Dissemination in pigtailed macaques after primary infection of dengue-3 virus. *Microbiol. Indones.* 5, 94–98. doi: 10.5454/mi.5.2.7

Paul, J. R., Melnick, J. L., and Sabin, A. B. (1948). Experimental attempts to transmit phlebotomus (sandfly, pappataci) and dengue fevers to chimpanzees. *Proc. Soc. Exp. Biol. Med.* 68, 193–198. doi: 10.3181/00379727-68-16431

Perng, G. C., and Chokephaibulkit, K. (2013). Immunologic hypo- or non-responder in natural dengue virus infection. *J. Biomed. Sci.* 20:34. doi: 10.1186/1423-0127-20-34

Porter, K. R., Ewing, D., Chen, L., Wu, S. J., Hayes, C. G., Ferrari, M., et al. (2012). Immunogenicity and protective efficacy of a vaxfectin-adjuvanted tetravalent dengue DNA vaccine. *Vaccine* 30, 336–341. doi: 10.1016/j.vaccine.2011.10.085

Putnak, R., Barvir, D. A., Burrous, J. M., Dubois, D. R., D'Andrea, V. M., Hoke, C. H., et al. (1996). Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. *J. Infect. Dis.* 174, 1176–1184. doi: 10.1093/infdis/174.6.1176

Raviprakash, K., Porter, K. R., Kochel, R. J., Ewing, D., Simmons, M., Phillips, I., et al. (2000). Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. *J. Gen. Virol.* 81, 1659–1667.

Reagan, K. L., Machain-Williams, C., Wang, T., and Blair, C. D. (2012). Immunization of mice with recombinant mosquito salivary protein D7 enhances mortality from subsequent West Nile virus infection via mosquito bite. *PLoS Negl. Trop. Dis.* 6:e1935. doi: 10.1371/journal.pntd.0001935

Robert Putnak, J., Coller, B. A., Voss, G., Vaughn, D. W., Clements, D., Peters, I., et al. (2005). An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. *Vaccine* 23, 4442–4452. doi: 10.1016/j.vaccine.2005.03.042

Roberts, R., and Sobel, B. E. (1973). Ediortial: Isoenzymes of creatine phosphokinase and diagnosis of myocardial infarction. *Ann. Intern. Med.* 79, 741–743. doi: 10.7326/0003-4819-79-5-741

Rosen, L. (1958). Experimental infection of New World monkeys with dengue and yellow fever viruses. *Am. J. Trop. Med. Hyg.* 7, 406–410.

Sabin, A. B. (1952). Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* 1, 30–50.

Sabin, A. B., and Schlesinger, R. W. (1945). Production of immunity to dengue with virus modified by propagation in mice. *Science* 101, 640–642. doi: 10.1126/science.101.2634.640

Saito, M., Oishi, K., Inoue, S., Dimaano, E. M., Alera, M. T., Robles, A. M., et al. (2004). Association of increased platelet-associated immunoglobulins with thrombocytopenia and the severity of disease in secondary dengue virus infections. *Clin. Exp. Immunol.* 138, 299–303. doi: 10.1111/j.1365-2249.2004.02626.x

Saks, V. A., Rosenshtraukh, L. V., Smirnov, V. N., and Chazov, E. I. (1978). Role of creatine phosphokinase in cellular function and metabolism. *Can. J. Physiol. Pharmacol.* 56, 691–706. doi: 10.1139/y78-113

Sariol, C. A., Munoz-Jordan, J. L., Abel, K., Rosado, L. C., Pantoja, P., Giavedoni, L., et al. (2007). Transcriptional activation of interferon-stimulated genes but not of cytokine genes after primary infection of rhesus macaques with dengue virus type 1. *Clin. Vaccine Immunol.* 14, 756–766. doi: 10.1128/CVI.00052-07

Scherer, W. F., Breakenridge, F. A., and Dickerman, R. W. (1972). Cross-protection studies and search for subclinical disease in new world monkeys infected sequentially with different immunologic types of Dengue viruses. *Am. J. Epidemiol.* 95, 67–79.

Scherer, W. F., Russell, P. K., Rosen, L., Casals, J., and Dickerman, R. W. (1978). Experimental infection of chimpanzees with dengue viruses. *Am. J. Trop. Med. Hyg.* 27, 590–599.

Schiavetta, A. M., Harre, J. G., Wagner, E., Simmons, M., and Raviprakash, K. (2003). Variable susceptibility of the owl monkey (Aotus nancymae) to four serotypes of dengue virus. *Contemp. Top. Lab. Anim. Sci.* 42, 12–20.

Schul, W., Liu, W., Xu, H. Y., Flamand, M., and Vasudevan, S. G. (2007). A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. *J. Infect. Dis.* 195, 665–674. doi: 10.1086/511310

Scott, R. M., Nisalak, A., Eckels, K. H., Tingpalapong, M., Harrison, V. R., Gould, D. J., et al. (1980). Dengue-2 vaccine: viremia and immune responses in rhesus monkeys. *Infect. Immun.* 27, 181–186.

Shresta, S., Sharar, K. L., Prigozhin, D. M., Beatty, P. R., and Harris, E. (2006). Murine model for dengue virus-induced lethal disease with increased vascular permeability. *J. Virol.* 80, 10208–10217. doi: 10.1128/JVI.00062-06

Simmons, J., St. John, H. F., and Reynolds, H. K. (1931). Experimental studies of dengue. *Phillipp. J. Sci.* 44, 189–244.

Smith, D. R., Carrara, A. S., Aguilar, P. V., and Weaver, S. C. (2005). Evaluation of methods to assess transmission potential of Venezuelan equine encephalitis virus by mosquitoes and estimation of mosquito saliva titers. *Am. J. Trop. Med. Hyg.* 73, 33–39.

Soundravally, R., and Hoti, S. L. (2007). Immunopathogenesis of dengue hemorrhagic fever and shock syndrome: role of TAP and HPA gene polymorphism. *Hum. Immunol.* 68, 973–979. doi: 10.1016/j.humimm.2007.09.007

Sprague, D. L., Elzey, B. D., Crist, S. A., Waldschmidt, T. J., Jensen, R. J., and Ratliff, T. L. (2008). Platelet-mediated modulation of adaptive immunity: unique delivery of CD154 signal by platelet-derived membrane vesicles. *Blood* 111, 5028–5036. doi: 10.1182/blood-2007-06-097410

Srichaikul, T., and Nimmannitya, S. (2000). Haematology in dengue and dengue haemorrhagic fever. *Baillieres. Best Pract. Res. Clin. Haematol.* 13, 261–276. doi: 10.1053/beha.2000.0073

Stephens, H. A. (2010). HLA and other gene associations with dengue disease severity. *Curr. Top. Microbiol. Immunol.* 338, 99–114. doi: 10.1007/978-3-642-02215-9_8

Stephens, H. A., Klaythong, R., Sirikong, M., Vaughn, D. W., Green, S., Kalayanarooj, S., et al. (2002). HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. *Tissue Antigens* 60, 309–318. doi: 10.1034/j.1399-0039.2002.600405.x

Stramer, S. L., Linnen, J. M., Carrick, J. M., Foster, G. A., Krysztof, D. E., Zou, S., et al. (2012). Dengue viremia in blood donors identified by RNA and detection of dengue transfusion transmission during the 2007 dengue outbreak in Puerto Rico. *Transfusion* 52, 1657–1666. doi: 10.1111/j.1537-2995.2012.03566.x

Styer, L. M., Kent, K. A., Albright, R. G., Bennett, C. J., Kramer, L. D., and Bernard, K. A. (2007). Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. *PLoS Pathog.* 3, 1262–1270. doi: 10.1371/journal.ppat.0030132

Sun, W., Eckels, K. H., Putnak, J. R., Lyons, A. G., Thomas, S. J., Vaughn, D. W., et al. (2013). Experimental dengue virus challenge of human subjects previously vaccinated with live attenuated tetravalent dengue vaccines. *J. Infect. Dis.* 207, 700–708. doi: 10.1093/infdis/jis744

Surasombatpattana, P., Patramool, S., Luplertlop, N., Yssel, H., and Misse, D. (2012). Aedes aegypti saliva enhances dengue virus infection of human keratinocytes by suppressing innate immune responses. *J. Invest. Dermatol.* 132, 2103–2105. doi: 10.1038/jid.2012.76

Togo, Y. (1964). Hemagglutination-inhibition test in the study of measles immunity. *Am. J. Hyg.* 79, 250–259.

Tsai, J. J., Liu, L. T., Chang, K., Wang, S. H., Hsiao, H. M., Clark, K. B., et al. (2012). The importance of hematopoietic progenitor cells in dengue. *Ther. Adv. Hematol.* 3, 59–71. doi: 10.1177/2040620711417660

Watts, D. M., Burke, D. S., Harrison, B. A., Whitmire, R. E., and Nisalak, A. (1987). Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. *Am. J. Trop. Med. Hyg.* 36, 143–152.

Weiskopf, D., Angelo, M. A., de Azeredo, E. L., Sidney, J., Greenbaum, J. A., Fernando, A. N., et al. (2013). Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2046–E2053. doi: 10.1073/pnas.1305227110

Whitehead, R. H., Chaicumpa, V., Olson, L. C., and Russell, P. K. (1970). Sequential dengue virus infections in the white-handed gibbon (Hylobates lar). *Am. J. Trop. Med. Hyg.* 19, 94–102.

Who. (2011). "Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)," in *WHO Technical Report*, (Geneva).

Who. (2012). *Dengue and Severe Dengue* [Online]. Available online at: http://www.who.int/media centre/factsheets/fs117/en/ [Accessed June 23, 2013].

Who, and Tdr. (2009). "Dengue: guidelines for diagnosis, treatment, prevention, and control," in *For Research on Disease of Poverty*, eds K. Ciceri and P. Tissot (Geneva: WHO press), 1–147.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* 6, 93–106. doi: 10.1038/nri1779

Wong, C. H., Jenne, C. N., Petri, B., Chrobok, N. L., and Kubes, P. (2013). Nucleation of platelets with blood-borne pathogens on Kupffer cells precedes other innate immunity and contributes to bacterial clearance. *Nat. Immunol.* 14, 785–792. doi: 10.1038/ni.2631

Yoshida, T., Omatsu, T., Saito, A., Katakai, Y., Iwasaki, Y., Iijima, S., et al. (2012). CD16(+) natural killer cells play a limited role against primary dengue virus infection in tamarins. *Arch. Virol.* 157, 363–368. doi: 10.1007/s00705-011-1178-6

Yoshida, T., Omatsu, T., Saito, A., Katakai, Y., Iwasaki, Y., Kurosawa, T., et al. (2013). Dynamics of cellular immune responses in the acute phase of dengue virus infection. *Arch. Virol.* 158, 1209–1220. doi: 10.1007/s00705-013-1618-6

Zhang, Z. S., Yan, Y. S., Weng, Y. W., Huang, H. L., Li, S. Q., He, S., et al. (2007). High-level expression of recombinant dengue virus type 2 envelope domain III protein and induction of neutralizing antibodies in BALB/C mice. *J. Virol. Methods* 143, 125–131. doi: 10.1016/j.jviromet.2007.02.012

Zompi, S., and Harris, E. (2012). Animal models of dengue virus infection. *Viruses* 4, 62–82. doi: 10.3390/v4010062

Zompi, S., Santich, B. H., Beatty, P. R., and Harris, E. (2011). Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. *J. Immunol.* 188, 404–416. doi: 10.4049/jimmunol.1102124

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2013; paper pending published: 19 August 2013; accepted: 24 September 2013; published online: 11 October 2013.

*Citation: Clark KB, Onlamoon N, Hsiao H-M, Perng GC and Villinger F (2013) Can non-human primates serve as models for investigating dengue disease pathogenesis? Front. Microbiol. **4**:305. doi: 10.3389/fmicb.2013.00305*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 08 March 2013
doi: 10.3389/fmicb.2013.00049

A transgenic mouse model of human T cell leukemia virus type 1-associated diseases

Takeo Ohsugi*

Division of Microbiology and Genetics, Institute of Resource Development and Analysis, Kumamoto University, Kumamoto, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Yuetsu Tanaka, University of the Ryukyus, Japan
Makoto Yamagishi, The University of Tokyo, Japan
Seiji Okada, Kumamoto University, Japan

***Correspondence:**
Takeo Ohsugi, Division of Microbiology and Genetics, Institute of Resource Development and Analysis, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan.
e-mail: ohsugi@gpo.kumamoto-u.ac.jp

Human T cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T cell leukemia/lymphoma (ATLL) and several inflammatory diseases. Tax, the protein encoded by HTLV-1, may be responsible for the development of the diseases caused by this virus. To investigate the pathogenic role of Tax, several transgenic mouse strains expressing Tax have been developed in recent years. These mice develop various tumors including large granular lymphocytic leukemia, as well as inflammatory diseases such as arthritis. These results suggest that Tax expression alone is sufficient to cause both malignant neoplastic diseases and inflammatory diseases. However, until recently, there were no *tax* transgenic mice that develop T cell leukemia and lymphoma resembling ATLL. The first successful induction of leukemia in T cells was pre-T cell leukemia generated in transgenic mice in which a mouse lymphocyte-specific protein tyrosine kinase $p56^{lck}$ (*lck*)-proximal promoter was used to express the *tax* gene in immature T cells. Subsequently, transgenic mice were established in which the *lck*-distal promoter was used to express Tax in mature T cells; these mice developed mature T cell leukemia and lymphoma that more closely resembled ATLL than did earlier mouse models.

Keywords: animal model, ATLL, HTLV-1, Tax, transgenic mice

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) was the first human retrovirus to be isolated (Poiesz et al., 1980). It is estimated that 10–20 million people worldwide are infected with HTLV-1, which is endemic in southwestern Japan, the Caribbean Islands, South America, and Africa. Infection with HTLV-1 can result in an aggressive malignancy known as adult T cell leukemia/lymphoma (ATLL) or in inflammatory diseases, such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), after a prolonged period of latency often lasting between 20 and 50 years (Watanabe, 1997). The lifetime incidence of ATLL among carriers of HTLV-1 was estimated to be 1–5%, whereas that of HAM/TSP was 0.3–4.0% (Verdonck et al., 2007). The lifetime incidence of HTLV-1-associated diseases in general, including ATLL, HAM/TSP, and other inflammatory diseases, such as uveitis, polymyositis, arthropathy, and infective dermatitis, may be close to 10% (Verdonck et al., 2007). However, the reasons why HTLV-1-infected individuals develop different types of diseases and the mechanisms through which HTLV-1 causes these diseases remain unclear. To address these questions, an appropriate animal model is required.

HTLV-1 GENOME

The HTLV-1 genome consists of a diploid plus-strand RNA. Like other retroviruses, the integrated HTLV-1 proviral genome contains long-terminal-repeat (LTR) regions flanking the genes encoding the major structural proteins, *gag*, *pol*, and *env*. The genome also has an extra sequence designated *pX*. The *pX* region has four partially overlapping open reading frames designated I, II, III, and IV, which encode the proteins p12, p13, and p30, Rex, and Tax, respectively (Grassmann et al., 2005; Matsuoka and Jeang, 2007). Tax and Rex act in combination to regulate HTLV-1 gene expression and replication in both positive and negative pathways (Yoshida, 2005). p12 is thought to facilitate persistent viral infection (Albrecht et al., 2000). p30 attenuates HTLV-1 transcription by suppressing Tax protein synthesis (Nicot et al., 2004). The role of p13 is currently unclear. The HTLV-1 minus-strand RNA encodes a basic leucine zipper factor (HBZ) and the protein is synthesized in an antisense fashion from the 3′ LTR (Larocca et al., 1989; Gaudray et al., 2002). HBZ inhibits Tax-mediated transactivation of viral transcription (Arnold et al., 2006; Lemasson et al., 2007; Clerc et al., 2008). However, several researchers have reported that HBZ mRNA, but not HBZ protein, could induce T cell proliferation and to promote cell survival (Satou et al., 2006; Arnold et al., 2008). At present, the role of HBZ in HTLV-1 infection is controversial. More recently, Satou et al. (2011) created *hbz* transgenic mice and reported that more than one-third of these mice developed T cell lymphoma after a long latent period.

The transcription activator protein, Tax, is one of the regulatory proteins encoded by the *pX* region that has been extensively studied *in vitro*. Tax is a 40 kDa phosphoprotein that is essential for both viral replication and cellular transformation (Yoshida, 2001; Jeang et al., 2004). Transactivation of Tax is thought to initiate the processes that lead to ATLL or inflammatory diseases (Sun and Yamaoka, 2005; Matsuoka and Jeang, 2007; Currer et al., 2012; Yamagishi and Watanabe, 2012).

tax TRANSGENIC MICE

One of the best ways to investigate the oncogenic role(s) of *tax in vivo* is to generate a transgenic mouse model expressing HTLV-1 Tax (**Table 1**). The first HTLV-1 *tax* transgenic mice, in which Tax was expressed under the control of the HTLV-1 LTR, developed

Table 1 | Representative *tax* transgenic mouse models.

Promoter	Gene	Diseases	ATLL-like	HAM/TSP-like	Reference
HTLV-1 LTR	*tax*	Thymic involution Neurofibroma Early death	None	None	Hinrichs et al. (1987) Nerenberg et al. (1987)
HTLV-1 LTR	*tax*	Sjögren-like syndrome (exocrinopathy)	None	None	Green et al. (1989)
HTLV-1 LTR	*env-pX*	Arthritis	None	None	Iwakura et al. (1991)
HTLV-1 LTR	*tax*	Arthritis	None	None	Habu et al. (1999)
CD4	*tax*	Arthritis	None	None	
HTLV-1 LTR	*tax*	Skeletal abnormalities	None	None	Ruddle et al. (1993)
Granzyme B	*tax*	Granular lymphocytic leukemia	Leukemia/lymphoma	None	Grossman et al. (1995)
Metallothionein	*tax*	Arthropathy	None	None	Saggioro et al. (1997)
lck-proximal	*tax*	$CD4^-CD8^-$ pre-T-cell leukemia	Leukemia/lymphoma	None	Hasegawa et al. (2006)
lck-distal	*tax*	$CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell leukemia Arthropathy Histiocytic sarcoma (spinal cord)	Leukemia/lymphoma	Symmetrical paraparesis of the hind limbs	Ohsugi et al. (2007)

thymic involution, neurofibroma, and early death (Hinrichs et al., 1987; Nerenberg et al., 1987). Studies of these mice indicated that Tax expression alone was sufficient to induce tumorigenesis in transgenic mice. Iwakura et al. subsequently reported a very high incidence of inflammatory arthritis in transgenic mice carrying the HTLV-1 *env–pX* region (*pX* transgenic mice) or *tax* with the HTLV-1 LTR promoter (Iwakura et al., 1991; Habu et al., 1999). Arthropathy develops in *pX* transgenic mice as early as 4 weeks of age, and inflammatory arthropathy was also reported in another *tax* transgenic mouse model (Saggioro et al., 1997). These reports suggest that Tax expression induces inflammatory diseases in mice. Other transgenic mice were reported to develop Sjögren's-like syndrome (Green et al., 1989) and skeletal abnormalities (Ruddle et al., 1993).

However, none of these transgenic mouse models developed leukemia and lymphoma. The HTLV-1 LTR was used to regulate *tax* expression in these models. Other promoters were used in transgenic constructs to restrict *tax* expression to the lymphoid compartment and establish a better model of ATLL-like malignancies. Grossman et al. (1995) used the granzyme B promoter to drive *tax* expression in the mature T cell compartment. Those mice developed large granular lymphocytic leukemia, demonstrating that Tax expression alone in the lymphocyte compartment is sufficient for the development of leukemia.

T CELL LEUKEMIA IN *tax* TRANSGENIC MICE

Tax expression in transgenic mice caused large granular lymphocytic leukemia, but none of the transgenic mice developed T cell leukemia and lymphoma resembling ATLL. Recently, Hasegawa et al. (2006) established transgenic mice in which *tax* expression was restricted to thymocytes by using the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ (*lck*)-proximal promoter. These mice developed pre-T cell ($CD4^-CD8^-CD44^+CD25^+$) leukemia and lymphoma. Histological analysis showed diffuse, large-cell lymphomas involving the spleen, lymph nodes, liver, thymus, bone marrow, kidney, lung, meninges, and skin. The histopathological findings were identical to those observed in ATLL patients. The mice were functionally immunocompromised and developed opportunistic infections, which are also characteristics of ATLL. The leukemic cells were transplantable to severe combined immunodeficient mice. These transgenic mice demonstrated that Tax expression in the lymphocyte compartment is sufficient for the development of T cell leukemia and lymphoma. One major difference between these mice and humans with the disease is in the phenotype of the tumor cells, as the most common phenotype in ATLL in humans is $CD4^+$ mature T cells (Watanabe et al., 1997; Matsuoka and Jeang, 2007; Verdonck et al., 2007).

We created a transgenic mouse model of HTLV-1 using the distal promoter of *lck* to express *tax* in mature T cells (Ohsugi et al., 2007). The expression of the *lck* gene is regulated by two distinct promoter elements, a proximal and a distal promoter (Voronova et al., 1987; Perlmutter et al., 1988; Takadera et al., 1989). The *lck*-proximal promoter is most active in immature thymocytes, whereas the activity of the distal promoter is higher in mature thymocytes and peripheral T lymphocytes (Reynolds et al., 1990; Wildin et al., 1991; Allen et al., 1992). Tax mRNA expression in various organs of the transgenic mice was examined by quantitative real-time RT-PCR. The thymus and spleen strongly expressed Tax mRNA. Over 2 years, 28.1% of the *tax* transgenic mice developed mature T cell leukemia/lymphoma compared with just 1% of non-transgenic littermates.

PHENOTYPE OF T CELL LEUKEMIA IN *tax* TRANSGENIC MICE

The final cell specification to the T cell lineage takes place within the thymus. An overview of thymic T cell development is illustrated in **Figure 1**, and involves a series of distinct stages that

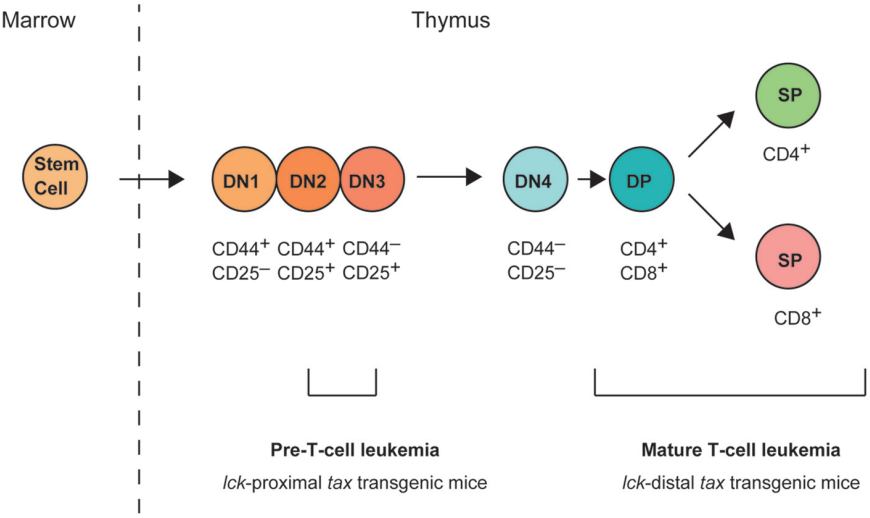


FIGURE 1 | T cell development in the thymus. Hematopoietic stem cells arise in the bone marrow and migrate to the thymus. Early committed T cells do not express CD4 or CD8, and are referred to as double-negative (DN; i.e., $CD4^-$ or $CD8^-$) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; and DN4, $CD44^-CD25^-$). At the DN3 stage, DN cells express a pre-T cell receptor (TCR) and its surface expression triggers the upregulation of CD4 and CD8 expression on the cell surface, and progression to the $CD4^+CD8^+$ double-positive (DP) stage. DP cells then mature into either $CD4^+$ or $CD8^+$ single-positive (SP) T cells. *lck*-proximal *tax* transgenic mice develop pre-T-cell leukemia originating from DN2/3 stage cells, whereas *lck*-distal *tax* transgenic mice develop mature leukemia originating after the progression to DP stage cells.

can be defined by the expression of cell-surface markers. Early T cells are $CD4^-CD8^-$ double-negative (DN) and can be further subdivided into DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) stages based on their expression of CD25 and CD44 (Koch and Radtke, 2011). The productive rearrangement of the T cell receptor (TCR) β chain locus occurs during the DN3 to DN4 transition, and leads to the expression of the pre-TCR. Only cells expressing functional pre-TCR proliferate and differentiate into $CD4^+CD8^+$ double-positive (DP) cells. However, most DP cells die through negative selection or neglect because their TCRs have too high or too low affinity for peptide–major histocompatibility complex molecule complexes. The cells that mature successfully migrate to the periphery as functional $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive T cells.

The phenotype of T cell leukemia in *lck*-proximal *tax* transgenic mice was $CD4^-CD8^-$, but $CD44^+$, $CD25^+$, and $CD117^-$ (also known as c-kit^-; Hasegawa et al., 2006). These results suggest that the malignant leukemic cells were derived from DN2/3 stage cells in the thymus. Transgenes controlled by the *lck*-proximal promoter are expressed very early in T cell development and can be detected in DN1 cells, although their expression in the early progenitors is not homogeneous until the DN3/4 stage (Cleverley et al., 1999; Buckland et al., 2000). Various studies have suggested that the Lck tyrosine kinase plays an important role in thymocyte development as a signaling molecule downstream from pre-TCR (Mombaerts et al., 1994; Wallace et al., 1995; van Oers et al., 1996; Fehling et al., 1997), while pre-TCR was first detected in the $CD44^-CD25^+$ DN3 subset (Saint-Ruf et al., 1994; Bruno et al., 1995). A green fluorescent protein (GFP) transgenic mouse was created in which *gfp* expression was under the control of the proximal promoter of *lck*, and the earliest GFP-positive cells were found among the $CD44^+CD25^-$ DN1 stage cells (Shimizu et al., 2001). Interestingly, pre-T cell leukemia in *lck*-proximal *tax* transgenic mice was probably derived from DN2/3 stage cells, even though the *lck*-proximal promoter is active in DN1–DN4 stage cells.

The phenotype of T cell leukemia in *lck*-distal *tax* transgenic mice displayed $CD4^+$, $CD8^+$, or $CD4^+CD8^+$ (DP) T cells. Overall, 60% of the leukemic cells were $CD8^+CD25^-$ T cells, 25% were $CD4^+CD25^-$ T cells, and 15% were $CD4^+CD8^+$ T cells (Ohsugi et al., 2007). As the Tax expression level did not vary in these cell populations, it remains unclear why $CD8^+$ T cells comprised approximately 60% of the total mature T cell leukemia cells in the *lck*-distal *tax* transgenic mice.

TAX-RELATED DISEASES IN *tax* TRANSGENIC MICE

ARTHROPATHY

tax transgenic mice develop an inflammatory arthropathy (Iwakura et al., 1991; Saggioro et al., 1997) that is pathologically similar to human rheumatoid arthritis and to mouse models of rheumatoid arthritis, with synovial proliferation and the expression of rheumatoid factor (Lee and Weinblatt, 2001; Luross and Williams, 2001; Firestein, 2003). By 24 months of age, our established *lck*-distal *tax* transgenic mice without leukemia developed severe arthropathy, with a cumulative incidence of 22.8% (Ohsugi and Kumasaka, 2011), but no arthropathic mouse was reported among the *lck*-proximal *tax* transgenic mice. The *lck*-distal *tax* transgenic mice with arthropathy differ in several aspects from other transgenic mice. They develop arthropathy after a prolonged latency period of at least 9 months, whereas the arthropathy that develops in the *pX* transgenic mice occurs as early as 4 weeks

of age. At 3 months of age, 60% (BALB/c background), 25% (C3H/He background), and 0% (C57BL/6 background) of *pX* transgenic mice displayed arthropathy (Iwakura et al., 1998). The genetic background of our established *lck*-distal *tax* transgenic mice was the F1 generation of a BDF1 (DBA/2 × C57BL/6) cross with C57BL/6. We attempted to generate *lck*-distal *tax* transgenic mice with the BALB/c background (backcross generation 8: N8), but did not observe a high incidence of arthropathy by 24 months of age. The expression of the cytokines interleukin-1β (IL-1β), IL-6, and macrophage migration inhibitory factor (MIF) was markedly enhanced in the joints of the *lck*-distal *tax* transgenic mice, but the expression of tumor necrosis factor-α (TNF-α) was not elevated. Ashino et al. (2007) also found that serum IL-1β and IL-6 concentrations were significantly higher in *pX* transgenic mice than those in non-transgenic or non-arthritic *pX* transgenic mice. Consistent with our arthropathic mice, their serum TNF-α concentrations were low, with no significant differences between the groups (Ashino et al., 2007). IL-6 is a key proinflammatory cytokine that is abundant in the synovium and synovial tissues of patients with rheumatoid arthritis (Okamoto et al., 1997). Taken together, these data suggest that proinflammatory cytokines, other than TNF-α, are important in the development of the inflammatory arthropathy associated with Tax expression.

HAM/TSP-LIKE DISEASE

Eight out of 297 *lck*-distal *tax* transgenic mice developed HAM/TSP-like disease with symmetrical paraparesis of the hind limbs, whereas these symptoms were absent in their non-transgenic littermates and in other mouse strains at our animal facilities (Ohsugi et al., in press). The *tax* transgenic mice with HAM/TSP-like disease had spinal cord lesions in the lumbar vertebrae that were caused by the infiltration of bone marrow-derived histiocytic sarcoma cells. Mice with HAM/TSP-like disease also displayed abnormal expression of cytokines and chemokines, including TNF-α and IL-6. Constitutive exposure to high levels of proinflammatory cytokines is thought to be protumorigenic (Balkwill, 2009; Grivennikov et al., 2009). Therefore, we speculated that *tax*-expressing T cells stimulate the proliferation of histiocytic cells in bone marrow through the activities of cytokines or chemokines. The transformed histiocytic cells may then predominantly invade the lumbar spinal cord (Ohsugi et al., in press).

To my knowledge, there have been no reports of spontaneous symmetrical paraparesis caused by histiocytic sarcoma in mice. However, the lesions in patients with HAM/TSP show marked T cell infiltration, and the disease is associated with an inflammatory state (Sakai et al., 2001; Kubota et al., 2002; Goon et al., 2003; Muraro et al., 2003). Therefore, it is important to note that the etiology of HAM/TSP-like disease in *lck*-distal *tax* transgenic mice differs substantially from that of HAM/TSP in humans. Nevertheless, the present results indicate that the relationship between HTLV-1 infection and histiocytic disorders should be the focus of future studies. In particular, those studies should examine whether the cytokines and chemokines secreted from HTLV-1-infected T cells induce the growth or oncogenic transformation of histiocytic cells in humans. A recent paper proposed that, in a murine model of multiple sclerosis, there is a gateway through which immune cells can enter the central nervous system (Arima et al., 2012). The authors described an entry site by the dorsal blood vessels of the fifth lumbar cord through which immune cells can enter the central nervous system. Histiocytic sarcoma grew predominantly in the spinal cord of the fifth to sixth lumbar vertebrae in HAM/TSP-like mice. Further studies are required to confirm that histiocytic sarcoma cells can access the central nervous system via the spinal cord at the fifth lumbar vertebra. Such studies may clarify the mechanisms underlying the movement of Tax-positive T cells into the central nervous system in humans with HAM/TSP.

CONCLUSION

Several strains of HTLV-1 *tax* transgenic mice have been developed over recent years. Studies in these mice have shown that Tax expression alone is sufficient to cause both malignant neoplastic diseases, including T cell leukemia and lymphoma, and inflammatory diseases, such as arthropathy. These mice will be widely used to study the pathogenesis of HTLV-1 and to evaluate new anticancer and anti-inflammatory agents for HTLV-1-related diseases. However, until now, none of the *tax* transgenic mice developed HAM/TSP-like disease, a systemic immune-mediated inflammatory disease that resembles the disease in humans. Further studies are required to establish a transgenic mouse model of HAM/TSP with the selection of appropriate promoters.

REFERENCES

Albrecht, B., Collins, N. D., Burniston, M. T., Nisbet, J. W., Ratner, L., Green, P. L., et al. (2000). Human T-lymphotropic virus type 1 open reading frame I $p12^{I}$ is required for efficient viral infectivity in primary lymphocytes. *J. Virol.* 74, 9828–9835.

Allen, J. M., Forbush, K. A., and Perlmutter, R. M. (1992). Functional dissection of the *lck* proximal promoter. *Mol. Cell. Biol.* 12, 2758–2768.

Arima, Y., Harada, M., Kamimura, D., Park, J. H., Kawano, F., Yull, F. E., et al. (2012). Regional neural activation defines a gateway for autoreactive T cells to cross the blood–brain barrier. *Cell* 148, 447–457.

Arnold, J., Yamamoto, B., Li, M., Phipps, A. J., Younis, I., Lairmore, M. D., et al. (2006). Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1. *Blood* 107, 3976–3982.

Arnold, J., Zimmerman, B., Li, M., Lairmore, M. D., and Green, P. L. (2008). Human T-cell leukemia virus type-1 antisense-encoded gene, *Hbz*, promotes T-lymphocyte proliferation. *Blood* 112, 3788–3797.

Ashino, T., Arima, Y., Shioda, S., Iwakura, Y., Numazawa, S., and Yoshida, T. (2007). Effect of interleukin-6 neutralization on CYP3A11 and metallothionein-1/2 expressions in arthritic mouse liver. *Eur. J. Pharmacol.* 558, 199–207.

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nat. Rev. Cancer* 9, 361–371.

Bruno, L., Rocha, B., Rolink, A., Von Boehmer, H., and Rodewald, H. R. (1995). Intra- and extra-thymic expression of the pre-T cell receptor α gene. *Eur. J. Immunol.* 25, 1877–1882.

Buckland, J., Pennington, D. J., Bruno, L., and Owen, M. J. (2000). Co-ordination of the expression of the protein tyrosine kinase $p56^{lck}$ with the pre-T cell receptor during thymocyte development. *Eur. J. Immunol.* 30, 8–18.

Clerc, I., Polakowski, N., Andre-Arpin, C., Cook, P., Barbeau, B., Mesnard, J. M., et al. (2008). An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CBP contributes to the down-regulation of tax-dependent viral transcription by HBZ. *J. Biol. Chem.* 283, 23903–23913.

Cleverley, S., Henning, S., and Cantrell, D. (1999). Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function. *Curr. Biol.* 9, 657–660.

Currer, R., Van Duyne, R., Jaworski, E., Guendel, I., Sampey, G., Das, R., et al. (2012). HTLV Tax:

a fascinating multifunctional co-regulator of viral and cellular pathways. *Front. Microbiol.* 3:406. doi: 10.3389/fmicb.2012.00406

Fehling, H. J., Iritani, B. M., Krotkova, A., Forbush, K. A., Laplace, C., Perlmutter, R. M., et al. (1997). Restoration of thymopoiesis in pT$\alpha^{-/-}$ mice by anti-CD3ε antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity* 6, 703–714.

Firestein, G. S. (2003). Evolving concepts of rheumatoid arthritis. *Nature* 423, 356–361.

Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2002). The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J. Virol.* 76, 12813–12822.

Goon, P. K., Igakura, T., Hanon, E., Mosley, A. J., Asquith, B., Gould, K. G., et al. (2003). High circulating frequencies of tumor necrosis factor alpha- and interleukin-2-secreting human T-lymphotropic virus type 1 (HTLV-1)-specific $CD4^+$ T cells in patients with HTLV-1-associated neurological disease. *J. Virol.* 77, 9716–9722.

Grassmann, R., Aboud, M., and Jeang, K. T. (2005). Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* 24, 5976–5985.

Green, J. E., Hinrichs, S. H., Vogel, J., and Jay, G. (1989). Exocrinopathy resembling Sjogren's syndrome in HTLV-1 tax transgenic mice. *Nature* 341, 72–74.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G. Y., Vallabhapurapu, S., et al. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 15, 103–113.

Grossman, W. J., Kimata, J. T., Wong, F. H., Zutter, M., Ley, T. J., and Ratner, L. (1995). Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1057–1061.

Habu, K., Nakayama-Yamada, J., Asano, M., Saijo, S., Itagaki, K., Horai, R., et al. (1999). The human T cell leukemia virus type I-tax gene is responsible for the development of both inflammatory polyarthropathy resembling rheumatoid arthritis and noninflammatory ankylotic arthropathy in transgenic mice. *J. Immunol.* 162, 2956–2963.

Hasegawa, H., Sawa, H., Lewis, M. J., Orba, Y., Sheehy, N., Yamamoto, Y., et al. (2006). Thymus-derived leukemia–lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. *Nat. Med.* 12, 466–472.

Hinrichs, S. H., Nerenberg, M., Reynolds, R. K., Khoury, G., and Jay, G. (1987). A transgenic mouse model for human neurofibromatosis. *Science* 237, 1340–1343.

Iwakura, Y., Itagaki, K., Ishitsuka, C., Yamasaki, Y., Matsuzawa, A., Yonehara, S., et al. (1998). The development of autoimmune inflammatory arthropathy in mice transgenic for the human T cell leukemia virus type-1 *env-pX* region is not dependent on H-2 haplotypes and modified by the expression levels of Fas antigen. *J. Immunol.* 161, 6592–6598.

Iwakura, Y., Tosu, M., Yoshida, E., Takiguchi, M., Sato, K., Kitajima, I., et al. (1991). Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I. *Science* 253, 1026–1028.

Jeang, K. T., Giam, C. Z., Majone, F., and Aboud, M. (2004). Life, death, and tax: role of HTLV-I oncoprotein in genetic instability and cellular transformation. *J. Biol. Chem.* 279, 31991–31994.

Koch, U., and Radtke, F. (2011). Mechanisms of T cell development and transformation. *Annu. Rev. Cell Dev. Biol.* 27, 539–562.

Kubota, R., Soldan, S. S., Martin, R., and Jacobson, S. (2002). Selected cytotoxic T lymphocytes with high specificity for HTLV-I in cerebrospinal fluid from a HAM/TSP patient. *J. Neurovirol.* 8, 53–57.

Larocca, D., Chao, L. A., Seto, M. H., and Brunck, T. K. (1989). Human T-cell leukemia virus minus strand transcription in infected T-cells. *Biochem. Biophys. Res. Commun.* 163, 1006–1013.

Lee, D. M., and Weinblatt, M. E. (2001). Rheumatoid arthritis. *Lancet* 358, 903–911.

Lemasson, I., Lewis, M. R., Polakowski, N., Hivin, P., Cavanagh, M. H., Thebault, S., et al. (2007). Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription. *J. Virol.* 81, 1543–1553.

Luross, J. A., and Williams, N. A. (2001). The genetic and immunopathological processes underlying collagen-induced arthritis. *Immunology* 103, 407–416.

Matsuoka, M., and Jeang, K. T. (2007). Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat. Rev. Cancer* 7, 270–280.

Mombaerts, P., Anderson, S. J., Perlmutter, R. M., Mak, T. W., and Tonegawa, S. (1994). An activated *lck* transgene promotes thymocyte development in RAG-1 mutant mice. *Immunity* 1, 261–267.

Muraro, P. A., Wandinger, K. P., Bielekova, B., Gran, B., Marques, A., Utz, U., et al. (2003). Molecular tracking of antigen-specific T cell clones in neurological immune-mediated disorders. *Brain* 126, 20–31.

Nerenberg, M., Hinrichs, S. H., Reynolds, R. K., Khoury, G., and Jay, G. (1987). The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. *Science* 237, 1324–1329.

Nicot, C., Dundr, M., Johnson, J. M., Fullen, J. R., Alonzo, N., Fukumoto, R., et al. (2004). HTLV-1-encoded $p30^{II}$ is a post-transcriptional negative regulator of viral replication. *Nat. Med.* 10, 197–201.

Ohsugi, T., and Kumasaka, T. (2011). Low CD4/CD8 T-cell ratio associated with inflammatory arthropathy in human T-cell leukemia virus type I Tax transgenic mice. *PLoS ONE* 6:e18518. doi: 10.1371/journal.pone.0018518

Ohsugi, T., Kumasaka, T., Okada, S., and Urano, T. (2007). The Tax protein of HTLV-1 promotes oncogenesis in not only immature T cells but also mature T cells. *Nat. Med.* 13, 527–528.

Ohsugi, T., Wakamiya, M., Morikawa, S., Matsuura, K., Kumar, J. M., Kumasaka, T., et al. (in press). Invasion of histiocytic sarcoma into the spinal cord of HTLV-1 Tax transgenic mice with HTLV-1-associated myelopathy/tropical spastic paraparesis-like disease. *Oncol. Res.* (in press).

Okamoto, H., Yamamura, M., Morita, Y., Harada, S., Makino, H., and Ota, Z. (1997). The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. *Arthritis Rheum.* 40, 1096–1105.

Perlmutter, R. M., Marth, J. D., Lewis, D. B., Peet, R., Ziegler, S. F., and Wilson, C. B. (1988). Structure and expression of *lck* transcripts in human lymphoid cells. *J. Cell. Biochem.* 38, 117–126.

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D., and Gallo, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 77, 7415–7419.

Reynolds, P. J., Lesley, J., Trotter, J., Schulte, R., Hyman, R., and Sefton, B. M. (1990). Changes in the relative abundance of type I and type II *lck* mRNA transcripts suggest differential promoter usage during T-cell development. *Mol. Cell. Biol.* 10, 4266–4270.

Ruddle, N. H., Li, C. B., Horne, W. C., Santiago, P., Troiano, N., Jay, G., et al. (1993). Mice transgenic for HTLV-I LTR-tax exhibit *tax* expression in bone, skeletal alterations, and high bone turnover. *Virology* 197, 196–204.

Saggioro, D., Rosato, A., Esposito, G., Rosenberg, M. P., Harrison, J., Felber, B. K., et al. (1997). Inflammatory polyarthropathy and bone remodeling in HTLV-I Tax-transgenic mice. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 14, 272–280.

Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H. J., and Von Boehmer, H. (1994). Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266, 1208–1212.

Sakai, J. A., Nagai, M., Brennan, M. B., Mora, C. A., and Jacobson, S. (2001). In vitro spontaneous lymphoproliferation in patients with human T-cell lymphotropic virus type I-associated neurologic disease: predominant expansion of $CD8^+$ T cells. *Blood* 98, 1506–1511.

Satou, Y., Yasunaga, J., Yoshida, M., and Matsuoka, M. (2006). HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 720–725.

Satou, Y., Yasunaga, J., Zhao, T., Yoshida, M., Miyazato, P., Takai, K., et al. (2011). *HTLV-1 bZIP factor* induces T-cell lymphoma and systemic inflammation in vivo. *PLoS Pathog.* 7:e1001274. doi: 10.1371/journal.ppat.1001274

Shimizu, C., Kawamoto, H., Yamashita, M., Kimura, M., Kondou, E., Kaneko, Y., et al. (2001). Progression of T cell lineage restriction in the earliest subpopulation of murine adult thymus visualized by the expression of *lck* proximal promoter activity. *Int. Immunol.* 13, 105–117.

Sun, S. C., and Yamaoka, S. (2005). Activation of NF-κB by HTLV-I and implications for cell transformation. *Oncogene* 24, 5952–5964.

Takadera, T., Leung, S., Gernone, A., Koga, Y., Takihara, Y., Miyamoto, N. G., et al. (1989). Structure of the two promoters of the human *lck* gene: differential accumulation of two classes of *lck* transcripts in T cells. *Mol. Cell. Biol.* 9, 2173–2180.

van Oers, N. S., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). αβ T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. *Immunity* 5, 429–436.

Verdonck, K., Gonzalez, E., Van Dooren, S., Vandamme, A. M., Vanham, G., and Gotuzzo, E. (2007). Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect. Dis.* 7, 266–281.

Voronova, A. F., Adler, H. T., and Sefton, B. M. (1987). Two *lck* transcripts containing different 5′ untranslated regions are present in T cells. *Mol. Cell. Biol.* 7, 4407–4413.

Wallace, V. A., Kawai, K., Levelt, C. N., Kishihara, K., Molina, T., Timms, E., et al. (1995). T lymphocyte development in $p56^{lck}$ deficient mice: allelic exclusion of the TcR β locus is incomplete but thymocyte development is not restored by TcR β or TcR αβ transgenes. *Eur. J. Immunol.* 25, 1312–1318.

Watanabe, T. (1997). HTLV-1-associated diseases. *Int. J. Hematol.* 66, 257–278.

Watanabe, T., Mochizuki, M., and Yamaguchi, K. (1997). HTLV-1 uveitis (HU). *Leukemia* 11(Suppl. 3), 582–584.

Wildin, R. S., Garvin, A. M., Pawar, S., Lewis, D. B., Abraham, K. M., Forbush, K. A., et al. (1991). Developmental regulation of *lck* gene expression in T lymphocytes. *J. Exp. Med.* 173, 383–393.

Yamagishi, M., and Watanabe, T. (2012). Molecular hallmarks of adult T cell leukemia. *Front. Microbiol.* 3:334. doi: 10.3389/fmicb.2012.00334

Yoshida, M. (2001). Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. *Annu. Rev. Immunol.* 19, 475–496.

Yoshida, M. (2005). Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis. *Oncogene* 24, 5931–5937.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 January 2013; accepted: 21 February 2013; published online: 08 March 2013.

Citation: Ohsugi T (2013) A transgenic mouse model of human T cell leukemia virus type 1-associated diseases. Front. Microbiol. ***4****:49. doi: 10.3389/fmicb.2013.00049*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 08 November 2013
doi: 10.3389/fmicb.2013.00328

Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus

***Yoko Aida*[1]*, *Hironobu Murakami*[1,2], *Masahiko Takahashi*[3] *and Shin-Nosuke Takeshima*[1]**

[1] *Viral Infectious Diseases Unit, RIKEN, Wako, Saitama, Japan*
[2] *Laboratory of Animal Health II, Azabu University, Sagamihara, Kanagawa, Japan*
[3] *Division of Virology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Toshiki Watanabe, The University of Tokyo, Japan
Takeo Ohsugi, Kumamoto University, Japan

***Correspondence:**
Yoko Aida, Viral Infectious Diseases Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
e-mail: aida@riken.jp

Bovine leukemia virus (BLV) and human T-cell leukemia virus type 1 (HTLV-1) make up a unique retrovirus family. Both viruses induce chronic lymphoproliferative diseases with BLV affecting the B-cell lineage and HTLV-1 affecting the T-cell lineage. The pathologies of BLV- and HTLV-induced infections are notably similar, with an absence of chronic viraemia and a long latency period. These viruses encode at least two regulatory proteins, namely, Tax and Rex, in the pX region located between the *env* gene and the 3′ long terminal repeat. The Tax protein is a key contributor to the oncogenic potential of the virus, and is also the key protein involved in viral replication. However, BLV infection is not sufficient for leukemogenesis, and additional events such as gene mutations must take place. In this review, we first summarize the similarities between the two viruses in terms of genomic organization, virology, and pathology. We then describe the current knowledge of the BLV model, which may also be relevant for the understanding of leukemogenesis caused by HTLV-1. In addition, we address our improved understanding of Tax functions through the newly identified BLV Tax mutants, which have a substitution between amino acids 240 and 265.

Keywords: BLV, HTLV-1, EBL, B-cell lymphoma, Tax, leukemogensis, transactivation, apoptosis

INTRODUCTION

Bovine leukosis was first reported in 1871 as the presence of slightly yellow nodules in the enlarged spleen of cattle (Leisering, 1871). Spleen disruption consecutive to tumor formation is one of the most important clinical manifestations of bovine leukemia. Bovine leukosis is classified into two types, sporadic bovine leukosis (SBL) and enzootic bovine leukosis (EBL), which are characterized by T- and B-cell leukosis, respectively (Gillet et al., 2007). The occurrence of EBL in cattle is much higher than that of SBL (Theilen and Dungworth, 1965; Onuma et al., 1979). Bovine leukemia virus (BLV), which belongs to the *Retroviridae* family and *Deltaretrovirus* genus, is the etiologic agent of EBL, although it remains unknown what causes SBL (Gillet et al., 2007). The natural hosts of BLV are domestic cattle and water buffaloes; however, experimental infection with BLV in sheep can lead to the development of lymphoma (Djilali and Parodi, 1989). Interestingly, BLV is consistently associated with leukemia only in cattle and sheep, even though it can infect many cell lines (Graves and Ferrer, 1976) and can be experimentally transmitted to rabbits (Wyatt et al., 1989; Onuma et al., 1990), rats (Altanerova et al., 1989), chickens (Altanerova et al., 1990), pigs, goats, and sheep (Mammerickx et al., 1981). Most BLV-infected cattle are asymptomatic, but approximately one-third of them suffer from persistent lymphocytosis (PL) characterized by non-malignant polyclonal B-cell expansion and 1–5% of them develop B-cell leukemia/lymphoma after a long latency period (Gillet et al., 2007). On the other hand, sheep that are experimentally inoculated with BLV develop B-cell tumors at a higher frequency and with a shorter latency period than those observed in naturally infected cattle (Ferrer et al., 1978; Burny et al., 1979; Kenyon et al., 1981; Aida et al., 1989). Interestingly, the transformed B-lymphocytes in cattle are $CD5^+$ IgM^+ B-cells (Aida et al., 1993), whereas in sheep they are $CD5^-$ IgM^+ B-cells (Murakami et al., 1994a,b), suggesting that the mechanisms of leukemogenesis induced by BLV may differ (Graves and Ferrer, 1976; Djilali and Parodi, 1989).

BLV is closely related to human T-cell leukemia virus type 1 (HTLV-1), which is the causative agent of adult T-cell leukemia (ATL) and a chronic neurological disorder known as tropical spastic paraparesis or HTLV-1-associated myelopathy HAM/TSP (Gessain et al., 1985; Osame et al., 1986; Gillet et al., 2007). Therefore, studies on BLV may facilitate our understanding of the mechanism of leukemogenesis induced by HTLV-1.

BLV AND HTLV-1

All retroviruses are encoded by *gag*, *pro*, *pol*, and *env* essential genes, which are necessary for the production of infectious virions, and are flanked by two identical long terminal repeats (LTRs; **Figure 1**). The *gag*, *pro*, *pol*, and *env* genes encode the internal structural proteins of the virion, the viral protease, the reverse transcriptase, and the envelope glycoproteins of the virion, respectively. The genome sequences of BLV and HTLV-1 are different, but have a unique sequence called the pX situated between the *env* gene and the 3′LTR and encoded by the regulatory gene (**Figure 1**). The pX sequence is not of host cell origin; that is, it is not an oncogene. It has been reported that both viruses have an ability to immortalize primary cells *in vitro* (Grassmann et al., 1989; Willems

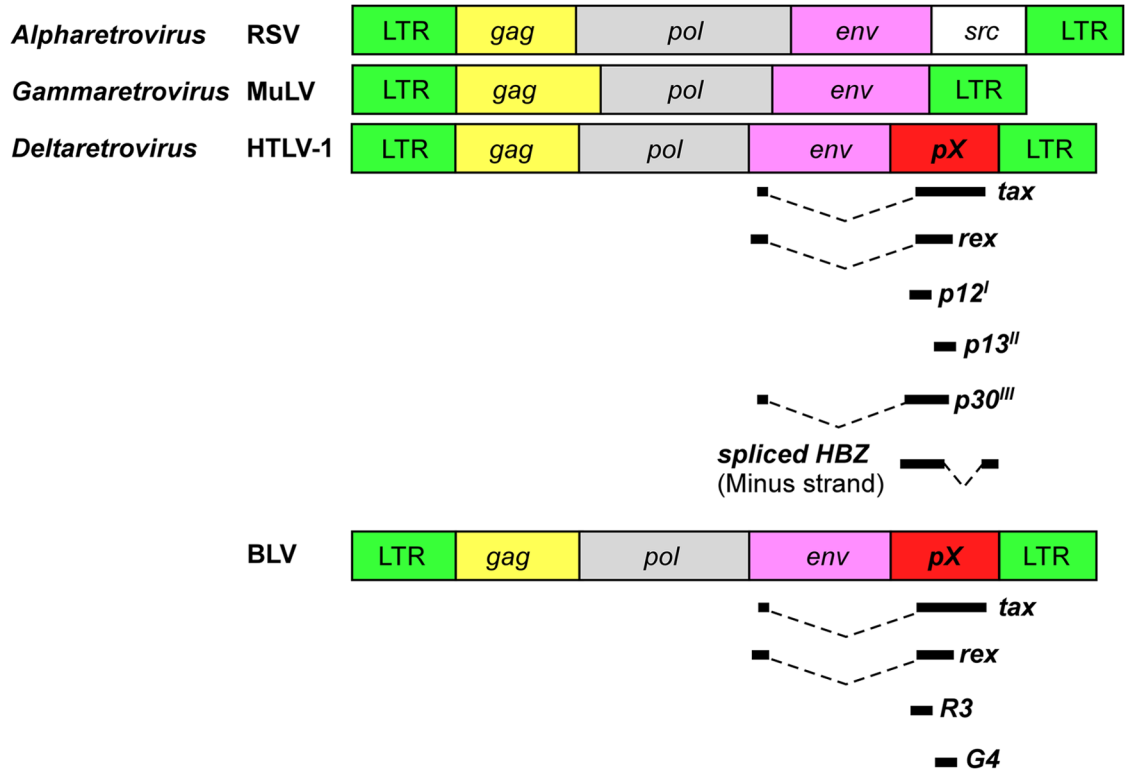


FIGURE 1 | Schematic representation of genome organization of retroviruses. HTLV-1 and BLV encode unique regulatory and accessory proteins in the pX region. RSV, Rous sarcoma virus; MuLV, murine leukemia virus.

et al., 1990). Because their structure and properties differ from any other class of retroviruses, BLV and HTLV-1 viruses were classified into a new group of retroviruses (Gillet et al., 2007). In both viruses the regulatory proteins Tax and Rex are encoded in the pX region. The R3 and G4 proteins are encoded in the BLV pX region, while $p12^{I}$, $p13^{II}$, and $p30^{II}$ are encoded in the HTLV-1 pX region (Sagata et al., 1984b; Franchini et al., 2003; **Figure 1**). Interestingly, the HTLV-1 genome codes for HBZ, a unique gene encoded by the minus strand chain (Gaudray et al., 2002; **Figure 1**). The major functions of the viral proteins encoded in the BLV and HTLV-1 pX regions are summarized in **Table 1**. The Tax protein has been extensively studied, and it is believed to play a critical role in leukemogenesis induced by BLV and HTLV-1 (Katoh et al., 1989; Tanaka et al., 1990; Willems et al., 1990). The Rex protein is responsible for nuclear export of viral RNA and promotes cytoplasmic accumulation and translation of viral messenger mRNA in BLV- and HTLV-1-infected cells (Felber et al., 1989). BLV R3 and G4 proteins contribute to the maintenance of high viral load (Willems et al., 1994; Florins et al., 2007). The G4 protein is particularly relevant to leukemogenesis, since it can immortalize primary rat embryo fibroblasts (REFs; Lefebvre et al., 2002). HTLV-1 $p12^{I}$ is similar to the R3 protein, in that it contributes to the maintenance of infectivity (Collins et al., 1998), and both proteins are located in the nucleus and cellular membranes (Gillet et al., 2007). On the other hand, HTLV-1 $p13^{II}$ protein resembles the G4 protein, since both proteins bind to farnesyl pyrophosphate synthetase, which farnesylates Ras (Lefebvre et al., 2002), and the $p13^{II}$ protein promotes Ras-dependent apoptosis (Hiraragi et al., 2005). HTLV-1 $p30^{II}$ protein regulates gene transcription through its interaction with the cAMP responsive element (CRE) binding protein (CREB)/p300 (Zhang et al., 2001). The HBZ protein plays a critical role in the leukemogenesis of HTLV-1, and HBZ knockdown inhibits the proliferation of ATL cells (Satou et al., 2006). However, since the BLV genome does not code for HBZ, it is assumed that the Tax protein plays a central role in the leukemogenesis of BLV.

The infection route of BLV and HTLV is by horizontal and vertical transmission. BLV is transmitted via direct contact (Kono et al., 1983), milk, and insect bites (Ferrer and Piper, 1978), while HTLV-1 is transmitted via milk and sexual intercourse (Bangham, 2003). Moreover, the artificial transmission of BLV is caused by iatrogenic procedures such as dehorning, ear tattooing, and reuse of needles (Hopkins and DiGiacomo, 1997), whereas the artificial transmission of HTLV-1 is caused by blood transfusion and needle sharing among drug abusers (Robert-Guroff et al., 1986). Since cell contact is required for the efficient transmission of both BLV and HTLV-1, cell-free infection by these viruses is believed to be very inefficient, most probably due to virion instability (Voneche et al., 1992; Johnston et al., 1996; Igakura et al., 2003).

As shown in **Figure 2**, an infection with BLV is characterized by three progressive stages of disease, including an asymptomatic

Table 1 | Viral proteins are encoded in BLV and HTLV-1 pX regions.

Virus	Viral protein	Major reported functions	Reference
BLV	Tax	Transcriptional activator of viral expression	Derse (1987), Willems et al. (1987), Katoh et al. (1989)
		Oncogenic potential	Willems et al. (1990)
		Activation of NF-kappa B (NF-κB) pathway	Szynal et al. (2003), Klener et al. (2006)
	Rex	Nuclear export of viral mRNAs	Felber et al. (1989)
	G4	The maintenance of high viral load	Willems et al. (1994), Florins et al. (2007)
		Oncogenic potential	Kerkhofs et al. (1998), Lefebvre et al. (2002)
	R3	The maintenance of high viral load	Willems et al. (1994), Florins et al. (2007)
HTLV-1	Tax	Transcriptional activator of viral expression	Kashanchi and Brady (2005)
		Oncogenic potential	Matsuoka and Jeang (2011)
		Induction of DNA damage, cellular senescence and apoptosis	Chlichlia and Khazaie (2010)
		Functional regulation of many cellular proteins by direct binding	Boxus et al. (2008)
	HBZ	Inhibition of HTLV-1 transcription	Lemasson et al. (2007)
		Suppression of the classical pathway of NF-κB	Zhao et al. (2009)
		Enhancement of TGF-β signaling	Zhao et al. (2011)
		Oncogenic potential	Satou et al. (2006, 2011)
	Rex	Nuclear export of viral mRNAs	Felber et al. (1989)
	$p12^{I}$	Maintenance of viral infectivity	Collins et al. (1998)
		Activation of nuclear factor of activated T-cells (NFAT) pathway	Ding et al. (2002)
	$p13^{II}$	Suppression of viral replication	Andresen et al. (2011)
		Interaction with farnesyl pyrophosphate synthetase	Lefebvre et al. (2002)
		Activation of Ras-mediated apoptosis	Hiraragi et al. (2005)
	$p30^{II}$	Suppression of viral replication	Nicot et al. (2004)
		Regulation of gene transcription by binding with p300	Zhang et al. (2001)
		Enhancement of Myc transforming potential	Zhang et al. (2001)

stage, PL, and lymphoma. Most BLV-infected cattle are asymptomatic, but approximately one-third of them suffer from PL characterized by a permanent and relatively stable increase in the number of B-lymphocytes in the peripheral blood. PL is considered to be a benign form of the disease resulting from the accumulation of untransformed B-lymphocytes. Finally, 1–5% of BLV-infected cattle develop B-lymphoma in various lymph nodes after a long latency period (Schwartz and Levy, 1994; Florins et al., 2008). Although BLV can also infect $CD4^+$ T-cells, $CD8^+$ T-cells, γ/δ T-cells, monocytes, and granulocytes in cattle (Williams et al., 1988; Stott et al., 1991; Schwartz et al., 1994; Mirsky et al., 1996; Wu et al., 1996; Panei et al., 2013), a large number of the tumor cells are derived from $CD5^+$ IgM^+ B-cell subpopulations (Schwartz and Levy, 1994). Interestingly, the full-length BLV proviral genome is maintained in each animal throughout the course of the disease (Tajima et al., 1998a). In addition, previous studies have shown that both large and small deletions of proviral genomes may be very rare events in BLV-infected cattle. Thus, the proviral loads were significantly increased at the PL stage compared with the aleukemic stage and were further increased at the lymphoma stage (Jimba et al., 2010, 2012; **Figure 2B**). These findings clearly demonstrated that the BLV proviral copy number increases with increasing severity of the disease. On the other hand, unlike BLV, HTLV-1 is associated with ATL and with the chronic neurological disorder, HAM/TSP, and induces not only a malignant tumor but also an inflammatory disease (Gessain et al., 1985; Osame et al., 1986). Although the pathogenesis of HTLV-1 is slightly different from BLV, HTLV-1, like BLV, can infect many cells in addition to T-cells, including B-cells and monocytes (Koyanagi et al., 1993; Schwartz and Levy, 1994). In contrast to BLV, defective HTLV-1 proviral genomes have been found in more than half of all examined patients with ATL (Konishi et al., 1984; Korber et al., 1991; Ohshima et al., 1991; Tsukasaki et al., 1997).

MECHANISM OF LEUKEMOGENESIS BY BLV

Animal retroviruses, which belong to the *Alpharetrovirus* and *Gammaretrovirus* genera, induce tumors by one of two mechanisms: either by activation of the "viral oncogene" or by "insertional activation" of a cellular gene such as a proto-oncogene (Weiss et al., 1985; **Figure 1**). By contrast, BLV lacks a known oncogene (Sagata et al., 1984a,b) and does not integrate into preferred sites in their host cell genomes, which related to the disruption of the host gene but not to the suppression of viral gene expression (Murakami et al., 2011b).

Most studies of BLV-induced leukemogenesis have focused on the Tax protein because it is believed to be a potent transcriptional

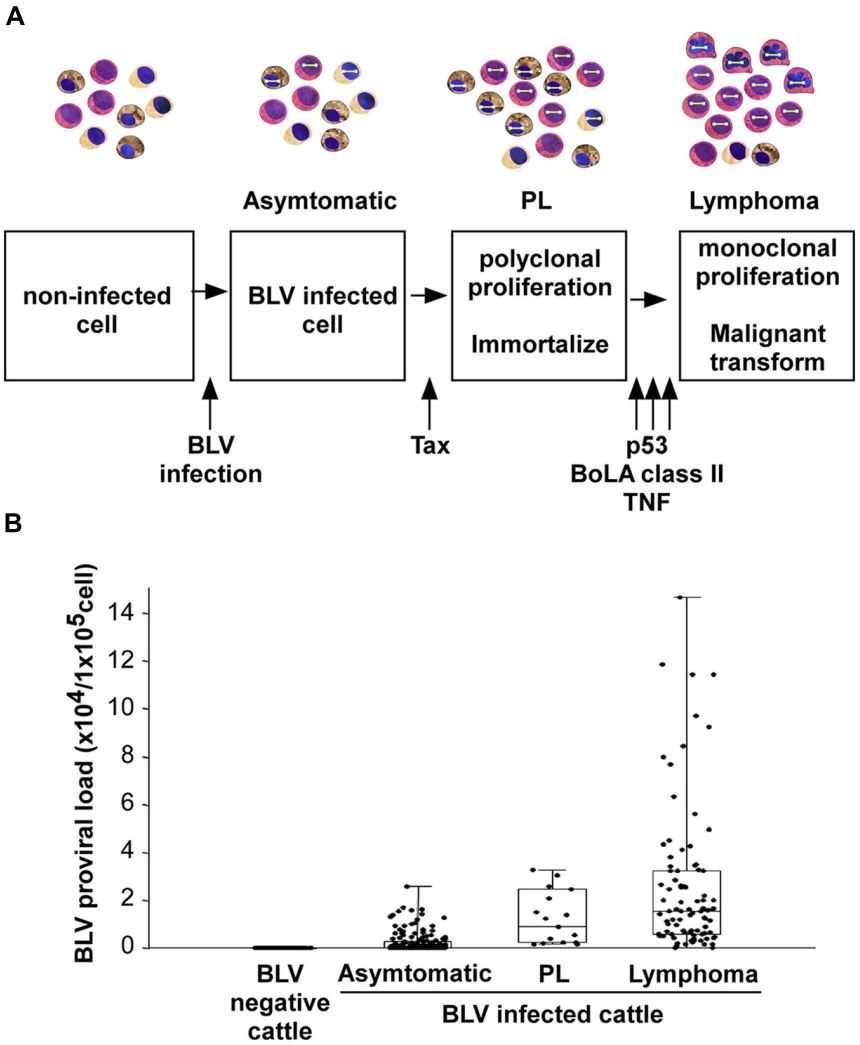


FIGURE 2 | BLV-induced leukemogenesis is a multistep process. (A) An infection with BLV is characterized by three progressive stages of disease: asymptomatic stage, persistent lymphocytosis (PL), and lymphoma. BLV infects to cells non-specifically. Among them, BLV Tax protein immortalizes a part of BLV-infected cells, probably only $CD5^+$ IgM^+ B-cells, and induces polyclonal proliferation of the cells. However, the Tax protein does not have the ability to transform the cells. For lymphoma to develop, a malignant transformation needs to occur with the help of host factors, such as p53 mutation, TNF-α activities or bovine leukocyte antigen (BoLA) class II phosphorylation. **(B)** The provirus load increases with disease progression.

activator of viral gene expression. In addition to its function as a transcriptional activator, Tax induces immortalization of primary REFs (Willems et al., 1990, 1998). Furthermore, Tax cooperates with the Harvey rat sarcoma viral oncoprotein (Ha-ras) for the induction of full transformation of primary REF (Willems et al., 1990). Importantly, the Tax transformed cells induce tumors in nude mice. The ability of the Tax protein to induce immortalization may be the first step in the BLV-mediated transformation process. Moreover, after the infection of cattle and during the latency period, the expression of BLV becomes blocked at the transcriptional level (Kettmann et al., 1982; Lagarias and Radke, 1989). Such repression appears to be very important for the escape of BLV from the host's immunosurveillance system, and later only a certain small proportion of infected animals rapidly develop a terminal disease (Gillet et al., 2007). Indeed, transcription of the BLV genome in fresh tumor cells or in fresh peripheral blood mononuclear cells (PBMCs) from infected individuals is almost undetectable by conventional techniques (Kettmann et al., 1982; Tajima et al., 2003b; Tajima and Aida, 2005). *In situ* hybridization has revealed the expression of viral RNA at low levels in many cells, and at a high level in only a few cells within PBMCs freshly isolated from BLV-infected asymptomatic animals (Lagarias and Radke, 1989). Thus, BLV infection is probably not sufficient for leukemogenesis and some additional events such as gene mutations might be involved in the leukemogenic process (**Figure 2A**). Taken together, Tax may induce immortalization of only $CD5^+$ IgM^+ B-cells among BLV-infected B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, γ/δ T-cells, monocytes, and granulocytes in cattle, thereby conferring a selective transformation advantage to the infected $CD5^+$ IgM^+ B-cells by a second event.

A mutation in the p53 tumor suppressor gene is one of several genetic changes known to be involved in the development of lymphoma (**Figure 2A**). The protein encoded by the p53 tumor suppressor gene plays a critical role in transducing a signal from the damaged DNA to genes that control cell cycle and apoptosis. Approximately half of the solid tumors induced by BLV in cattle (Dequiedt et al., 1995; Ishiguro et al., 1997; Zhuang et al., 1997; Tajima et al., 1998b) and three of four bovine B-cell lymphoma lines (Komori et al., 1996) were shown to harbor missense mutations in p53. By contrast, very few mutations were found in B-cells from cows with PL and none of the uninfected cattle harbored a mutated p53 gene. These observations indicate that p53 mutations frequently occur at the final stage of lymphoma in cattle. A previous study of the molecular mechanism of mutations at codons 206, 207, 241, and 242, which were identified in lymphoma, showed that these mutations may potentially alter the wild-type function of the bovine p53 protein, including the conformation and transactivator and growth suppressor activities, and then cause lymphoma (Tajima et al., 1998b). These four mutations were clearly divided into two functionally distinct groups: (i) the mutant forms with substitutions at codons 241 and 242, which were mapped within an evolutionarily conserved region and corresponded to the human "hot-spot" mutations, and had completely lost the capacity for transactivation and growth suppression while gaining transdominant repression activity in p53-null SAOS-2 cells; and (ii) the mutations at codons 206 and 207, which were located outside the evolutionarily conserved regions and partially retained the capacity for transactivation and growth suppression. Collectively, these naturally occurring mutations may potentially alter the wild-type function, and in addition, out of the four missense mutations, at least two mutations may be sufficient to cause lymphoma. However, since the other two mutations may be insufficient to induce lymphoma, it is possible that other cancer-related genes may contribute to lymphoma in concert with the p53 mutations.

A major factor involved in the clinical progression of BLV-infected animals is the bovine leukocyte antigen (BoLA; **Figure 2A**), which plays a crucial role in determining immune responsiveness (Lewin and Bernoco, 1986; Lewin et al., 1988; Zanotti et al., 1996; Takeshima and Aida, 2006). Several studies have shown that genetic variations in *BoLA-DRB3*, which is a functionally important and the most polymorphic BoLA class II locus in cattle, influence resistance and susceptibility to a wide variety of infectious diseases, including lymphoma (Aida, 2001) and PL (Xu et al., 1993; Sulimova et al., 1995; Starkenburg et al., 1997; Juliarena et al., 2008), and affect BLV proviral load (Miyasaka et al., 2013). For example, the presence of the amino acids Glu–Arg (ER) at positions 70–71 of the BoLA-DRβ chain was associated with resistance to PL in BLV-infected cattle (Xu et al., 1993). Furthermore, the *BoLA-DRB3* alleles encoding Glu, Arg, and Val at positions 74, 77, and 78, respectively, of the BoLA-DRβ chain might be associated with resistance to tumor development (Aida, 2001). In a related study, Nagaoka et al. (1999) and Konnai et al. (2003) found that the ovine leukocyte antigen (*OLA*)-*DRB1* alleles encoding the Arg–Lys (RK) and the Ser–Arg (SR) motifs at positions 70–71 of the OLA-DRβ chain are associated with resistance (RK motif) and susceptibility (SR motif) to the development of lymphoma after experimental infection of sheep with BLV. The sheep with alleles encoding the RK motif produced neutralizing antibodies against BLV and interferon-γ, eliminated BLV completely, and did not develop lymphoma (Konnai et al., 2003). The susceptibility to the monoclonal expansion of BLV-infected B-lymphocytes is thus associated with specific alleles of BoLA system.

A polymorphism in the promoter region of the tumor necrosis factor (TNF)-α gene is one of several genetic changes involved in the development of lymphoma (**Figure 2A**). A previous study found that, in sheep experimentally infected with BLV, the frequency of the TNF-α-824G allele, which has been associated with low transcription activity of the promoter/predicted enhancer region of the bovine TNF-α gene, was higher in animals with lymphoma than in asymptomatic carrier animals. In addition, a tendency was observed for increased BLV-provirus load in cattle homozygous for the TNF-α-824G/G allele compared to cattle homozygous for the TNF-α-824A/A or TNF-α-824A/G alleles. These data suggest that the observed polymorphism in the promoter region of the TNF-α gene could at least in part contribute to the progression of lymphoma in BLV infection (Konnai et al., 2006).

The BLV studies have also focused on understanding the process of signal transduction such as B-cell receptor (BCR) signaling (Alber et al., 1993), since many signal transduction factors have been implicated in leukemogenesis of B-cells in humans (Murakami et al., 2011a). For example, the immunoreceptor tyrosine-based activation (ITAM) motifs present in the transmembrane gp30 proteins of the BLV envelope are important for the incorporation of envelope proteins into the virion (Inabe et al., 1999) and are required for infectivity *in vivo* (Willems et al., 1995). In addition to the viral signaling motif, the spleen tyrosine kinase (Syk) mRNA expression was significantly increased in PL samples, whereas it was decreased in tumor samples, suggesting that Syk mRNA expression dynamics is closely related to the progression of BLV-induced disease (Murakami et al., 2011a).

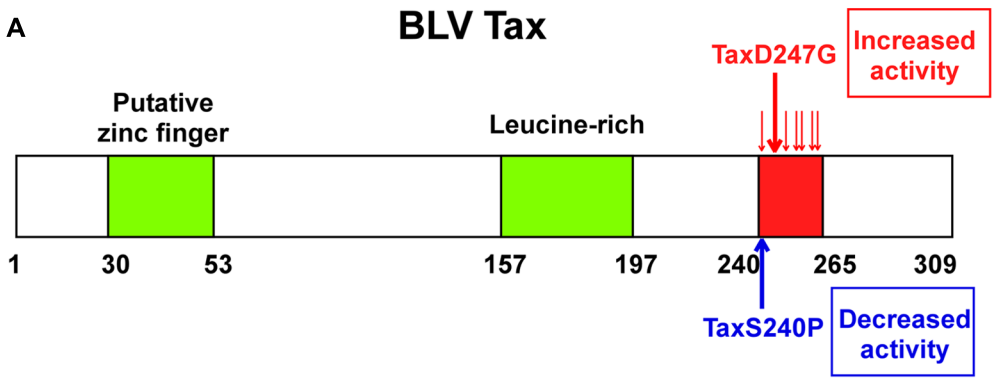
BLV Tax FUNCTION

As mentioned above, the Tax gene is a key contributor to the oncogenic potential, as well as a key protein involved in the replication of the virus. **Table 1** summarizes the functions of the Tax protein. The Tax open reading frame is mainly encoded in the pX region, and its translation occurs upstream of the *pol* stop codon. The Tax protein is modified by phosphorylation of two serine residues and is detected as a 34–38 kDa product (Chen et al., 1989; Willems et al., 1998). In addition, the Tax protein has T- and B-cell epitopes corresponding to regions 110–130/131–150 and 261–280, respectively (Sakakibara et al., 1998). One of the best characterized functions of Tax is the activation of viral transcription. The Tax protein acts on a triplicate 21 bp enhancer motif known as the Tax-responsive element (TxRE) in the U3 region of the 5′LTR, and it stimulates transactivation of the viral genome (Derse, 1987; Willems et al., 1987; Katoh et al., 1989). The TxRE consists of a cyclic AMP-response element (CRE)-like sequence, and it has been suggested that Tax binds to this element

indirectly through cellular factors, such as the members of the CREB/activating transcription factor (ATF) family of basic leucine zipper proteins that have been shown to bind to the CRE-like sequence (Adam et al., 1994, 1996; Boros et al., 1995). Furthermore, the Tax protein modulates the expression of cellular genes that are involved in the regulation of cell growth (Tajima and Aida, 2002). In addition to its function in the regulation of cellular and viral transcription, the Tax protein can induce immortalization of primary REF and cooperates with Ha-Ras oncogene to fully transform the primary cells (Willems et al., 1990). On the other hand, the transactivation and transformation of Tax may be independently induced by each mechanism, since phosphorylation of Tax is required for its transformation but not for its activation (Willems et al., 1998). Moreover, the expression of Tax in primary ovine B-cells, which depends on CD154 and interleukin-4, affects B-cell proliferation, cell cycle phase distribution, and survival, leading to cytokine-independent growth (Szynal et al., 2003). This immortalization process is also associated with increased B cell leukemia/lymphoma 2 (Bcl-2) protein levels, nuclear factor kappa B (NF-κB) accumulation, and a series of intracellular pathways that remain to be characterized (Klener et al., 2006). In addition, Tax inhibits base-excision DNA repair of oxidative damage, thereby potentially increasing the accumulation of ambient mutations in cellular DNA (Philpott and Buehring, 1999).

NEGATIVE REGULATION OF BLV Tax BY THE REGION BETWEEN RESIDUES 240–265

Our studies (Tajima and Aida, 2000) demonstrated new functions of the region between amino acids 240 and 265 of BLV Tax. As shown in **Figure 3**, a series of mutants with at least one amino acid substitution between amino acids 240 and 265 of BLV Tax were identified, including TaxD247G and TaxS240P, which exhibit an enhanced ability to stimulate and reduce viral LTR-directed transcription respectively, compared to the wild-type protein (Tajima and Aida, 2000). Transient expression analysis revealed that the TaxD247G mutant increased the production of viral protein and particles from a defective recombinant proviral BLV clone to a greater extent than the wild-type Tax (TaxWT). Conversely, the TaxS240P mutant was unable to induce the release of viral particles. The microarray data in human HeLa cells and its validation of differentially expressed genes at the RNA and protein levels in bovine 23CLN cells revealed several alterations in genes involved in many cellular functions such as transcription, signal transduction, cell growth, apoptosis, and the immune response (Arainga et al., 2012). In both of human HeLa cells and bovine 23CLN cells, the TaxD247G mutant induced higher gene expression compared with TaxWT and TaxS240P and many of these genes were expressed at the lowest level in the TaxS240P-transfected cells. In particular, our results showed that Tax activates the proteins which are involved in activator protein 1 (AP-1) signaling pathway [FBJ osteosarcoma



B **The region between amino acids 240 and 265 of BLV Tax regulates the level of:**

1. Activation of BLV-LTR promoter (Tajima and Aida, 2000)
2. Expression and propagation of BLV *in vitro* but not *in vivo* (Tajima and Aida, 2000)
3. Induction of apoptosis *in vitro* but not *ex vivo* (Takahashi et al., 2005)
4. Expression of many cellular genes, such as AP-1 signaling pathway-related proteins, G-proteins, GTP-binding proteins, and immune response-related proteins (Arainga et al., 2012)

Restricts the potential of:

1. Activation of other retroviral enhancers (Tajima and Aida, 2000)
2. Induction of *c-fos* expression (Tajima and Aida, 2000)

FIGURE 3 | Schematic representations of BLV Tax protein mutants and function. (A) Missense mutations between amino acids 240 and 265 containing D247G and S240P influence the transactivation activity of the BLV Tax protein. A putative zinc finger structure (amino acids 30–53) and a leucine-rich activation domain (amino acids 157–197) are also indicated. **(B)** Multiple functions of the region between amino acids 240 and 265.

oncogene (FOS), jun proto-oncogene (JUN), etc.] via interactions with other transcriptional pathways (G-protein, GTP-binding proteins, etc.). Likewise, the TaxD247G mutant induced apoptosis in transfected cells more effectively than the TaxWT (Takahashi et al., 2005). These results suggest that the region between amino acids 240 and 265 of the Tax protein might act as a negative regulatory domain, and missense mutations in this region might lead to enhanced transactivation activity of Tax, expression of many cellular genes and induction of apoptosis. Our results raise the possibility that the target sequence specificity of retroviral enhancers of Tax might be limited by this region because TaxD247G, but not TaxS240P, was found to activate other retroviral enhancers such as HTLV-1, HIV-1, and mouse mammary tumor virus (MMTV) and Moloney murine leukemia virus (M-MuLV), and *c-fos*, which are not activated by TaxWT (Tajima and Aida, 2000; **Figure 3B**). The microarray data also raised the possibility that BLV Tax regulates the innate immune response (**Figure 3B**): the largest group of downregulated genes was related to the immune response, and the majority of these genes belonged to the interferon family of antiviral factors, such as interferon-induced protein with tetratricopeptide repeats 1 (IFIT1; Arainga et al., 2012). Interferons are major components of the innate immune system, and are recognized for their antiviral function in addition to their antiproliferative and immunomodulatory effects on cells (Hu et al., 1993). It is likely that BLV Tax downregulates the innate immune response, thereby increasing the production of viral protein.

An infectious molecular clone of BLV encoding the TaxD247G was examined for the viral expression and propagation, as well as for the induction of apoptosis in a sheep model (Tajima et al., 2003a; Takahashi et al., 2004, 2005). Interestingly, the infectious molecular clone of BLV encoding the TaxD247G produced more viral particles and was transmitted at an elevated rate *in vitro*, but with no significant differences in the proviral load and the expression of viral RNA between sheep experimentally injected with BLVs encoding the TaxWT or the mutant TaxD247G proteins (Tajima et al., 2003a). These findings suggest the presence of a dominant host defense mechanism regulating BLV–LTR-directed transcription by Tax that may play an important role in viral silencing *in vivo* (**Figure 4**). Likewise, although the transient expression of TaxD247G induced apoptosis in transfected cells *in vitro* more effectively than TaxWT, higher level of protection against apoptosis was observed in PBMCs isolated from sheep infected with TaxD247G-encoded BLV compared to TaxWT-encoded BLV (Takahashi et al., 2005; **Figure 4**). These findings demonstrate that TaxD247G has an increased potential to induce apoptosis, which could be beneficial for BLV propagation like other viruses (Wurzer et al., 2003; Richard and Tulasne, 2012). One possible explanation for our results might be that TaxD247G-induced apoptosis is modulated by a dominant mechanism *ex vivo*, so the function might be suppressed.

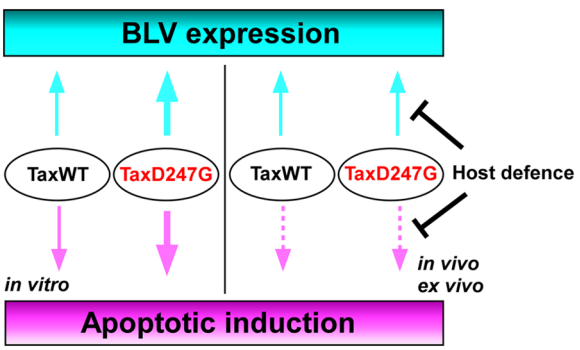


FIGURE 4 | Proposed mechanism for the regulation of BLV expression and apoptosis induction in TaxD247G-encoded BLV-infected sheep.

CONCLUSION

BLV is the etiologic agent of EBL, which is the most common neoplastic disease in cattle. It infects cattle worldwide, thereby imposing a severe economic burden on the dairy cattle industry. In this review, we evaluated existing information on the mechanism of BLV-induced leukemogenesis. We propose that, since BLV Tax induces immortalization of only $CD5^+$ IgM^+ B-cells within BLV-infected B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, γ/δ T-cells, monocytes, and granulocytes in cattle, it may confer a selective transformation advantage to the infected $CD5^+$ IgM^+ B-cells by a second event, such as p53 mutation, polymorphisms of BoLA, or the promoter region of the TNF-α gene. We also propose new functions of the region between amino acids 240 and 265 of BLV Tax (**Figure 3**). Namely, the transactivation activity and target sequence specificity of BLV Tax might be limited or negatively regulated by this region. The most interesting point regarding the ability of TaxD247G to enhance BLV expression and apoptotic induction *in vitro* is that it might be suppressed *in vivo* or *ex vivo*. Thus, we hypothesize that there could be dominant mechanisms controlling the functions of TaxD247G *ex vivo* and *in vivo*, as shown in **Figure 4**. For HTLV-1, it has been reported that $CD8^+$ cell-mediated cytotoxic T-lymphocytes (CTLs) target Tax-expressing cells, thereby reducing the number of infected cells (Hanon et al., 2000). Likewise, BLV-infected cells expressing Tax may be exposed to the host defense system, and BLV may evolve in a manner that promotes the shielding of their potential abilities. Therefore, a strong transactivation activity of BLV Tax might not be advantageous for the propagation of BLV *in vivo*. Taken together, the findings discussed in this review suggest that there might be a dominant mechanism involved in the induction of apoptosis and expression of HTLV-1 *in vivo*. To address our hypothesis, it seems necessary to evaluate whether possible host responses against BLV infection, such as the induction of CTLs, genetic, and epigenetic alterations in apoptosis-regulatory genes, and DNA and chromatin modifications of BLV promoter for the suppression of viral expression, could be enhanced in animals infected with TaxD247G-encoded BLV. Thus, future investigations of the relationship between apoptosis and viral expression using BLV containing the mutant D247G Tax as a model will broaden our understanding of the replication and propagation of HTLV-1, and leukemia progression.

ACKNOWLEDGMENTS

The studies on BLV were supported by Grants-in-Aid for Scientific Research (A, B, and C) from the Japan Society for the Promotion of

Science (JSPS), and by a grant from the Program for the Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry.

REFERENCES

Adam, E., Kerkhofs, P., Mammerickx, M., Burny, A., Kettmann, R., and Willems, L. (1996). The CREB, ATF-1, and ATF-2 transcription factors from bovine leukemia virus-infected B lymphocytes activate viral expression. *J. Virol.* 70, 1990–1999.

Adam, E., Kerkhofs, P., Mammerickx, M., Kettmann, R., Burny, A., Droogmans, L., et al. (1994). Involvement of the cyclic AMP-responsive element binding protein in bovine leukemia virus expression *in vivo*. *J. Virol.* 68, 5845–5853.

Aida, Y. (2001). Influence of host genetic differences on leukemogenesis induced by bovine leukemia virus. *AIDS Res. Hum. Retroviruses* 17, S12.

Aida, Y., Miyasaka, M., Okada, K., Onuma, M., Kogure, S., Suzuki, M., et al. (1989). Further phenotypic characterization of target cells for bovine leukemia virus experimental infection in sheep. *Am. J. Vet. Res.* 50, 1946–1951.

Aida, Y., Okada, K., and Amanuma, H. (1993). Phenotype and ontogeny of cells carrying a tumor-associated antigen that is expressed on bovine leukemia virus-induced lymphosarcoma. *Cancer Res.* 53, 429–437.

Alber, G., Kim, K. M., Weiser, P., Riesterer, C., Carsetti, R., and Reth, M. (1993). Molecular mimicry of the antigen receptor signalling motif by transmembrane proteins of the Epstein–Barr virus and the bovine leukaemia virus. *Curr. Biol.* 3, 333–339. doi: 10.1016/0960-9822(93)90196-U

Altanerova, V., Ban, J., Kettmann, R., and Altaner, C. (1990). Induction of leukemia in chicken by bovine leukemia virus due to insertional mutagenesis. *Arch. Geschwulstforsch.* 60, 89–96.

Altanerova, V., Portetelle, D., Kettmann, R., and Altaner, C. (1989). Infection of rats with bovine leukaemia virus: establishment of a virus-producing rat cell line. *J. Gen. Virol.* 70(Pt 7), 1929–1932. doi: 10.1099/0022-1317-70-7-1929

Andresen, V., Pise-Masison, C. A., Sinha-Datta, U., Bellon, M., Valeri, V., Washington Parks, R., et al. (2011). Suppression of HTLV-1 replication by Tax-mediated rerouting of the p13 viral protein to nuclear speckles. *Blood* 118, 1549–1559. doi: 10.1182/blood-2010-06-293340

Arainga, M., Takeda, E., and Aida, Y. (2012). Identification of bovine leukemia virus tax function associated with host cell transcription, signaling, stress response and immune response pathway by microarray-based gene expression analysis. *BMC Genomics* 13:121. doi: 10.1186/1471-2164-13-121

Bangham, C. R. (2003). Human T-lymphotropic virus type 1 (HTLV-1): persistence and immune control. *Int. J. Hematol.* 78, 297–303. doi: 10.1007/BF02983553

Boros, I. M., Tie, F., and Giam, C. Z. (1995). Interaction of bovine leukemia virus transactivator Tax with bZip proteins. *Virology* 214, 207–214. doi: 10.1006/viro.1995.9939

Boxus, M., Twizere, J. C., Legros, S., Dewulf, J. F., Kettmann, R., and Willems, L. (2008). The HTLV-1 Tax interactome. *Retrovirology* 5, 76. doi: 10.1186/1742-4690-5-76

Burny, A., Bex, F., Bruck, C., Cleuter, Y., Dekegel, D., Ghysdael, J., et al. (1979). Biochemical and epidemiological studies on bovine leukemia virus (BLV). *Haematol. Blood Transfus.* 23, 445–452.

Chen, G., Willems, L., Portetelle, D., Willard-Gallo, K. E., Burny, A., Gheysen, D., et al. (1989). Synthesis of functional bovine leukemia virus (BLV) p34tax protein by recombinant baculoviruses. *Virology* 173, 343–347. doi: 10.1016/0042-6822(89)90254-7

Chlichlia, K., and Khazaie, K. (2010). HTLV-1 Tax: linking transformation, DNA damage and apoptotic T-cell death. *Chem. Biol. Interact.* 188, 359–365. doi: 10.1016/j.cbi.2010.06.005

Collins, N. D., Newbound, G. C., Albrecht, B., Beard, J. L., Ratner, L., and Lairmore, M. D. (1998). Selective ablation of human T-cell lymphotropic virus type 1 p12I reduces viral infectivity *in vivo*. *Blood* 91, 4701–4707.

Dequiedt, F., Kettmann, R., Burny, A., and Willems, L. (1995). Mutations in the p53 tumor-suppressor gene are frequently associated with bovine leukemia virus-induced leukemogenesis in cattle but not in sheep. *Virology* 209, 676–683. doi: 10.1006/viro.1995.1303

Derse, D. (1987). Bovine leukemia virus transcription is controlled by a virus-encoded trans-acting factor and by *cis*-acting response elements. *J. Virol.* 61, 2462–2471.

Ding, W., Albrecht, B., Kelley, R. E., Muthusamy, N., Kim, S. J., Altschuld, R. A., et al. (2002). Human T-cell lymphotropic virus type 1 p12(I) expression increases cytoplasmic calcium to enhance the activation of nuclear factor of activated T cells. *J. Virol.* 76, 10374–10382. doi: 10.1128/JVI.76.20.10374-10382.2002

Djilali, S., and Parodi, A. L. (1989). The BLV-induced leukemia–lymphosarcoma complex in sheep. *Vet. Immunol. Immunopathol.* 22, 233–244. doi: 10.1016/0165-2427(89)90010-X

Felber, B. K., Derse, D., Athanassopoulos, A., Campbell, M., and Pavlakis, G. N. (1989). Cross-activation of the Rex proteins of HTLV-I and BLV and of the Rev protein of HIV-1 and nonreciprocal interactions with their RNA responsive elements. *New Biol.* 1, 318–328.

Ferrer, J. F., Marshak, R. R., Abt, D. A., and Kenyon, S. J. (1978). Persistent lymphocytosis in cattle: its cause, nature and relation to lymphosarcoma. *Ann. Rech. Vet.* 9, 851–857.

Ferrer, J. F., and Piper, C. E. (1978). An evaluation of the role of milk in the natural transmission of BLV. *Ann. Rech. Vet.* 9, 803–807.

Florins, A., Boxus, M., Vandermeers, F., Verlaeten, O., Bouzar, A. B., Defoiche, J., et al. (2008). Emphasis on cell turnover in two hosts infected by bovine leukemia virus: a rationale for host susceptibility to disease. *Vet. Immunol. Immunopathol.* 125, 1–7. doi: 10.1016/j.vetimm.2008.04.007

Florins, A., Gillet, N., Boxus, M., Kerkhofs, P., Kettmann, R., and Willems, L. (2007). Even attenuated bovine leukemia virus proviruses can be pathogenic in sheep. *J. Virol.* 81, 10195–10200. doi: 10.1128/JVI.01058-07

Franchini, G., Fukumoto, R., and Fullen, J. R. (2003). T-cell control by human T-cell leukemia/lymphoma virus type 1. *Int. J. Hematol.* 78, 280–296. doi: 10.1007/BF02983552

Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2002). The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J. Virol.* 76, 12813–12822. doi: 10.1128/JVI.76.24.12813-12822.2002

Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., et al. (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* 2, 407–410. doi: 10.1016/S0140-6736(85)92734-5

Gillet, N., Florins, A., Boxus, M., Burteau, C., Nigro, A., Vandermeers, F., et al. (2007). Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology* 4, 18. doi: 10.1186/1742-4690-4-18

Grassmann, R., Dengler, C., Muller-Fleckenstein, I., Fleckenstein, B., Mcguire, K., Dokhelar, M. C., et al. (1989). Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc. Natl. Acad. Sci. U.S.A.* 86, 3351–3355. doi: 10.1073/pnas.86.9.3351

Graves, D. C., and Ferrer, J. F. (1976). *In vitro* transmission and propagation of the bovine leukemia virus in monolayer cell cultures. *Cancer Res.* 36, 4152–4159.

Hanon, E., Hall, S., Taylor, G. P., Saito, M., Davis, R., Tanaka, Y., et al. (2000). Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. *Blood* 95, 1386–1392.

Hiraragi, H., Michael, B., Nair, A., Silic-Benussi, M., Ciminale, V., and Lairmore, M. (2005). Human T-lymphotropic virus type 1 mitochondrion-localizing protein p13II sensitizes Jurkat T cells to Ras-mediated apoptosis. *J. Virol.* 79, 9449–9457. doi: 10.1128/JVI.79.15.9449-9457.2005

Hopkins, S. G., and DiGiacomo, R. F. (1997). Natural transmission of bovine leukemia virus in dairy and beef cattle. *Vet. Clin. North Am. Food Anim. Pract.* 13, 107–128.

Hu, R., Gan, Y., Liu, J., Miller, D., and Zoon, K. C. (1993). Evidence for multiple binding sites for several components of human lymphoblastoid interferon-alpha. *J. Biol. Chem.* 268, 12591–12595.

Igakura, T., Stinchcombe, J. C., Goon, P. K., Taylor, G. P., Weber, J. N., Griffiths, G. M., et al. (2003). Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. *Science* 299, 1713–1716. doi: 10.1126/science.1080115

Inabe, K., Nishizawa, M., Tajima, S., Ikuta, K., and Aida, Y. (1999). The YXXL sequences of a transmembrane protein of bovine leukemia virus are required for viral entry and incorporation of viral envelope protein into virions. *J. Virol.* 73, 1293–1301.

Ishiguro, N., Furuoka, H., Matsui, T., Horiuchi, M., Shinagawa, M., Asahina, M., et al. (1997). p53 mutation as a potential cellular factor for tumor development

in enzootic bovine leukosis. *Vet. Immunol. Immunopathol.* 55, 351–358. doi: 10.1016/S0165-2427(96)05721-2

Jimba, M., Takeshima, S. N., Matoba, K., Endoh, D., and Aida, Y. (2010). BLV-CoCoMo-qPCR: quantitation of bovine leukemia virus proviral load using the CoCoMo algorithm. *Retrovirology* 7, 91. doi: 10.1186/1742-4690-7-91

Jimba, M., Takeshima, S. N., Murakami, H., Kohara, J., Kobayashi, N., Matsuhashi, T., et al. (2012). BLV-CoCoMo-qPCR: a useful tool for evaluating bovine leukemia virus infection status. *BMC Vet. Res.* 8:167. doi: 10.1186/1746-6148-8-167

Johnston, E. R., Powers, M. A., Kidd, L. C., and Radke, K. (1996). Peripheral blood mononuclear cells from sheep infected with a variant of bovine leukemia virus synthesize envelope glycoproteins but fail to induce syncytia in culture. *J. Virol.* 70, 6296–6303.

Juliarena, M. A., Poli, M., Sala, L., Ceriani, C., Gutierrez, S., Dolcini, G., et al. (2008). Association of BLV infection profiles with alleles of the BoLA-DRB3.2 *gene. Anim. Genet.* 39, 432–438. doi: 10.1111/j.1365-2052.2008.01750.x

Kashanchi, F., and Brady, J. N. (2005). Transcriptional and post-transcriptional gene regulation of HTLV-1. *Oncogene* 24, 5938–5951. doi: 10.1038/sj.onc.1208973

Katoh, I., Yoshinaka, Y., and Ikawa, Y. (1989). Bovine leukemia virus trans-activator p38tax activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. *EMBO J.* 8, 497–503.

Kenyon, S. J., Ferrer, J. F., Mcfeely, R. A., and Graves, D. C. (1981). Induction of lymphosarcoma in sheep by bovine leukemia virus. *J. Natl. Cancer Inst.* 67, 1157–1163.

Kerkhofs, P., Heremans, H., Burny, A., Kettmann, R., and Willems, L. (1998). *In vitro* and *in vivo* oncogenic potential of bovine leukemia virus G4 protein. *J. Virol.* 72, 2554–2559.

Kettmann, R., Deschamps, J., Cleuter, Y., Couez, D., Burny, A., and Marbaix, G. (1982). Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3′ proximate cellular sequences. *Proc. Natl. Acad. Sci. U.S.A.* 79, 2465–2469. doi: 10.1073/pnas.79.8.2465

Klener, P., Szynal, M., Cleuter, Y., Merimi, M., Duvillier, H., Lallemand, F., et al. (2006). Insights into gene expression changes impacting B-cell transformation: cross-species microarray analysis of bovine leukemia virus tax-responsive genes in ovine B cells. *J. Virol.* 80, 1922–1938. doi: 10.1128/JVI.80.4.1922-1938.2006

Komori, H., Ishiguro, N., Horiuchi, M., Shinagawa, M., and Aida, Y. (1996). Predominant p53 mutations in enzootic bovine leukemic cell lines. *Vet. Immunol. Immunopathol.* 52, 53–63. doi: 10.1016/0165-2427(95)05538-X

Konishi, H., Kobayashi, N., and Hatanaka, M. (1984). Defective human T-cell leukemia virus in adult T-cell leukemia patients. *Mol. Biol. Med.* 2, 273–283.

Konnai, S., Takeshima, S. N., Tajima, S., Yin, S. A., Okada, K., Onuma, M., et al. (2003). The influence of ovine MHC class II DRB1 alleles on immune response in bovine leukemia virus infection. *Microbiol. Immunol.* 47, 223–232.

Konnai, S., Usui, T., Ikeda, M., Kohara, J., Hirata, T., Okada, K., et al. (2006). Tumor necrosis factor-alpha genetic polymorphism may contribute to progression of bovine leukemia virus-infection. *Microbes Infect.* 8, 2163–2171. doi: 10.1016/j.micinf.2006.04.017

Kono, Y., Sentsui, H., Arai, K., Ishida, H., and Irishio, W. (1983). Contact transmission of bovine leukemia virus under insect-free conditions. *J. Vet. Med. Sci.* 45, 799–802.

Korber, B., Okayama, A., Donnelly, R., Tachibana, N., and Essex, M. (1991). Polymerase chain reaction analysis of defective human T-cell leukemia virus type I proviral genomes in leukemic cells of patients with adult T-cell leukemia. *J. Virol.* 65, 5471–5476.

Koyanagi, Y., Itoyama, Y., Nakamura, N., Takamatsu, K., Kira, J., Iwamasa, T., et al. (1993). *In vivo* infection of human T-cell leukemia virus type I in non-T cells. *Virology* 196, 25–33. doi: 10.1006/viro.1993.1451

Lagarias, D. M., and Radke, K. (1989). Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. *J. Virol.* 63, 2099–2107.

Lefebvre, L., Vanderplasschen, A., Ciminale, V., Heremans, H., Dangoisse, O., Jauniaux, J. C., et al. (2002). Oncoviral bovine leukemia virus G4 and human T-cell leukemia virus type 1 p13(II) accessory proteins interact with farnesyl pyrophosphate synthetase. *J. Virol.* 76, 1400–1414. doi: 10.1128/JVI.76.3.1400-1414.2002

Leisering, A. (1871). Hypertrophy der Malpigischen Korperchen der Milz. *Bericht uber das Veterinarwesen im Konigreich Sachsen* 16, 15–16.

Lemasson, I., Lewis, M. R., Polakowski, N., Hivin, P., Cavanagh, M. H., Thebault, S., et al. (2007). Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription. *J. Virol.* 81, 1543–1553. doi: 10.1128/JVI.00480-06

Lewin, H. A., and Bernoco, D. (1986). Evidence for BoLA-linked resistance and susceptibility to subclinical progression of bovine leukaemia virus infection. *Anim. Genet.* 17, 197–207. doi: 10.1111/j.1365-2052.1986.tb03191.x

Lewin, H. A., Wu, M. C., Stewart, J. A., and Nolan, T. J. (1988). Association between BoLA and subclinical bovine leukemia virus infection in a herd of Holstein–Friesian cows. *Immunogenetics* 27, 338–344. doi: 10.1007/BF00395129

Mammerickx, M., Portetelle, D., and Burny, A. (1981). Experimental cross-transmissions of bovine leukemia virus (BLV) between several animal species. *Zbl. Vet. Med. B* 28, 69–81. doi: 10.1111/j.1439-0450.1981.tb01740.x

Matsuoka, M., and Jeang, K. T. (2011). Human T-cell leukemia virus type 1 (HTLV-1) and leukemic transformation: viral infectivity, Tax, HBZ and therapy. *Oncogene* 30, 1379–1389. doi: 10.1038/onc.2010.537

Mirsky, M. L., Olmstead, C. A., Da, Y., and Lewin, H. A. (1996). The prevalence of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection. *J. Virol.* 70, 2178–2183.

Miyasaka, T., Takeshima, S. N., Jimba, M., Matsumoto, Y., Kobayashi, N., Matsuhashi, T., et al. (2013). Identification of bovine leukocyte antigen class II haplotypes associated with variations in bovine leukemia virus proviral load in Japanese Black cattle. *Tissue Antigens* 81, 72–82. doi: 10.1111/tan.12041

Murakami, H., Kuroiwa, T., Suzuki, K., Miura, Y., and Sentsui, H. (2011a). Analysis of Syk expression in bovine lymphoma and persistent lymphocytosis induced by bovine leukemia virus. *J. Vet. Med. Sci.* 73, 41–45. doi: 10.1292/jvms.10-0225

Murakami, H., Yamada, T., Suzuki, M., Nakahara, Y., Suzuki, K., and Sentsui, H. (2011b). Bovine leukemia virus integration site selection in cattle that develop leukemia. *Virus Res.* 156, 107–112. doi: 10.1016/j.virusres.2011.01.004

Murakami, K., Aida, Y., Kageyama, R., Numakunai, S., Ohshima, K., Okada, K., et al. (1994a). Immunopathologic study and characterization of the phenotype of transformed cells in sheep with bovine leukemia virus-induced lymphosarcoma. *Am. J. Vet. Res.* 55, 72–80.

Murakami, K., Okada, K., Ikawa, Y., and Aida, Y. (1994b). Bovine leukemia virus induces $CD5^-$ B cell lymphoma in sheep despite temporarily increasing $CD5^+$ B cells in asymptomatic stage. *Virology* 202, 458–465. doi: 10.1006/viro.1994.1362

Nagaoka, Y., Kabeya, H., Onuma, M., Kasai, N., Okada, K., and Aida, Y. (1999). Ovine MHC class II DRB1 alleles associated with resistance or susceptibility to development of bovine leukemia virus-induced ovine lymphoma. *Cancer Res.* 59, 975–981.

Nicot, C., Dundr, M., Johnson, J. M., Fullen, J. R., Alonzo, N., Fukumoto, R., et al. (2004). HTLV-1-encoded p30II is a post-transcriptional negative regulator of viral replication. *Nat. Med.* 10, 197–201. doi: 10.1038/nm984

Ohshima, K., Kikuchi, M., Masuda, Y., Kobari, S., Sumiyoshi, Y., Eguchi, F., et al. (1991). Defective provirus form of human T-cell leukemia virus type I in adult T-cell leukemia/lymphoma: clinicopathological features. *Cancer Res.* 51, 4639–4642.

Onuma, M., Honma, T., Mikami, T., Ichijo, S., and Konishi, T. (1979). Studies on the sporadic and enzootic forms of bovine leukosis. *J. Comp. Pathol.* 89, 159–167. doi: 10.1016/0021-9975(79)90055-0

Onuma, M., Wada, M., Yasutomi, Y., Yamamoto, M., Okada, H. M., and Kawakami, Y. (1990). Suppression of immunological responses in rabbits experimentally infected with bovine leukemia virus. *Vet. Microbiol.* 25, 131–141. doi: 10.1016/0378-1135(90)90072-4

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., et al. (1986). HTLV-I associated myelopathy, a new clinical entity. *Lancet* 1, 1031–1032. doi: 10.1016/S0140-6736(86)91298-5

Panei, C. J., Takeshima, S. N., Omori, T., Nunoya, T., Davis, W. C., Ishizaki, H., et al. (2013). Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR. *BMC Vet. Res.* 9:95. doi: 10.1186/1746-6148-9-95

Philpott, S. M., and Buehring, G. C. (1999). Defective DNA repair in cells with human T-cell leukemia/bovine leukemia viruses: role of tax gene. *J. Natl. Cancer Inst.* 91, 933–942. doi: 10.1093/jnci/91.11.933

Richard, A., and Tulasne, D. (2012). Caspase cleavage of viral proteins, another way for viruses to make the best of apoptosis. *Cell Death Dis.* 3, e277. doi: 10.1038/cddis.2012.18

Robert-Guroff, M., Weiss, S. H., Giron, J. A., Jennings, A. M., Ginzburg, H. M., Margolis, I. B., et al. (1986). Prevalence of antibodies to HTLV-I, -II, and -III in intravenous drug abusers from an AIDS endemic region. *JAMA* 255, 3133–3137. doi: 10.1001/jama.1986.03370220095034

Sagata, N., Yasunaga, T., Ogawa, Y., Tsuzuku-Kawamura, J., and Ikawa, Y. (1984a). Bovine leukemia virus: unique structural features of its long terminal repeats and its evolutionary relationship to human T-cell leukemia virus. *Proc. Natl. Acad. Sci. U.S.A.* 81, 4741–4745. doi: 10.1073/pnas.81.15.4741

Sagata, N., Yasunaga, T., Ohishi, K., Tsuzuku-Kawamura, J., Onuma, M., and Ikawa, Y. (1984b). Comparison of the entire genomes of bovine leukemia virus and human T-cell leukemia virus and characterization of their unidentified open reading frames. *EMBO J.* 3, 3231–3237.

Sakakibara, N., Kabeya, H., Ohashi, K., Sugimoto, C., and Onuma, M. (1998). Epitope mapping of bovine leukemia virus transactivator protein tax. *J. Vet. Med. Sci.* 60, 599–605. doi: 10.1292/jvms.60.599

Satou, Y., Yasunaga, J., Yoshida, M., and Matsuoka, M. (2006). HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 720–725. doi: 10.1073/pnas.05076 31103

Satou, Y., Yasunaga, J., Zhao, T., Yoshida, M., Miyazato, P., Takai, K., et al. (2011). HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation *in vivo*. *PLoS Pathog.* 7:e1001274. doi: 10.1371/journal.ppat.1001274

Schwartz, I., Bensaid, A., Polack, B., Perrin, B., Berthelemy, M., and Levy, D. (1994). *In vivo* leukocyte tropism of bovine leukemia virus in sheep and cattle. *J. Virol.* 68, 4589–4596.

Schwartz, I., and Levy, D. (1994). Pathobiology of bovine leukemia virus. *Vet. Res.* 25, 521–536.

Starkenburg, R. J., Hansen, L. B., Kehrli, M. E. Jr., and Chester-Jones, H. (1997). Frequencies and effects of alternative DRB3.2 alleles of bovine lymphocyte antigen for Holsteins in milk selection and control lines. *J. Dairy Sci.* 80, 3411–3419. doi: 10.3168/jds.S0022-0302(97)76316-1

Stott, M. L., Thurmond, M. C., Dunn, S. J., Osburn, B. I., and Stott, J. L. (1991). Integrated bovine leukosis proviral DNA in T helper and T cytotoxic/suppressor lymphocytes. *J. Gen. Virol.* 72(Pt 2), 307–315. doi: 10.1099/0022-1317-72-2-307

Sulimova, G. E., Udina, I. G., Shaikhaev, G. O., and Zakharov, I. A. (1995). DNA polymorphism of the BoLA-DRB3 gene in cattle in connection with resistance and susceptibility to leukemia. *Genetika* 31, 1294–1299.

Szynal, M., Cleuter, Y., Beskorwayne, T., Bagnis, C., Van Lint, C., Kerkhofs, P., et al. (2003). Disruption of B-cell homeostatic control mediated by the BLV-Tax oncoprotein: association with the upregulation of Bcl-2 and signaling through NF-kappaB. *Oncogene* 22, 4531–4542. doi: 10.1038/sj.onc.1206546

Tajima, S., and Aida, Y. (2000). The region between amino acids 245 and 265 of the bovine leukemia virus (BLV) tax protein restricts transactivation not only via the BLV enhancer but also via other retrovirus enhancers. *J. Virol.* 74, 10939–10949. doi: 10.1128/JVI.74.23.10939-10949.2000

Tajima, S., and Aida, Y. (2002). Mutant tax protein from bovine leukemia virus with enhanced ability to activate the expression of c-fos. *J. Virol.* 76, 2557–2562. doi: 10.1128/jvi.76.5.2557-2562.2002

Tajima, S., and Aida, Y. (2005). Induction of expression of bovine leukemia virus (BLV) in blood taken from BLV-infected cows without removal of plasma. *Microbes Infect.* 7, 1211–1216. doi: 10.1016/j.micinf.2005.04.010

Tajima, S., Ikawa, Y., and Aida, Y. (1998a). Complete bovine leukemia virus (BLV) provirus is conserved in BLV-infected cattle throughout the course of B-cell lymphosarcoma development. *J. Virol.* 72, 7569–7576.

Tajima, S., Zhuang, W. Z., Kato, M. V., Okada, K., Ikawa, Y., and Aida, Y. (1998b). Function and conformation of wild-type p53 protein are influenced by mutations in bovine leukemia virus-induced B-cell lymphosarcoma. *Virology* 243, 735–746. doi: 10.1006/viro.1998.9051

Tajima, S., Takahashi, M., Takeshima, S. N., Konnai, S., Yin, S. A., Watarai, S., et al. (2003a). A mutant form of the tax protein of bovine leukemia virus (BLV), with enhanced transactivation activity, increases expression and propagation of BLV *in vitro* but not *in vivo*. *J. Virol.* 77, 1894–1903. doi: 10.1128/JVI.77.3.1894-1903.2003

Tajima, S., Tsukamoto, M., and Aida, Y. (2003b). Latency of viral expression *in vivo* is not related to CpG methylation in the U3 region and part of the R region of the long terminal repeat of bovine leukemia virus. *J. Virol.* 77, 4423–4430. doi: 10.1128/JVI.77.7.4423-4430.2003

Takahashi, M., Tajima, S., Okada, K., Davis, W. C., and Aida, Y. (2005). Involvement of bovine leukemia virus in induction and inhibition of apoptosis. *Microbes Infect.* 7, 19–28. doi: 10.1016/j.micinf.2004.09.014

Takahashi, M., Tajima, S., Takeshima, S. N., Konnai, S., Yin, S. A., Okada, K., et al. (2004). Ex vivo survival of peripheral blood mononuclear cells in sheep induced by bovine leukemia virus (BLV) mainly occurs in $CD5^-$ B cells that express BLV. *Microbes Infect.* 6, 584–595. doi: 10.1016/j.micinf.2004.02.014

Takeshima, S-N., and Aida, Y. (2006). Structure, function and disease susceptibility of the bovine major histocompatibility complex. *Anim. Sci. J.* 77, 138–150. doi: 10.1111/j.1740-0929.2006.00332.x

Tanaka, A., Takahashi, C., Yamaoka, S., Nosaka, T., Maki, M., and Hatanaka, M. (1990). Oncogenic transformation by the tax gene of human T-cell leukemia virus type I *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1071–1075. doi: 10.1073/pnas.87.3.1071

Theilen, G. H., and Dungworth, D. L. (1965). Bovine lymphosarcoma in California. 3. The calf form. *Am. J. Vet. Res.* 26, 696–709.

Tsukasaki, K., Tsushima, H., Yamamura, M., Hata, T., Murata, K., Maeda, T., et al. (1997). Integration patterns of HTLV-I provirus in relation to the clinical course of ATL: frequent clonal change at crisis from indolent disease. *Blood* 89, 948–956.

Voneche, V., Portetelle, D., Kettmann, R., Willems, L., Limbach, K., Paoletti, E., et al. (1992). Fusogenic segments of bovine leukemia virus and simian immunodeficiency virus are interchangeable and mediate fusion by means of oblique insertion in the lipid bilayer of their target cells. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3810–3814. doi: 10.1073/pnas.89.9.3810

Weiss, R., Teich, N., Varmus, H., and Coffin, J. (1985). *RNA Tumor Viruses*. New York: Cold Spring Harbor Laboratory.

Willems, L., Gatot, J. S., Mammerickx, M., Portetelle, D., Burny, A., Kerkhofs, P., et al. (1995). The YXXL signalling motifs of the bovine leukemia virus transmembrane protein are required for *in vivo* infection and maintenance of high viral loads. *J. Virol.* 69, 4137–4141.

Willems, L., Gegonne, A., Chen, G., Burny, A., Kettmann, R., and Ghysdael, J. (1987). The bovine leukemia virus p34 is a transactivator protein. *EMBO J.* 6, 3385–3389.

Willems, L., Grimonpont, C., Kerkhofs, P., Capiau, C., Gheysen, D., Conrath, K., et al. (1998). Phosphorylation of bovine leukemia virus Tax protein is required for *in vitro* transformation but not for transactivation. *Oncogene* 16, 2165–2176. doi: 10.1038/sj.onc.1201765

Willems, L., Heremans, H., Chen, G., Portetelle, D., Billiau, A., Burny, A., et al. (1990). Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation. *EMBO J.* 9, 1577–1581.

Willems, L., Kerkhofs, P., Dequiedt, F., Portetelle, D., Mammerickx, M., Burny, A., et al. (1994). Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11532–11536. doi: 10.1073/pnas.91.24.11532

Williams, D. L., Barta, O., and Amborski, G. F. (1988). Molecular studies of T-lymphocytes from cattle infected with bovine leukemia virus. *Vet. Immunol. Immunopathol.* 19, 307–323. doi: 10.1016/0165-2427(88)90117-1

Wu, D., Takahashi, K., Murakami, K., Tani, K., Koguchi, A., Asahina, M., et al. (1996). B-1a, B-1b and conventional B cell lymphoma from enzootic bovine leukosis. *Vet. Immunol. Immunopathol.* 55, 63–72. doi: 10.1016/S0165-2427(96)05631-0

Wurzer, W. J., Planz, O., Ehrhardt, C., Giner, M., Silberzahn, T., Pleschka, S., et al. (2003). Caspase 3 activation is essential for efficient influenza virus propagation. *EMBO J.* 22, 2717–2728. doi: 10.1093/emboj/cdg279

Wyatt, C. R., Wingett, D., White, J. S., Buck, C. D., Knowles, D., Reeves, R., et al. (1989). Persistent infection of rabbits with bovine leukemia virus associated with development of immune dysfunction. *J. Virol.* 63, 4498–4506.

Xu, A., Van Eijk, M. J., Park, C., and Lewin, H. A. (1993). Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus. *J. Immunol.* 151, 6977–6985.

Zanotti, M., Poli, G., Ponti, W., Polli, M., Rocchi, M., Bolzani, E., et al. (1996). Association of BoLA class II haplotypes with subclinical progression of bovine leukaemia virus infection in Holstein–Friesian cattle. *Anim. Genet.* 27, 337–341.

Zhang, W., Nisbet, J. W., Albrecht, B., Ding, W., Kashanchi, F., Bartoe, J. T., et al. (2001). Human T-lymphotropic virus type 1 p30(II) regulates gene transcription by binding CREB binding protein/p300. *J. Virol.* 75, 9885–9895. doi: 10.1128/JVI.75.20.9885-9895.2001

Zhao, T., Satou, Y., Sugata, K., Miyazato, P., Green, P. L., Imamura, T., et al. (2011). HTLV-1 bZIP factor enhances TGF-beta signaling through p300 coactivator. *Blood* 118, 1865–1876. doi: 10.1182/blood-2010-12-326199

Zhao, T., Yasunaga, J., Satou, Y., Nakao, M., Takahashi, M., Fujii, M., et al. (2009). Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113, 2755–2764. doi: 10.1182/blood-2008-06-161729

Zhuang, W., Tajima, S., Okada, K., Ikawa, Y., and Aida, Y. (1997). Point mutation of p53 tumor suppressor gene in bovine leukemia virus-induced lymphosarcoma. *Leukemia* 11(Suppl 3), 344–346.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 July 2013; accepted: 17 October 2013; published online: 08 November 2013.

Citation: Aida Y, Murakami H, Takahashi M and Takeshima S-N (2013) Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. Front. Microbiol. ***4****:328. doi: 10.3389/fmicb.2013.00328*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 28 June 2013
doi: 10.3389/fmicb.2013.00176

Macaques as model hosts for studies of HIV-1 infection

Anisha Misra, Rajesh Thippeshappa and Jason T. Kimata*

Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Francois Villinger, Emory University School of Medicine, USA
Frank Kirchhoff, University Clinic Ulm, Germany

***Correspondence:**
Jason T. Kimata, Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, BCM385, Houston, TX 77030, USA
e-mail: jkimata@bcm.edu

Increasing evidence indicates that the host range of primate lentiviruses is in part determined by their ability to counteract innate restriction factors that are effectors of the type 1 interferon (IFN-1) response. For human immunodeficiency virus type 1 (HIV-1), *in vitro* experiments have shown that its tropism may be narrow and limited to humans and chimpanzees because its replication in other non-human primate species is hindered by factors such as TRIM5α (tripartite motif 5 alpha), APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3), and tetherin. Based on these data, it has been hypothesized that primate lentiviruses will infect and replicate in a new species if they are able to counteract and evade suppression by the IFN-1 response. Several studies have tested whether engineering HIV-1 recombinants with minimal amounts of simian immunodeficiency virus sequences would enable replication in $CD4^+$ T cells of non-natural hosts such as Asian macaques and proposed that infection of these macaque species could be used to study transmission and pathogenesis. Indeed, infection of macaques with these viruses revealed that Vif-mediated counteraction of APOBEC3G function is central to cross-species tropism but that other IFN-induced factors may also play important roles in controlling replication. Further studies of these macaque models of infection with HIV-1 derivatives could provide valuable insights into the interaction of lentiviruses and the innate immune response and how lentiviruses adapt and cause disease.

Keywords: HIV-1, SIV, AIDS, macaque models, tropism, innate restriction

INTRODUCTION

Early studies on primate lentiviruses identified key host cell factors required for replication (Hatziioannou and Evans, 2012). More recent investigations have shown that overcoming the suppressive effects of innate restriction factors is also necessary for human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency viruses (SIVs) to replicate in human and non-human primate hosts, respectively. Viral accessory proteins play key roles in antagonizing these inhibitory factors, which are effectors of the type 1 interferon (IFN-1) response (Harris et al., 2012). However, their functional activities are commonly limited to susceptible host species, suggesting that innate immunity may be a significant barrier to transmission of lentiviruses. We, and others, have engineered HIV-1 recombinants with minimal SIV sequences conferring resistance to specific restriction factors and infected macaques to experimentally test this hypothesis. Investigations utilizing these macaque-tropic HIV-1 derivatives may lead to a greater understanding of inter-species transmission of primate lentiviruses as well as the development of a macaque model of HIV-1 infection and disease.

MACAQUE AIDS MODEL DEVELOPMENT AND SPECIES TROPISM OF PRIMATE LENTIVIRUSES

The development of non-human primate acquired immunodeficiency syndrome (AIDS) models provided initial insights into the species tropism of lentiviruses. In particular, these experiments demonstrated a narrow species tropism for HIV-1. Gibbons and chimpanzees are susceptible to HIV-1 (Gardner and Luciw, 1989; Fultz, 1993). However, due to their endangered status and maintenance cost, they are not reasonable model hosts. On the other hand, Asian macaques, including *Macaca mulatta* (rhesus macaques, RM) and *M. fascicularis* (cynomolgus monkeys, CM) and cells from these species appear to be resistant to HIV-1 (Agy et al., 1992; Cowan et al., 2002; Munk et al., 2002), suggesting genetic barriers to infection. In retrospect, these findings are not surprising given that HIV-1 evolved from a novel recombinant SIV infecting chimpanzees (SIVcpz; Gao et al., 1999; Bailes et al., 2003). Uniquely, one species, *M. nemestrina* (pigtailed macaques, PTM), has been found to be susceptible to transient infection but not disease (Agy et al., 1992, 1997; Gartner et al., 1994), demonstrating that a potent resistance mechanism(s) may indeed control viral replication.

With the absence of a susceptible non-human primate host for HIV-1, a SIV-AIDS macaque model was developed accidently following the discovery that Asian macaques housed with sooty mangabeys at a US primate center had developed AIDS like disease (Gardner, 1996; Apetrei et al., 2005). Although African monkey species harbor SIVs and live with high virus loads without developing disease (Klatt et al., 2012b), SIVs isolated from sooty mangabeys (SM, *Cercocebus atys*) cause AIDS at varying rates when inoculated into Asian macaques. As a result, SIV infection of macaques has become the most widely used model for studies of AIDS immunopathogenesis and viral fitness (Kimata, 2006; Hatziioannou and Evans, 2012). Quite interestingly, PTMs appear to be more susceptible to infection and disease induced by SIV than RMs, which may be due to a higher level of immune activation and gastrointestinal immune dysfunction (Klatt et al., 2012a; Canary et al., 2013).

Genetic differences in reverse transcriptase and protease of HIV-1 and SIVmac make it difficult to evaluate the efficacy of antiretroviral drugs that target these proteins using the SIVmac-RM model. Evaluating vaccines against HIV-1 is also impossible since cytotoxic T cell epitopes may differ and neutralizing antibodies are not cross-reactive. These shortcomings have been partially addressed by constructing chimeric SIV/HIV-1 viruses (SHIVs) that include certain HIV genes in the SIVmac239 backbone (Shibata et al., 1991; **Figure 1**).

Aside from the obvious utility for translational studies, the development of SHIVs revealed important clues about the functional activity of HIV-1 proteins in macaques. SIVmac based chimeras that include HIV-1 gene substitutions in *env*, *tat*, and *rev* (Env-SHIV) or *nef* (Nef-SHIV) are pathogenic in macaques (Li et al., 1995; Luciw et al., 1995; Reimann et al., 1996; Sinclair et al., 1997; Alexander et al., 1999). Chimeras with HIV-1 *rt* substitutions (RT-SHIVs) also persistently replicate in macaque hosts (Uberla et al., 1995; Ambrose et al., 2007). While not required, *vpu* of HIV-1 enhances the pathogenicity of Env-SHIV (Stephens et al., 2002). Thus, a significant amount of HIV-1 sequences can functionally replace SIV sequences, but determinants within *gag–pol* and *vif* of SIV appear necessary for infection of Asian macaques.

INNATE RESTRICTION FACTORS OF PRIMATE LENTIVIRUSES

Several cellular restriction factors have been identified that can limit replication of primate lentiviruses in different species, but whose activities are specifically inhibited or evaded (**Table 1**). These include apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) proteins, tripartite motif 5 alpha (TRIM5α) and related TRIM5–cyclophilin A fusion proteins (TRIMcyp), tetherin/BST2/CD317, and sterile alpha motif (SAM) domain and HD domain-containing protein 1 (SAMHD1; Thippeshappa et al., 2012). All are regulated by IFN-1, suggesting that innate immunity plays a critical role in preventing infection and that viral adaptations that antagonize or escape the effects of the factors may be required for successful transmission of lentiviruses.

The APOBEC3 (A3) proteins belong to a seven-member family of cytidine deaminases (Jarmuz et al., 2002). A3G was identified as a Vif-targeted inhibitory factor of HIV-1 during a screen for cellular factors that blocked post-entry steps of infection prior to integration (Sheehy et al., 2002). In the absence of Vif, it interferes with viral replication by incorporating into the virion and disrupting reverse transcription or causing accumulation of deleterious G to A mutations (Mangeat et al., 2003; Zhang et al., 2003; Bishop et al., 2008). Hypermutated viral genomes may be degraded or produce non-functional truncated or misfolded viral proteins that are processed and serve as antigens for cellular immune responses (Casartelli et al., 2010).

In virus producing cells, Vif binds A3G and links it to an E3 ubiquitin ligase complex, thereby redirecting it for degradation by the proteasome (Conticello et al., 2003) and preventing its incorporation into assembling virions. Interestingly, Vif function appears to be species-specific. For example, the HIV-1 Vif antagonizes the human A3G protein but not A3G of other non-human primate species. By contrast, the Vif protein of SIVagm antagonizes African green monkey (AGM) A3G but not human A3G (Mariani et al., 2003). These findings suggest that Vif-mediated inhibition of the A3G proteins is likely essential for transmission of a virus to a new host species.

Of the innate restriction factors, only TRIM5α was initially discovered as an inhibitory factor of HIV-1 in Old World Monkeys (OWMs; Stremlau et al., 2006; Grutter and Luban, 2012). TRIM5α blocks a post-entry stage of HIV-1 replication through an interaction with the capsid protein. It belongs to the tripartite family of proteins, and contains a RING finger, B-box2, and coiled coil domain, which are responsible for E3 ubiquitin ligase activity and higher order self-association. It also has a B30.2/SPRY domain that detects the incoming viral capsid proteins, linking the viral core to an ubiquitin-proteasome-dependent pathway. This disrupts the preintegration complex, thereby blocking reverse transcription. However, in cases where the proteasome pathway is inhibited, nuclear entry of viral DNA is impaired. Recent studies also establish TRIM5α as an innate immune sensor of the retroviral capsid (Pertel et al., 2011). Sequence variation in B30.2/SPRY of TRIM5α and amino acid variations in the viral capsid are responsible for species-specific restriction and evasion, respectively (Nakayama et al., 2005; Sawyer et al., 2005). Additionally, allelic variation in TRIM5 influences transmission and modulates disease progression in SIV-infected RM (Kirmaier et al., 2010; Lim et al., 2010b; Reynolds et al., 2011). Interestingly, PTMs do not express a TRIM5α isoform, partially explaining their unique susceptibility to HIV-1 (Brennan et al., 2007).

Novel TRIMcyp also interfere with post-entry steps in HIV/SIV infection. First identified in New World Owl Monkeys (Sayah et al., 2004), the fusion protein appears to have arisen via line-mediated retrotransposition of the cyclophilin A gene into the TRIM5 locus. Subsequent studies have also identified TRIMcyp fusion proteins in RM, CM, and PTM that evolved independently (Brennan et al., 2008; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008; Dietrich et al., 2011). Allelic variation in the cyclophilin A domain of the macaque TRIMcyp proteins affects recognition and inhibition of HIV-1 and 2 and SIVagm but not SIVmac. Interestingly, RMs and CMs are polymorphic for TRIM5α alleles and TRIMcyp, although geographically distinct CM populations show different frequencies of TRIMcyp. PTMs, on the other hand, are homozygous for TRIMcyp, again demonstrating a unique genotype for PTMs in comparison to other Asian macaques (Brennan et al., 2008; Newman et al., 2008; Kuang et al., 2009; Dietrich et al., 2011; Saito et al., 2012).

Tetherin or BST2 is interferon inducible type II membrane protein that interferes with the release of HIV-1 progeny virions from the surface of infected human T cells and also functions as an innate immune sensor of viral infection to promote inflammatory responses (Neil et al., 2008; Van Damme et al., 2008; Galao et al., 2012). Initially, it was discovered that the HIV-1 protein Vpu inhibits tetherin and is required for the efficient release of progeny virions (Neil et al., 2008; Van Damme et al., 2008). Subsequent studies have shown that primate lentiviruses that do not encode Vpu evolved other strategies to antagonize tetherin. For example, HIV-2 and SIV use Env- and Nef-dependent mechanisms to counteract the restrictive effect of tetherin (Jia et al., 2009; Le Tortorec and Neil, 2009), respectively. Additionally, the effects of the viral antagonists are specific for the host species in which they evolved.

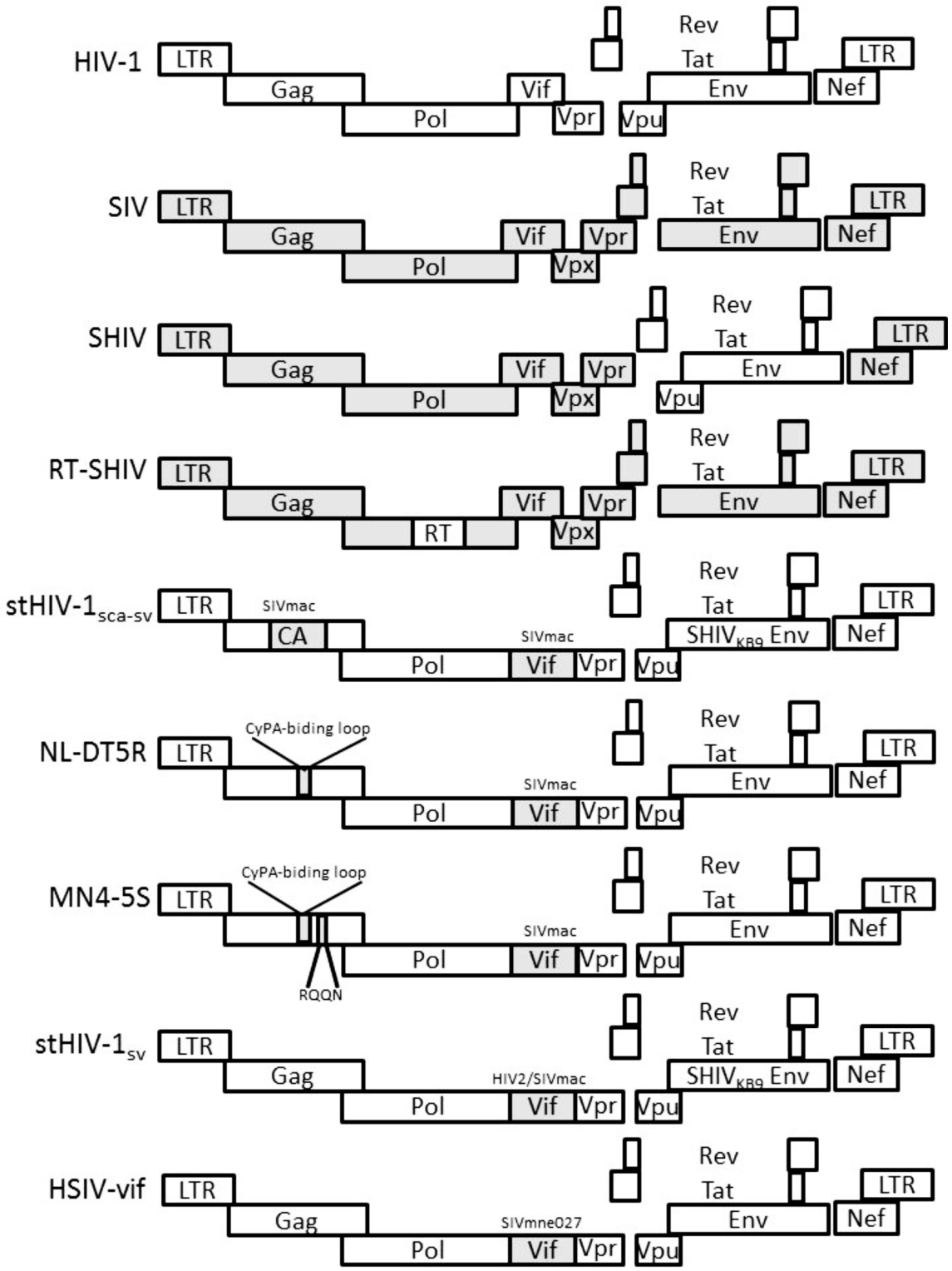


FIGURE 1 | Genetic organization of HIV-1, SIV, and HIV-1/SIV chimeric proviruses. HIV-1 sequences are in white. SIV sequences are shaded gray.

Table 1 | Restriction factors and primate lentivirus infection.

Restriction factors	Mechanism of inhibition	Inhibitory activity in different species
TRIM5α	Binds viral capsid and blocks infection at or before reverse transcription; innate immune sensing of retroviral infection	TRIM5α blocks HIV-1 infection of Asian macaques, except PTM, which do not express TRIM5α; Allelic variation in TRIM5α influences control of SIVs in RM; viral capsid mutations confer resistance to TRIM5α
TRIMcyp		RM and PTM TRIMcyp do not inhibit HIV-1; variation in CM TRIMcyp influences inhibition of HIV-1, HIV-2, and SIVagm, but not SIVmac; viral capsid mutations enable evasion in susceptible hosts
APOBEC3 family proteins	Introduce G to A mutations, reduce infectivity, interfere with reverse transcription	Blocks HIVs and SIVs in the absence of Vif in non-human primates and humans; Vif inhibitory activity against APOBEC3 proteins is limited to virus-adapted host species
BST2/tetherin	Restricts release of virions from the cell surface; innate sensing of infection and promotion of inflammatory responses	Inhibits virion release from human and non-human primate cells; HIV-1 Vpu, SIV Nef, and HIV-2 Env antagonize tetherin only in virus-adapted species
SAMHD1	Reduces dNTP pool required for cDNA synthesis	SAMHD1 proteins from different non-human primate species and humans inhibit HIV and SIV infection of myeloid derived cells and resting T cells; Vpx and Vpr proteins from some SIVs direct proteasome-mediated degradation of SAMHD1 of virus-adapted non-human primate species and humans; HIV-1 does not antagonize SAMHD1

The HIV-1 Vpu evolved to overcome the activity of human tetherin, but it is ineffective against tetherin from chimpanzees, RM, AGM, and mustached monkeys (Jia et al., 2009; Sauter et al., 2009; Lim et al., 2010a; Yang et al., 2010). Despite the close relatedness of HIV-1 and SIVcpz, Vpu of SIVcpz does not antagonize chimpanzee tetherin. Instead it uses Nef to downregulate chimpanzee tetherin expression like other SIVs, which also exhibits species-specific activity (Jia et al., 2009; Zhang et al., 2009).

SAMHD1 is a restriction factor that inhibits HIV-1 infection of myeloid cells (Hrecka et al., 2011; Laguette et al., 2011). Although its exact biological function is unclear, mutations in SAMHD1 can result in Aicardi Goutieres syndrome whose symptoms mimic that of a viral infection (Rice et al., 2009). Vpx protein from either HIV-2 or the SIVsm lineage inhibit human SAMHD1, resulting in its degradation through the proteasome. It has been noted that Vpx expression or SAMHD1 depletion increases the amount of dNTP's in macrophages, which suggests that SAMHD1 decreases the dNTP pool required for viral cDNA synthesis (Lahouassa et al., 2012). Structural studies also indicate that SAMHD1 is a dNTP triphosphate triphosphohydrolase (Goldstone et al., 2011). Interestingly, SAMHD1 only restricts infection of HIV-1 in non-dividing cells such as macrophages and resting T cells but not activated proliferating T cells (Baldauf et al., 2012; Descours et al., 2012). New data also indicate that phosphorylation may regulate SAMHD1's restriction activity (Cribier et al., 2013; White et al., 2013).

Like the other restriction factors, Vpx appears to antagonize SAMHD1 in a species-specific manner since human and gibbon SAMHD1 can be degraded by Vpx proteins from HIV-2rod, SIVmac, and SIVsm but not by Vpx from SIVrcm and SIVmnd2. However, Vpx proteins from different SIV and HIV-2 strains can induce degradation of RM and SM SAMHD1 (Laguette et al., 2012; Lim et al., 2012). Interestingly, some SIVs inhibit SAMHD1 of their natural hosts via Vpr. Thus, targeting SAMHD1 appears critical for replication and persistence of SIVs in OWMs. It is therefore interesting that HIV-1 does not have a mechanism to antagonize SAMHD1 in human cells. One hypothesis is that this may help the virus avoid immune sensing.

Other innate restriction factors such as interferon inducible transmembrane proteins (IFITM), and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (Lu et al., 2011; Wilson et al., 2012) have been shown to interfere with early and late stages of the viral life cycle, respectively. However, whether these factors have species-specific activity against primate lentiviruses is unknown.

ENGINEERING MACAQUE-TROPIC HIV-1 DERIVATIVES

The species-specific effects of innate restriction factors and requirement for particular SIV sequences for replication competent SHIV chimeras suggested that engineering macaque-tropic recombinant viruses consisting of mainly HIV-1 sequences may be possible as long as the virus can evade or antagonize key host restriction factors (**Figure 1**). Hatziioannou et al. (2006) generated the initial HIV-1 chimera with minimal SIV sequences that could replicate in RM peripheral blood mononuclear cells (PBMCs; stHIV-$1_{sca\text{-}sv}$). The virus included *ca* and *vif* substitutions from SIVmac in order to escape restriction by RM TRIM5α and A3G, respectively. In other studies, a macaque-tropic HIV-1 derivative with the SIV *vif* gene and a short 21 base pair segment corresponding to the HIV-1 cyclophilin A binding loop from SIV was constructed (NL-DT5R; Kamada et al., 2006; Igarashi et al., 2007).

The virus showed increased infectivity in both CM and PTM T cells. However, only after passaging in a CM T cell line was the virus able to replicate efficiently in $CD8^+$ cell-depleted PBMCs from either PTM or RM. While these HIV-1 derivatives infected PTM, they were rapidly controlled and did not cause disease. Additional studies selected *gag* variants better able to escape restriction by CM TRIMcyp (e.g., MN4-5S), but replication only modestly improved in CMs (Kuroishi et al., 2009; Saito et al., 2011).

Because of the absence of a post-entry block to HIV-1 infection and potential for more rapid AIDS progression, PTMs were hypothesized to be the most susceptible to macaque-tropic HIV-1 derivatives. Indeed, substituting *vif* in HIV-1 with alleles from SIVmne (HSIV-vif) or SIVmac or HIV-2 (stHIV-1) is sufficient for HIV-1 to replicate in PTM $CD4^+$ T cells (Hatziioannou et al., 2009; Thippeshappa et al., 2011). Infection of PTMs with mtHIV-1 resulted in acute infection and viremia that was controlled within 25 weeks post-infection. Interestingly, replication of HSIV-vif in PTMs extended for over 90 weeks post-infection, although plasma viral loads were low. Moreover, one animal demonstrated a steep drop in $CD4^+$ T cell counts, persistent but low viremia, and opportunistic infections after three years of infection (unpublished observations). It will be important to reisolate variants from this animal and examine the genetic and phenotypic changes that have occurred during infection. Since the different variants of HIV-1 and SIV used in these studies seem to make a difference in persistence and disease, other variants should be considered for future *in vivo* infection experiments.

What accounts for virological control in the PTMs remains unclear. There is suggestion from $CD8^+$ cell-depletion studies that cellular immune responses may be limiting replication of the macaque-tropic HIV-1 clones (Hatziioannou et al., 2009). Additionally, the IFN-1 response might restrict viral replication. IFNs are upregulated during HIV-1 and SIV infections (Neil and Bieniasz, 2009; Thippeshappa et al., 2012). Thus, these viruses must be able to overcome the induction of restrictive interferon-stimulated genes (ISGs) in order to replicate to high levels and cause disease. Indeed, new studies demonstrate that the prototype macaque-tropic HIV-1 derivatives are inhibited by IFNα in PTM cells. By contrast, pathogenic SIVmne and SIVmac clones are highly resistant to IFNα-induced inhibition (Bitzegeio et al., 2013; Thippeshappa et al., 2013). Interestingly, suppression of replication of the HIV-1 derivatives by IFNα may not be due to the induction of known restriction factors such as tetherin, TRIM5α, TRIMcyp, A3G, or SAMHD1, indicating that other ISGs may be responsible for potently blocking replication of macaque-tropic HIV-1 in PTMs. Furthermore, IFNα resistance may be acquired by mutations in *env*, enabling escape from an early block in replication (Thippeshappa et al., 2013). Infection of PTMs with this variant could provide insight into whether evasion of IFNα is critical for viral replication in the host.

SUMMARY AND CONCLUSIONS

The engineering of macaque-tropic HIV-1 derivatives has shed light on the significance of counteracting or escaping restriction factors of the innate immune response for cross-species transmission. Macaque models have provided experimental *in vivo* systems to demonstrate the importance of Vif-mediated antagonism of A3 proteins and evasion of TRIM5 isoforms. Indeed, in the absence of inhibitory TRIM5α or TRIMcyp alleles in the PTM, Vif-mediated inhibition of A3G is necessary and sufficient for transmission and persistence of HIV-1 in PTMs. However, the SIV Vif is not sufficient for robust replication of macaque-tropic HIV-1 chimeras in PTMs because these viruses fail to adequately overcome the IFNα-induced antiviral state. Additional adaptations like those we have identified in an *env* sequence may be necessary for HIV-1 to replicate to high levels in the PTM or other macaque hosts. What other restriction factors might play a role in controlling HIV-1 replication in OWMs like Asian Macaques is unclear, but the IFNα resistance mutations may help identify new mechanisms of escape. Finally, it is curious that lentiviruses of OWMs target SAMHD1 for degradation via Vpx or Vpr, and that Vpx enhances transmission and pathogenesis of SIV in PTMs (Hirsch et al., 1998; Belshan et al., 2012), but HIV-1 did not evolve a mechanism to inhibit this protein in humans. Macaque-tropic HIV-1 derivatives provide a way to test whether antagonizing the activity of SAMHD1 is necessary for replication in OWM species.

ACKNOWLEDGMENTS

Support provided by NIH grant AI099007 to Jason T. Kimata.

REFERENCES

Agy, M. B., Frumkin, L. R., Corey, L., Coombs, R. W., Wolinsky, S. M., Koehler, J., et al. (1992). Infection of *Macaca nemestrina* by human immunodeficiency virus type-1. *Science* 257, 103–106. doi: 10.1126/science.1621083

Agy, M. B., Schmidt, A., Florey, M. J., Kennedy, B. J., Schaefer, G., Katze, M. G., et al. (1997). Serial in vivo passage of HIV-1 infection in *Macaca nemestrina*. *Virology* 238, 336–343. doi: 10.1006/viro.1997.8832

Alexander, L., Du, Z., Howe, A. Y., Czajak, S., and Desrosiers, R. C. (1999). Induction of AIDS in rhesus monkeys by a recombinant simian immunodeficiency virus expressing nef of human immunodeficiency virus type 1. *J. Virol.* 73, 5814–5825.

Ambrose, Z., Palmer, S., Boltz, V. F., Kearney, M., Larsen, K., Polacino, P., et al. (2007). Suppression of viremia and evolution of human immunodeficiency virus type 1 drug resistance in a macaque model for antiretroviral therapy. *J. Virol.* 81, 12145–12155. doi: 10.1128/JVI.01301-07

Apetrei, C., Kaur, A., Lerche, N. W., Metzger, M., Pandrea, I., Hardcastle, J., et al. (2005). Molecular epidemiology of simian immunodeficiency virus SIVsm in U.S. primate centers unravels the origin of SIVmac and SIVstm. *J. Virol.* 79, 8991–9005. doi: 10.1128/JVI.79.14.8991-9005.2005

Bailes, E., Gao, F., Bibollet-Ruche, F., Courgnaud, V., Peeters, M., Marx, P. A., et al. (2003). Hybrid origin of SIV in chimpanzees. *Science* 300, 1713. doi: 10.1126/science.1080657

Baldauf, H. M., Pan, X., Erikson, E., Schmidt, S., Daddacha, W., Burggraf, M., et al. (2012). SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells. *Nat. Med.* 18, 1682–1687. doi: 10.1038/nm.2964

Belshan, M., Kimata, J. T., Brown, C., Cheng, X., McCulley, A., Larsen, A., et al. (2012). Vpx is critical for SIVmne infection of pigtail macaques. *Retrovirology* 9, 32. doi: 10.1186/1742-4690-9-32

Bishop, K. N., Verma, M., Kim, E. Y., Wolinsky, S. M., and Malim, M. H. (2008). APOBEC3G inhibits elongation of HIV-1 reverse transcripts. *PLoS Pathog.* 4:e1000231. doi: 10.1371/journal.ppat.1000231

Bitzegeio, J., Sampias, M., Bieniasz, P. D., Hatziioannou, T., and Sampias, M. (2013). Adaptation to the interferon-induced antiviral state by human and simian immunodeficiency viruses. *J. Virol.* 87, 3549–3560. doi: 10.1128/JVI.03219-12

Brennan, G., Kozyrev, Y., and Hu, S. L. (2008). TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3569–3574. doi: 10.1073/pnas.0709511105

Brennan, G., Kozyrev, Y., and Kodama, T. (2007). Novel TRIM5 isoforms

expressed by *Macaca nemestrina*. *J. Virol.* 81, 12210–12217. doi: 10.1128/JVI.02499-06

Canary, L. A., Vinton, C. L., Morcock, D. R., Pierce, J. B., Estes, J. D., Brenchley, J., et al. (2013). Rate of AIDS progression is associated with gastrointestinal dysfunction in simian immunodeficiency virus-infected pigtail macaques. *J. Immunol.* 190, 2959–2965. doi: 10.4049/jimmunol.1202319

Casartelli, N., Guivel-Benhassine, F., Bouziat, R., Brandler, S., Schwartz, O., and Moris, A. (2010). The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. *J. Exp. Med.* 207, 39–49. doi: 10.1084/jem.20091933

Conticello, S. G., Harris, R. S., and Neuberger, M. S. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. *Curr. Biol.* 13, 2009–2013. doi: 10.1016/j.cub.2003.10.034

Cowan, S., Hatziioannou, T., Cunningham, T., Muesing, M. A., Gottlinger, H. G., and Bieniasz, P. D. (2002). Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11914–11919. doi: 10.1073/pnas.162299499

Cribier, A., Descours, B., Valadão, A. L., Laguette, N., and Benkirane, M. (2013). Phosphorylation of SAMHD1 by Cyclin A2/CDK1 Regulates Its Restriction Activity toward HIV-1. *Cell Rep.* 3, 1036–1043. doi: 10.1016/j.celrep.2013.03.017

Descours, B., Cribier, A., Chable-Bessia, C., Ayinde, D., Rice, G., Crow, Y., et al. (2012). SAMHD1 restricts HIV-1 reverse transcription in quiescent CD4(+) T-cells. *Retrovirology* 9, 87. doi: 10.1186/1742-4690-9-87

Dietrich, E. A., Brennan, G., Ferguson, B., Wiseman, R. W., O'Connor, D., and Hu, S. L. (2011). Variable prevalence and functional diversity of the antiretroviral restriction factor TRIMCyp in *Macaca fascicularis*. *J. Virol.* 85, 9956–9963. doi: 10.1128/JVI.00097-11

Fultz, P. N. (1993). Nonhuman primate models for AIDS. *Clin. Infect. Dis.* 17(Suppl. 1), S230–S235. doi: 10.1093/clinids/17.Supplement_1.S230

Galao, R. P., Le Tortorec, A., Pickering, S., Kueck, T., and Neil, S. J. (2012). Innate sensing of HIV-1 assembly by Tetherin induces NFkappaB-dependent proinflammatory responses. *Cell Host Microbe* 12, 633–644. doi: 10.1016/j.chom.2012.10.007

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., et al. (1999). Origin of HIV-1 in the *chimpanzee Pan troglodytes troglodytes*. *Nature* 397, 436–441. doi: 10.1038/17130

Gardner, M. B. (1996). The history of simian AIDS. *J. Med. Primatol.* 25, 148–157. doi: 10.1111/j.1600-0684.1996.tb00011.x

Gardner, M. B., and Luciw, P. A. (1989). Animal models of AIDS. *FASEB J.* 3, 2593–2606.

Gartner, S., Liu, Y., Polonis, V., Lewis, M. G., Elkins, W. R., et al. (1994). Adaptation of HIV-1 to pig-tailed macaques. *J. Med. Primatol.* 23, 155–163. doi: 10.1111/j.1600-0684.1994.tb00117.x

Goldstone, D. C., Ennis-Adeniran, V., Hedden, J. J., Groom, H. C., Rice, G. I., Christodoulou, E., et al. (2011). HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature* 480, 379–382. doi: 10.1038/nature10623

Grutter, M. G., and Luban, J. (2012). TRIM5 structure, HIV-1 capsid recognition, and innate immune signaling. *Curr. Opin. Virol.* 2, 142–150. doi: 10.1016/j.coviro.2012.02.003

Harris, R. S., Hultquist, J. F., and Evans, D. T. (2012). The restriction factors of human immunodeficiency virus. *J. Biol. Chem.* 287, 40875–40883. doi: 10.1074/jbc.R112.416925

Hatziioannou, T., Ambrose, Z., Chung, N. P. Y., Piatak, M., Yuan, F., Trubey, C. M., et al. (2009). A macaque model of HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4425–4429. doi: 10.1073/pnas.0812587106

Hatziioannou, T., and Evans, D. T. (2012). Animal models for HIV/AIDS research. *Nat. Rev. Microbiol.* 10, 852–867. doi: 10.1038/nrmicro2911

Hatziioannou, T., Princiotta, M., Piatak, M. Jr., Yuan, F., Zhang, F., Lifson, J. D., et al. (2006). Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* 314, 95. doi: 10.1126/science.1130994

Hirsch, V. M., Sharkey, M. E., Brown, C. R., Brichacek, B., Goldstein, S., Wakefield, J., et al. (1998). Vpx is required for dissemination and pathogenesis of SIV(SM) PBj: evidence of macrophage-dependent viral amplification. *Nat. Med.* 4, 1401–1408. doi: 10.1038/3992

Hrecka, K., Hao, C., Gierszewska, M., Swanson, S. K., Kesik-Brodacka, M., Srivastava, S., et al. (2011). Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 474, 658–661. doi: 10.1038/nature10195

Igarashi, T., Iyengar, R., Byrum, R. A., Buckler-White, A., Dewar, R. L., Buckler, C. E., et al. (2007). Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques. *J. Virol.* 81, 11549–11552. doi: 10.1128/JVI.00960-07

Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., et al. (2002). An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* 79, 285–296. doi: 10.1006/geno.2002.6718

Jia, B., Serra-Moreno, R., Neidermyer, W., Rahmberg, A., Mackey, J., Fofana, I. B., et al. (2009). Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. *PLoS Pathog.* 5:e1000429. doi: 10.1371/journal.ppat.1000429

Kamada, K., Igarashi, T., Martin, M. A., Khamsri, B., Hatcho, K., Yamashita, T., et al. (2006). Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16959–16964. doi: 10.1073/pnas.0608289103

Kimata, J. T. (2006). HIV-1 fitness and disease progression: insights from the SIV-macaque model. *Curr. HIV Res.* 4, 65–77. doi: 10.2174/157016206775197628

Kirmaier, A., Wu, F., Newman, R. M., Hall, L. R., Morgan, J. S., O'Connor, S., et al. (2010). TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. *PLoS Biol.* 8:e1000462. doi: 10.1371/journal.pbio.1000462

Klatt, N. R., Canary, L. A., Vanderford, T. H., Vinton, C. L., Engram, J. C., Dunham, R. M., et al. (2012a). Dynamics of simian immunodeficiency virus SIVmac239 infection in pigtail macaques. *J. Virol.* 86, 1203–1213. doi: 10.1128/JVI.06033-11

Klatt, N. R., Silvestri, G., and Hirsch, V. (2012b). Nonpathogenic simian immunodeficiency virus infections. *Cold Spring Harb. Perspect. Med.* 2, a007153. doi: 10.1101/cshperspect.a007153

Kuang, Y. Q., Tang, X., Liu, F. L., Jiang, X. L., Zhang, Y. P., Gao, G., et al. (2009). Genotyping of TRIM5 locus in northern pig-tailed macaques (*Macaca leonina*), a primate species susceptible to Human Immunodeficiency Virus type 1 infection. *Retrovirology* 6, 58. doi: 10.1186/1742-4690-6-58

Kuroishi, A., Saito, A., Shingai, Y., Shioda, T., Nomaguchi, M., Adachi, A., et al. (2009). Modification of a loop sequence between alpha-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) vif and CA alpha-helices 4 and 5 loop improves replication in cynomolgus monkey cells. *Retrovirology* 6, 70. doi: 10.1186/1742-4690-6-70

Laguette, N., Rahm, N., Sobhian, B., Chable-Bessia, C., Münch, J., Snoeck, J., et al. (2012). Evolutionary and functional analyses of the interaction between the myeloid restriction factor SAMHD1 and the lentiviral Vpx protein. *Cell Host Microbe* 11, 205–217. doi: 10.1016/j.chom.2012.01.007

Laguette, N., Sobhian, B., Casartelli, N., Ringeard, M., Chable-Bessia, C., Ségéral, E., et al. (2011). SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 474, 654–657. doi: 10.1038/nature10117

Lahouassa, H., Daddacha, W., Hofmann, H., Ayinde, D., Logue, E. C., Dragin, L., et al. (2012). SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat. Immunol.* 13, 223–228. doi: 10.1038/ni.2236

Le Tortorec, A., and Neil, S. J. (2009). Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *J. Virol.* 83, 11966–11978. doi: 10.1128/JVI.01515-09

Li, J. T., Halloran, M., Lord, C. I., Watson, A., Ranchalis, J., Fung, M., et al. (1995). Persistent infection of macaques with simian-human immunodeficiency viruses. *J. Virol.* 69, 7061–7067.

Lim, E. S., Fregoso, O. I., McCoy, C. O., Matsen, F. A., Malik, H. S., Emerman, M., et al. (2012). The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. *Cell Host Microbe* 11, 194–204. doi: 10.1016/j.chom.2012.01.004

Lim, E. S., Malik, H. S., and Emerman, M. (2010a). Ancient adaptive evolution of tetherin shaped the functions of Vpu and Nef in human immunodeficiency virus and primate lentiviruses. *J. Virol.* 84, 7124–7134. doi: 10.1128/JVI.00468-10

Lim, S. Y., Rogers, T., Chan, T., Whitney, J. B., Kim, J., Sodroski, J., et al. (2010b). TRIM5alpha modulates immunodeficiency virus control in rhesus monkeys. *PLoS Pathog.* 6:e1000738. doi: 10.1371/journal.ppat.1000738

Lu, J., Pan, Q., Rong, L., He, W., Liu, S. L., and Liang, C. (2011). The IFITM proteins inhibit HIV-1 infection. *J. Virol.* 85, 2126–2137. doi: 10.1128/JVI.01531-10

Luciw, P. A., Pratt-Lowe, E., Shaw, K. E., Levy, J. A., and Cheng-Mayer, C. (1995). Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). *Proc. Natl. Acad. Sci. U.S.A.* 92, 7490–7494. doi: 10.1073/pnas.92.16.7490

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* 424, 99–103. doi: 10.1038/nature01709

Mariani, R., Chen, D., Schröfelbauer, B., Navarro, F., König, R., Bollman, B., et al. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114, 21–31.

Munk, C., Brandt, S. M., Lucero, G., and Landau, N. R. (2002). A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13843–13848. doi: 10.1073/pnas.212400099

Nakayama, E. E., Miyoshi, H., Nagai, Y., and Shioda, T. (2005). A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5alpha determines species-specific restriction of simian immunodeficiency virus SIVmac infection. *J. Virol.* 79, 8870–8877. doi: 10.1128/JVI.79.14.8870-8877.2005

Neil, S., and Bieniasz, P. (2009). Human immunodeficiency virus, restriction factors, and interferon. *J. Interferon Cytokine Res.* 29, 569–580. doi: 10.1089/jir.2009.0077

Neil, S. J., Zang, T., and Bieniasz, P. D (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425–430. doi: 10.1038/nature06553

Newman, R. M., Hall, L., Kirmaier, A., Pozzi, L. A., Pery, E., Farzan, M., et al. (2008). Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS Pathog.* 4:e1000003. doi: 10.1371/journal.ppat.1000003

Pertel, T., Hausmann, S., Morger, D., Züger, S., Guerra, J., Lascano, J., et al. (2011). TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472, 361–365. doi: 10.1038/nature09976

Reimann, K. A., Li, J. T., Veazey, R., Halloran, M., Park, I. W., Karlsson, G. B., et al. (1996). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70, 6922–6928.

Reynolds, M. R., Sacha, J. B., Weiler, A. M., Borchardt, G. J., Glidden, C. E., Sheppard, N. C., et al. (2011). The TRIM5{alpha} genotype of rhesus macaques affects acquisition of simian immunodeficiency virus SIVsmE660 infection after repeated limiting-dose intrarectal challenge. *J. Virol.* 85, 9637–9640. doi: 10.1128/JVI.05074-11

Rice, G. I., Bond, J., Asipu, A., Brunette, R. L., Manfield, I. W., Carr, I. M., et al. (2009). Mutations involved in Aicardi–Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response. *Nat. Genet.* 41, 829–832. doi: 10.1038/ng.373

Saito, A., Kono, K., Nomaguchi, M., Yasutomi, Y., Adachi, A., Shioda, T., et al. (2012). Geographical, genetic and functional diversity of antiretroviral host factor TRIMCyp in cynomolgus macaque (*Macaca fascicularis*). *J. Gen. Virol.* 93(Pt 3), 594–602. doi: 10.1099/vir.0.038075-0

Saito, A., Nomaguchi, M., Iijima, S., Kuroishi, A., Yoshida, T., Lee, Y. J., et al. (2011). Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications. *Microbes Infect.* 13, 58–64. doi: 10.1016/j.micinf.2010.10.001

Sauter, D., Schindler, M., Specht, A., Landford, W. N., Münch, J., Kim, K. A., et al. (2009). Tetherin-driven adaptation of Vpu and Nef function and the evolution of pandemic and nonpandemic HIV-1 strains. *Cell Host Microbe* 6, 409–421. doi: 10.1016/j.chom.2009.10.004

Sawyer, S. L., Wu, L. I., Emerman, M., and Malik, H. S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2832–2837. doi: 10.1073/pnas.0409853102

Sayah, D. M., Sokolskaja, E., Berthoux, L., and Luban, J. (2004). Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 430, 569–573. doi: 10.1038/nature02777

Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418, 646–650. doi: 10.1038/nature00939

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., and Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* 65, 3514–3520.

Sinclair, E., Barbosa, P., and Feinberg, M. B. (1997). The nef gene products of both simian and human immunodeficiency viruses enhance virus infectivity and are functionally interchangeable. *J. Virol.* 71, 3641–3651.

Stephens, E. B., McCormick, C., Pacyniak, E., Griffin, D., Pinson, D. M., Sun, F., et al. (2002). Deletion of the vpu sequences prior to the env in a simian-human immunodeficiency virus results in enhanced Env precursor synthesis but is less pathogenic for pig-tailed macaques. *Virology* 293, 252–261. doi: 10.1006/viro.2001.1244

Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., et al. (2006). Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5514–5519. doi: 10.1073/pnas.0509996103

Thippeshappa, R., Polacino, P., Yu Kimata, M. T., Siwak, E. B., Anderson, D., Wang, W., et al. (2011). Vif substitution enables persistent infection of pig-tailed macaques by human immunodeficiency virus type 1. *J. Virol.* 85, 3767–3779. doi: 10.1128/JVI.02438-10

Thippeshappa, R., Ruan, H., and Kimata, J. T. (2012). Breaking barriers to an AIDS model with macaque-tropic HIV-1 derivatives. *Biology (Basel)* 1, 134–164. doi: 10.3390/biology1020134

Thippeshappa, R., Ruan, H., Wang, W., Zhou, P., and Kimata, J. T. (2013). A variant macaque-tropic human immunodeficiency virus type 1 is resistant to interferon alpha induced restriction in pig-tailed macaque $CD4^+$ T-cells. *J. Virol.* 87, 6678–6692 doi: 10.1128/JVI.00338-13

Uberla, K., Stahl-Hennig, C., Böttiger, D., Mätz-Rensing, K., Kaup, F. J., Li, J., et al. (1995). Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8210–8214.

Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., et al. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 3, 245–252. doi: 10.1016/j.chom.2008.03.001

Virgen, C. A., Kratovac, Z., Bieniasz, P. D., and Hatziioannou, T. (2008). Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3563–3568. doi: 10.1073/pnas.0709258105

White, T. E., Brandariz-Nunez, A., Valle-Casuso, J. C., Amie, S., Anh Nguyen, L., Kim, B., et al. (2013). The retroviral restriction ability of SAMHD1, but not its deoxynucleotide triphosphohydrolase activity, is regulated by phosphorylation. *Cell Host Microbe* 13, 441–451. doi: 10.1016/j.chom.2013.03.005

Wilson, S. J., Schoggins, J. W., Zang, T., Kutluay, S. B., Jouvenet, N., Alim, M. A., et al. (2012). Inhibition of HIV-1 particle assembly by 2′,3′-cyclic-nucleotide 3′-phosphodiesterase. *Cell Host Microbe* 12, 585–597. doi: 10.1016/j.chom.2012.08.012

Wilson, S. J., Webb, B. L., Ylinen, L. M., Verschoor, E., Heeney, J. L., and Towers, G. J. (2008). Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3557–3562. doi: 10.1073/pnas.0709003105

Yang, S. J., Lopez, L. A., Hauser, H., Exline, C. M., Haworth, K. G., and Cannon, P. M. (2010). Anti-tetherin activities in Vpu-expressing primate lentiviruses. *Retrovirology* 7, 13. doi: 10.1186/1742-4690-7-13

Zhang, F., Wilson, S. J., Landford, W. C., Virgen, B., Gregory, D., Johnson, M. J., et al. (2009). Nef proteins from simian immunodeficiency viruses are tetherin antagonists. *Cell Host Microbe* 6, 54–67. doi: 10.1016/j.chom.2009.05.008

Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* 424, 94–98. doi: 10.1038/nature01707

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 April 2013; accepted: 11 June 2013; published online: 28 June 2013.
*Citation: Misra A, Thippeshappa R and Kimata JT (2013) Macaques as model hosts for studies of HIV-1 infection. Front. Microbiol. **4**:176. doi: 10.3389/fmicb.2013.00176*
This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 09 July 2013
doi: 10.3389/fmicb.2013.00187

Macaque-tropic human immunodeficiency virus type 1: breaking out of the host restriction factors

Akatsuki Saito*[1,2] ***and Hirofumi Akari*[1]**

[1] *Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Japan*
[2] *Japan Foundation for AIDS Prevention, Chiyoda-ku, Japan*

Edited by:
Tomoyuki Miura, Kyoto University, Japan

Reviewed by:
Emi E. Nakayama, Osaka University, Japan
Yoshiaki Nishimrua, National Institutes of Health, USA

****Correspondence:***
Akatsuki Saito, Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan
e-mail: akatsuki.saito@gmail.com

Macaque monkeys serve as important animal models for understanding the pathogenesis of lentiviral infections. Since human immunodeficiency virus type 1 (HIV-1) hardly replicates in macaque cells, simian immunodeficiency virus (SIV) or chimeric viruses between HIV-1 and SIV (SHIV) have been used as challenge viruses in this research field. These viruses, however, are genetically distant from HIV-1. Therefore, in order to evaluate the efficacy of anti-HIV-1 drugs and vaccines in macaques, the development of a macaque-tropic HIV-1 (HIV-1mt) having the ability to replicate efficiently in macaques has long been desired. Recent studies have demonstrated that host restriction factors, such as APOBEC3 family and TRIM5, impose a strong barrier against HIV-1 replication in macaque cells. By evading these restriction factors, others and we have succeeded in developing an HIV-1mt that is able to replicate in macaques. In this review, we have attempted to shed light on the role of host factors that affect the susceptibility of macaques to HIV-1mt infection, especially by focusing on TRIM5-related factors.

Keywords: macaques, HIV-1, animal model, host factors, genetic background

INTRODUCTION

It is estimated that about 2.5 million individuals per year get infected with human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS; UNAIDS Global report 2012, http://www.unaids.org/). To contain the disastrous epidemic, we need to consider effective approaches. For the pre-clinical evaluation of the anti-HIV-1 vaccines and therapy, it is necessary to have suitable animal models. Moreover, animal models would also aid for the understanding of the underlying mechanisms of HIV-1 pathogenicity. Since HIV-1 shows very narrow species specificity, being limited to human and apes, it has been quite challenging to develop an ideal animal model in which HIV-1 efficiently replicates and induces pathogenicity. Instead, many kinds of surrogate models developed as alternative strategy have provided us many important insights. In this decade, the molecular characterization of antiviral host restriction factors has dramatically progressed and shed light on the understanding of the viral specificity. These findings encouraged us to develop a novel non-human primate model for HIV-1 infection on the basis of a new concept (i.e., introduction of minimal modification to HIV-1 genome), by which the resultant virus would overcome a number of restriction factors. In this review, we summarize the history of the identification of the restriction factors and also discuss its impact and future direction on the development of HIV-1 animal models.

HISTORY OF HIV-1 ANIMAL MODELS

HIV-1 INFECTION IN SMALL ANIMALS

After the identification of HIV-1 as a causative agent of AIDS, many investigators sought to develop animal models for further research (reviewed in Gardner and Luciw, 1989). Although many efforts were performed in small animals, HIV-1 did not infect rodents, such as mice and rats, due to a number of restrictions, including the inability of HIV-1 Env to use the surface molecules in these animals as binding and entry receptors (Atchison et al., 1996) and the defect of murine cyclin T1 protein to associate with HIV-1 Tat (Kwak et al., 1999). Although rabbits were once expected to show susceptibility to HIV-1 infection (Filice et al., 1988; Kulaga et al., 1989), the reproducibility of this model remains to be elucidated (Reina et al., 1993; Speck et al., 1998; Tervo and Keppler, 2010). In an attempt to overcome the limitation in using these animals, several versions of humanized mice such as SCID-hu-PBL (severe combined immunodeficiency-human peripheral blood lymphocytes) mice (Mosier et al., 1988), Rag2$^{-/-}$ $\gamma c^{-/-}$ mice (Traggiai et al., 2004), NOG (NOD/Shi-scid/IL-2Rγnull) mice (Ito et al., 2002), NSG (NOD scid gamma) mice (Shultz et al., 2005), and NOD/SCID-hu BLT mice (Melkus et al., 2006), have been generated (reviewed in Berges and Rowan, 2011). To generate this model, human immune cells were implanted into immunocompromised mice. After reconstitution of engrafted immune cells, HIV-1 replication in these animals was investigated. Generally, robust HIV-1 replication and loss of peripheral CD4^{+} T cells is observed in infected animals. Therefore, this model system would be useful for evaluation of drugs and neutralizing antibodies against HIV-1 (Denton et al., 2008). Moreover, this model provides important insight about the viral latency and the role of accessory genes *in vivo* (Denton et al., 2012; Marsden et al., 2012; Sato et al., 2012). However, none or weak immune response is observed in these animals. Moreover, this model requires special surgical skills and facilities to perform experiments.

HIV-1 INFECTION IN NON-HUMAN PRIMATES

Differently from other pathogenic viruses for human such as measles and mumps, HIV-1 does not replicate in New World

monkeys (NWMs) and Old World monkeys (OWMs). In cells from NWMs, such as squirrel monkey (*Saimiri sciureus*) and common marmoset (*Callithrix jacchus*), the cluster of differentiation 4 (CD4) and C-C chemokine receptor type 5 (CCR5) molecules function insufficiently as binding and entry receptors (LaBonte et al., 2002). On the other hand, in OWM cells, most HIV-1 enters target cells as efficiently as human cells. Interestingly, recent studies revealed that some subtypes of HIV-1 are unable to efficiently utilize macaque CD4 because of the difference in the C-terminus of the D1 domain of CD4 between human and OWMs, and therefore adaptive mutation was required for optimal efficiency (Humes and Overbaugh, 2011; Humes et al., 2012). After entering target cells, the subsequent steps of HIV-1 life cycle (i.e., uncoating and reverse transcription) are strongly abolished in OWM cells (Shibata et al., 1995; Hofmann et al., 1999). Although pigtailed macaque (*Macaca nemestrina*; hereafter denoted as PM) was once believed to be promising because of its higher susceptibility to HIV-1 infection as compared to other OWMs (Agy et al., 1992), the HIV-1 replication in those animals was weak and the trial of serial *in vivo* passage was shown to be unsuccessful (Agy et al., 1997). Among the animals examined for their susceptibility to HIV-1 infection, chimpanzees and gibbon apes were identified to have high susceptibility (Fultz et al., 1986; Lusso et al., 1988). In 1980s and 1990s, many chimpanzees were experimentally infected with HIV-1, including clinically isolated viruses and molecularly cloned viruses, resulting in a robust viral replication (Alter et al., 1984; Fultz et al., 1987, 1999; Nara et al., 1987; Prince et al., 1988). These experiments provided many important insights, including the roles of neutralizing antibody in protective immunity. While some of the infected chimpanzees experienced AIDS-related symptoms (Fultz et al., 1991; Novembre et al., 1997; O'Neil et al., 2000), most of them seemed not to develop apparent clinical symptoms (Gardner and Luciw, 1989; Johnson et al., 1993). Furthermore, there are many concerns about using chimpanzees, including ethical issues and their quite high rearing cost; therefore, researchers finally decided not to use this ape for HIV-1 research (Cohen, 2007). Therefore, the need for the development of other non-human primate models has been increasing.

SIV INFECTION IN NON-HUMAN PRIMATES

As a surrogate model, simian immunodeficiency virus (SIV) infection in Asian macaques, such as rhesus macaque (*Macaca mulatta*; hereafter denoted as RM) and cynomolgus macaque (*Macaca fascicularis*; hereafter denoted as CM) has been developed. While SIV efficiently replicates in its natural host [e.g., sooty mangabey (*Cercocebus atys*; hereafter denoted as SM) for SIVsm and African green monkey (*Chlorocebus sabaeus*; hereafter denoted as AGM) for SIVagm, respectively; Ohta et al., 1988; Kraus et al., 1989], infected animals generally do not develop immunodeficiency, unlike the course of HIV-1 infection in humans. In the 1980s, accidental transmission of SIVsm to RMs caused a lethal disease, and the symptoms were quite similar to those seen in AIDS patients (Daniel et al., 1985; Letvin et al., 1985). Thereafter, the pathogenic virus was molecularly cloned as SIVmac (Naidu et al., 1988; Kestler et al., 1990). The combination of SIVmac and RMs has been broadly utilized as a surrogate model for HIV-1 infection because of its similarity in the genome structure and pathogenicity. Specifically, this model dramatically advanced our understanding in terms of the functional roles of the viral accessory genes *in vivo* (Kestler et al., 1991; Gibbs et al., 1995; Hirsch et al., 1998). Moreover, this model provided the important finding that the acquired protective immunity induced by live-attenuated vaccines was effective against homologous and heterologous SIV challenges (Daniel et al., 1992; Wyand et al., 1996, 1999).

INFECTION OF CHIMERIC VIRUS BETWEEN HIV-1 AND SIV IN NON-HUMAN PRIMATES

Accumulating evidence has demonstrated the inability of intact HIV-1 to replicate in OWM cells. Then, what kind of viral components in HIV-1 and SIV determine their host tropism? In an effort to answer this profound question, many researchers constructed chimeric viruses between HIV-1 and SIV and analyzed their viral replication in human and OWM cells. It was shown that chimeric viruses containing HIV-1-derived *gag* and/or *vif* were unable to replicate in macaque cells and that a chimeric virus encoding HIV-1-derived *env* on the SIVmac backbone was able to replicate in primary OWM cells (Shibata et al., 1991; Shibata and Adachi, 1992), indicating that the step of entry was not the determinant for the species specificity of HIV-1. As a consequence of vigorous investigation, Shibata et al. finally succeeded to construct a prototypic simian–human immunodeficiency virus (hereafter denoted as SHIV) clone that encodes HIV-1-derived *tat*, *rev*, *vpu*, and *env* genes on the SIVmac239 backbone (Shibata et al., 1991; Shibata and Adachi, 1992). This SHIV clone was shown to efficiently replicate in primary macaque cells. Thereafter, many groups developed several versions of SHIV. Of note, by serial passaging of apathogenic SHIV-89.6 in monkeys, Reimann et al. (1996) successfully obtained a highly pathogenic virus (SHIV-89.6P) that caused rapid and complete depletion of peripheral $CD4^+$ T cells, leading to simian AIDS. These chimeric viruses not only enabled us to evaluate the efficacy of antiviral immunity against HIV-1 Env but also supplied us important insights on what kind of SIVmac-derived genes are necessary to replicate in macaque cells. This SHIV model became a huge breakthrough for HIV-1 investigators; by using SHIV, the mechanism and efficacy of passive immunization (Shibata et al., 1999; Baba et al., 2000; Nishimura et al., 2002) as well as vaccine candidates (Igarashi et al., 1997; Letvin et al., 1997; Cafaro et al., 1999) were vigorously investigated. Incidentally, the lower sequence homology in RT between SIV and HIV-1 limited this model for the evaluation of antiretroviral drugs especially against RT. To overcome this limitation, RT-SHIV, which encodes HIV-1 RT in the place of SIVmac RT, was developed and used for the assessment of RT inhibitors (Uberla et al., 1995; Ambrose et al., 2004; North et al., 2005). SHIV carrying HIV-1 integrase (IN) in addition to RT was also constructed (Akiyama et al., 2008). These efforts have dramatically advanced the basic research related to HIV-1. However, since these viruses were constructed on the basis of SIVmac backbone, SHIVs are still far from HIV-1. Moreover, some pathogenic SHIV clones, such as SHIV-89.6P, show quite different phenotypes in macaques, unlike those in HIV-1 infection of humans and SIVmac infection of macaques (Feinberg and Moore, 2002). First, these SHIVs induced abnormally rapid, profound, and irreversible loss of $CD4^+$ T cells in macaques, differently from the gradual

decline of $CD4^+$ T cells observed in most HIV-1-infected patients (McCune, 2001). Second, these SHIVs were somehow highly susceptible to neutralizing antibodies, while most HIV-1 isolates and pathogenic SIVs were resistant to neutralization. Therefore, earlier vaccine studies using SHIV as a challenge virus succeeded in controlling viral replication by immunization with vaccine candidates (Amara et al., 2001; Barouch et al., 2001). Notably, these outcomes were frequently observed in experiments with SHIV using C-X-C chemokine receptor type 4 (CXCR4; X4-tropic virus), or SHIV using both CXCR4 and CCR5 as co-receptors (dual-tropic virus). Since HIV-1 in human population usually uses CCR5 as a co-receptor during transmission (Schuitemaker et al., 1992), it will be straightforward to develop an R5-tropic SHIV in order to reproduce the transmission, latency, and pathogenicity of HIV-1 in macaques. In fact, R5-tropic SHIVs were recently constructed and their phenotype seemed different from those of X4-tropic SHIVs and dual-tropic SHIVs. It is thought that X4-tropic SHIV selectively infects $CXCR4^+$ naive $CD4^+$ T cells that are enriched in secondary lymph nodes, while most SIV and R5-tropic SHIV mainly target $CCR5^+$ memory $CD4^+$ T cells in extra-lymphoid immune effector sites such as gut, lung and genital tract, explaining the divergent clinical sequel (Harouse, 1999; Nishimura et al., 2004; Ho et al., 2005). Especially, mucosal infection with R5-tropic SHIV would be a promising tool for investigating protection and transmission of immunodeficiency viruses (Matsuda et al., 2010; Bomsel et al., 2011; Gautam et al., 2012; Moldt et al., 2012).

In spite of the usefulness of these SHIVs in experiments targeting HIV-1 *env*, the low similarity in other genes, especially *gag* and *pol*, still limits the use of this virus as a challenge virus. Since cytotoxic T lymphocyte (CTL) response against Gag protein is thought to play a central role in controlling viral replication (Kiepiela et al., 2007), the absence of HIV-1-derived *gag* in current SHIV hampers evaluation of vaccine candidate against HIV-1 Gag. To solve this problem, we need to proceed to construct more relevant animal models of HIV-1. In this decade, our knowledge about host factors that form species barrier against HIV-1 has dramatically increased. This knowledge would permit us to develop an HIV-1 clone having the potential to replicate in macaques. Many efforts to develop a more feasible model were made by several groups as described below. Here, we summarize the role of anti-HIV-1 restriction factors in macaque cells and the viral antagonists against these factors.

INTRINSIC HOST FACTORS

APOBEC3 FAMILY

It has long been observed that the infectivity of *vif* gene-deficient HIV-1 in certain T cell lines such as H9 and CEM, as well as primary lymphocytes, was strongly decreased (Gabuzda et al., 1992; Sakai et al., 1993; Tervo and Keppler, 2010). Virions produced from these restrictive cells have less infectivity as compared to the wild-type virus. Many efforts were made to identify a cellular factor that conferred this restrictive activity. In particular, the fact that heterokaryons between permissive and restrictive cells suppressed the infectivity of the *vif*-deficient HIV-1 clearly suggested the existence of a potent endogenous inhibitor of HIV-1 replication in restrictive cells (Madani and Kabat, 1998; Simon et al., 1998). Finally, in 2002, the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 G (APOBEC3G; hereafter denoted as A3G) was identified as a novel host restriction factor in human cells (Sheehy et al., 2002). A3G is expressed in various tissues including testis, ovary, spleen, and peripheral blood mononuclear cells (PBMCs; Jarmuz et al., 2002). Since A3G is a member of the cytidine deaminase enzyme, the *vif*-deficient virus contains many G-to-A mutations in its minus-strand genome, leading to disruption of infectivity. Moreover, the fact that deamination-deficient mutant A3G can still inhibit *vif*-deficient HIV-1 implied that A3G exerts its antiviral activity with deamination-dependent and deamination-independent fashion (Newman et al., 2005). In order to counteract the A3G-mediated restriction, HIV-1 has equipped its genome with *vif* gene and the resultant protein, Vif, efficiently inhibits A3G incorporation into progeny virions by inducing proteasome-dependent degradation of A3G (Conticello et al., 2003; Kao et al., 2003; Mehle et al., 2004). Recently, it was reported that core-binding factor beta (CBFβ), a transcription regulator through RUNX binding, was required for HIV-1 Vif to degrade A3G (Hultquist et al., 2012; Jager et al., 2012). SIVmac Vif similarly recruits CBFβ in order to neutralize the RM A3G (Hultquist et al., 2012; Jager et al., 2012). It was also proposed that HIV-1 Vif suppresses human A3G activity by inhibiting the translation of A3G (Mercenne et al., 2010). Although the human genome encodes other six A3 members (A3A, B, C, DE, F, and H) in addition to A3G, the precise antiviral activity of the A3 proteins remains to be elucidated. Human A3F was also reported to have anti-HIV-1 activity and susceptibility to HIV-1 Vif (Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). In contrast, Miyagi et al. (2010) suggested that the antiviral activity of endogenous level of human A3F was negligible as compared to the activity of A3G. It is known that human A3DE and A3F, in addition to A3G, are also sensitive to counteraction by HIV-1 Vif (Goila-Gaur and Strebel, 2008). As seen in humans, the RM genome also encodes seven A3 members (Schmitt et al., 2011). Virgen and Hatziioannou (2007) investigated the susceptibility of HIV-1 to each RM A3 family member and showed that A3B, A3F, A3G, and A3H had the ability to restrict HIV-1 and were resistant to HIV-1 Vif activity. It should be noted that Vif-A3G interaction shows species specificity (Mariani et al., 2003). HIV-1 Vif is able to counteract A3G from humans but not from RM and AGM (Zennou and Bieniasz, 2006; Virgen and Hatziioannou, 2007). Conversely, SIVagm Vif is effective against A3G from AGM and RM, but unable to antagonize A3G from human and chimpanzee (Mariani et al., 2003). SIVmac Vif efficiently counteracts A3G from human, chimpanzee, AGM, and RM (Mariani et al., 2003). Are there any polymorphisms in the *A3* family? In case of humans, a polymorphism in *A3B* deletion was reported (Kidd et al., 2007). In RMs, a polymorphism in A3DE was observed and was reported to affect the antiviral activity (Virgen and Hatziioannou, 2007). How can we obtain HIV-1 with the ability to overcome macaque A3s? Many efforts have been made to evade from the restriction by the macaque A3 family. Schrofelbauer et al. (2006) showed that mutations of HIV-1 Vif at positions 14–17 from DRMR into SEMQ allowed HIV-1 Vif interaction with A3G from RM. However, this HIV-1 Vif harboring SEMQ remained susceptible to A3B, A3F, and A3H from RM (Virgen and Hatziioannou, 2007), suggesting that the introduction of this sequence

in HIV-1 Vif was not sufficient for evading from A3s other than A3G. Besides, since the replication of HIV-1 in OWM cells was suppressed, at least at two steps (early and late stages of HIV-1 lifecycle), it is reasonable to speculate that just a modification of *vif* is insufficient for HIV-1 to overcome the restriction in various OWM cells.

BONE MARROW STROMAL ANTIGEN 2

It had been observed that the production of *vpu*-deficient HIV-1 in certain cell lines was severely diminished (Klimkait et al., 1990; Sakai et al., 1995). Specifically, while permissive cells, such as HEK293T and HT1080 cells, allowed comparative levels of virion production, non-permissive cells, such as Jurkat and HeLa cells, decreased the amount of virion production in the absence of *vpu*. It was also reported that interferon (IFN) treatment led to phenotype switch from permissive to non-permissive (Neil et al., 2007). Thus, the existence of unknown IFN-inducible, Vpu-sensitive cellular factors, was predicted. In 2008, bone marrow stromal antigen 2 (BST-2), also known as tetherin, CD317, and HM1.24, was identified by two independent groups (Neil et al., 2008; Van Damme et al., 2008). BST-2 is a type 2 integral membrane protein, with the N-terminus located in the cytoplasm, one membrane-spanning domain, and a C-terminus modified by the addition of a glycosyl-phosphatidylinositol (GPI) anchor (Kupzig et al., 2003). Erikson et al. (2011) analyzed the expression profile of BST-2 *in vivo* and demonstrated that BST-2 was expressed in various tissues, especially spleen and alimentary system. They also showed that among PBMCs, monocytes express high levels of BST-2 as compared to T and B cells. Furthermore, like tripartite motif-containing protein 5 (TRIM5α), hominid BST-2, but not other primate BST-2, has been recently reported to function as an innate sensor, leading to the transforming growth factor β activated kinase-1 (TAK1)-dependent activation of NFκB and subsequent production of pro-inflammatory cytokines (Galao et al., 2012). Cocka and Bates (2012) recently showed that human *BST-2* gene expressed alternative splice isoforms that led to different antiviral activity as well as sensing activity from the wild-type one. To overcome BST-2-mediated restriction, HIV-1 downregulates BST-2 from the cell surface by expressing Vpu protein, a viral protein absent in most of the SIVsm/HIV-2 lineage (Neil et al., 2008; Van Damme et al., 2008). On the other hand, HIV-2 utilizes Env protein as an antagonist for human BST-2 (Le Tortorec and Neil, 2009). In the case of SIVmac, Nef protein confers the ability to overcome BST-2-mediated restriction in RM cells (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009). It is also reported that Env protein of SIVtan [SIV from Tantalus monkeys (*Chlorocebus tantalus*)] was effective against BST-2 from human and RM (Gupta et al., 2009). It should be noted that the antagonistic activity of these viral proteins against BST-2 is thought to function in a species-specific manner. While Vpu from the HIV-1 group M is able to counteract human and chimpanzee BST-2, most of these Vpus are ineffective against BST-2 from RM and AGM (McNatt et al., 2009; Sauter et al., 2009). In contrast, Nef from SIVmac is effective for BST-2 from RM and SM but ineffective for BST-2 from human (Jia et al., 2009). This characteristic resistance of human BST-2 to SIV Nef was proven to have an association with the deletion in human BST-2 of 5 amino acid residues, to which SIV Nef binds (Jia et al., 2009; Zhang et al., 2009). Although most SIVsm/HIV-2 lineage does not encode *vpu* gene, SIVcpz, SIVgor [SIV from gorillas (*Gorilla gorilla gorilla*)], SIVgsn [SIV from greater spot-nosed guenons (*Cercopithecus nictitans*)], SIVmon [SIV from mona monkeys (*Cercopithecus mona*)], SIVmus [SIV from moustached monkey (*Cercopithecus cephus*)], and SIVden [SIV from Dent's mona monkey (*Cercopithecus denti*)] were shown to harbor the *vpu* gene (Courgnaud et al., 2003; Dazza et al., 2005). Recently, Sauter et al. (2009) demonstrated that Vpus from SIVgsn and SIVden potently counteracted the BST-2 from RM. Moreover, Shingai et al. (2011) showed that Vpu from $SHIV_{DH12}$ potently counteracted BST-2 from RM. It is therefore possible that the exchange of present HIV-1_{NL4-3}-derived-Vpu with these Vpus might lead to efficient evasion from the BST-2-mediated restriction in macaque cells. It was reported that a *nef*-deleted SIVmac239 inoculated to RM became pathogenic after *in vivo* passage (Alexander et al., 2003; Serra-Moreno et al., 2011). Serra-Moreno et al. (2011) showed that the *nef*-deleted SIVmac239 gained the ability to antagonize BST-2 by utilizing its Env gp41 as a consequence of adaptive mutations in the *env* gene. In addition, Vpu from the less pathogenic HIV-1 group O was reported to lose anti-BST-2 activity (Sauter et al., 2009). It was shown that $SHIV_{DH12}$ lacking intact Vpu inefficiently replicated *in vivo* as compared to the wild-type virus (Shingai et al., 2011). These findings indicate the importance of evasion from BST-2-mediated restriction for lentiviral pathogenesis *in vivo*. Although detailed genetic information is limited, the *BST-2* gene is reported to be polymorphic at least in RM (McNatt et al., 2009). Therefore, when using macaques for HIV-1 research, we should also appreciate the polymorphisms in *BST-2* gene.

SAMHD1

It has long been observed that HIV-1 replication in myeloid linage cells, such as macrophages and dendritic cells (DCs) was impaired and the expression of HIV-2/SIV Vpx *in trans* was shown to rescue this inhibition (Goujon et al., 2007, 2008; Kaushik et al., 2009). The sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain containing protein 1 (SAMHD1) was identified as an HIV-1 restriction factor in myeloid cells that were degraded by the HIV-2/SIV Vpx protein (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 was reported to restrict HIV-1 replication in resting $CD4^+$ T cells as well (Baldauf et al., 2012; Descours et al., 2012). Historically, SAMHD1 is shown to be associated with the Aicardi–Goutières autoimmune-mediated neurodevelopmental syndrome. Patients having a mutation in *SAMHD1* gene would have symptoms of abnormal immune activation likely due to the excessive production of IFNα (Crow and Rehwinkel, 2009; Rice et al., 2009). Since SAMHD1 functions as a deoxyguanosine triphosphate (dGTP)-regulated deoxynucleoside triphosphate (dNTP) triphosphohydrolase (Powell et al., 2011), it exerts its anti-HIV-1 activity via the depletion of dNTP pools in virus-infected cells, leading to the inhibition of the reverse transcription (Lahouassa et al., 2012). The fact that SAMHD1-deficient $CD14^+$ monocytes efficiently permit HIV-1 replication supports this notion (Berger et al., 2011). It is noteworthy that SAMHD1 exerts its antiviral activity against various retroviruses ranging from alpha, beta and gamma retrovirus,

except for prototype foamy virus and Human T cell leukemia virus type I (HTLV-1; Gramberg et al., 2013). As described above, the SAMHD1-mediated restriction would be counteracted by HIV-2/SIV Vpx. Hofmann et al. (2012) showed that Vpx recruits SAMHD1 to a cullin4 A-RING E3 ubiquitin ligase, leading to proteasomal degradation. The importance of Vpx *in vivo* was based on the fact that the replication of *vpx*-deleted SIV in monkeys was significantly weaker than that in wild-type SIV (Gibbs et al., 1995; Hirsch et al., 1998; Belshan et al., 2012). However, *vpx*-deleted SIV still had the ability to induce simian AIDS in macaques, suggesting a limited role of SAMHD1-mediated restriction in SIV pathogenesis (Gibbs et al., 1995). It is of note that while HIV-2 as well as most of SIV linage such as SIVmac encodes *vpx*, HIV-1 as well as some SIV lineage such as SIVcpz and SIVgor does not encode *vpx* in its genome. Similar to the relationship between A3G and Vif, SAMHD1 is also antagonized by viral proteins in a species-specific manner. For instance, Vpxs from SIVmac and SIVsm are effective against SAMHD1 from human, OWMs, and NWMs (Laguette et al., 2012), while those from SIVrcm [SIV from red-capped mangabey (*Cercocebus torquatus*)] or SIVmnd [SIV from mandrill (*Mandrillus sphinx*)] are effective against SAMHD1 from OWMs and NWMs but not from humans (Lim et al., 2012). Lim et al. (2012) also found that Vpr from some SIV lineage, such as SIVdeb [SIV from De Brazza's monkey (*Cercopithecus neglectus*)], SIVmus, and a part of SIVagm (SIV from AGM), has the potency of degrading SAMHD1 from RM and AGM. It would be of great interest to introduce these *vpr*s into HIV-1mt and examine whether this modification would enhance the viral replication in myeloid linage cells from macaques.

TRIM5

It was demonstrated that the replication of HIV-1 in OWMs cells was severely abolished before reverse transcription (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002). An experiment using interspecies heterokaryons between OWM and human cells suggested the existence of an inhibitory factor in OWM cells (Munk et al., 2002). Stremlau et al. (2004) by screening the RM cDNA library, successfully identified TRIM5α as a restriction factor in RM cells that confer permissive cells resistance to HIV-1 infection. They also demonstrated that RM TRIM5α, but not human TRIM5α, could restrict HIV-1 infection. On the other hand, human TRIM5α potently restricts the N-tropic murine leukemia virus (N-MLV) as well as the equine infectious anemia virus (EIAV) but not B-tropic murine leukemia virus (B-MLV; Hatziioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004), indicating the importance of TRIM5α as a host factor restricting the cross-species transmission of retroviruses. TRIM5α is ubiquitously expressed and consists of a RING domain, a B-box domain, a coiled coil domain, and a PRYSPRY (B30.2) domain (Reymond et al., 2001). The characteristic PRYSPRY domain recognizes the capsid of incoming retroviruses, leading to the restriction of the infection at the post-entry step. This domain is also responsible for the species-specific function of TRIM5α (Nakayama and Shioda, 2010). It was shown that TRIM5α was IFN-inducible and that IFN treatment of cells led to the augmentation of antiviral activity (Asaoka et al., 2005; Sakuma et al., 2007). An additional role of TRIM5α as a pattern recognition receptor was recently identified (Pertel et al., 2011). TRIM5α binds to the incoming viral capsid and then activates its E3 ligase activity, together with the UBC13–UEV1A enzyme complex, resulting in the synthesis of free ubiquitin chains. The chains stimulate TAK1 phosphorylation and the expression of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)- and MPK (mitogen-activated protein kinase)-responsive genes, leading to an antiviral state (De Silva and Wu, 2011). Among the restriction factors discussed here, *TRIM5* gene might be most polymorphic in primates. At what degree does this polymorphism in *TRIM5* gene affect the susceptibility to retroviral infection? A length polymorphism in *TRIM5*α, in which the TFP residues from position 339 to 341 of TRIM5α were replaced with a single glutamine (Q), was identified in some RM individuals (Newman et al., 2006). This TFP/Q polymorphism affects the anti-lentiviral activity of RM TRIM5α against SIVsmE543-3 and SIVsmE041 but not against SIVmac (Kirmaier et al., 2010). Similarly, this polymorphism in RM *TRIM5*α is associated with the different antiviral activity against HIV-2 (Kono et al., 2008).

Although most cell lines from NWMs were susceptible to VSV-G pseudotyped HIV-1, cell lines from owl monkey (*Aotus trivirgatus*) exceptionally showed high resistance to infection by HIV-1 (Hofmann et al., 1999). As the reason for this discrepancy, Sayah et al. (2004) successfully identified TRIM5-Cyclophilin A (CypA) chimeric protein (referred to as TRIMCyp) in owl monkey, which was derived from LINE-1-mediated retrotransposition of CypA cDNA into the region between *TRIM5* exons 7 and 8. In the case of OWMs, the higher susceptibility of PM to HIV-1 infection was, at least in part, explained by the fact that PM exclusively have the *TRIMCyp* genotype instead of TRIM5α (Liao et al., 2007; Brennan et al., 2008; Virgen et al., 2008). Differently from owl monkey TRIMCyp, the *TRIMCyp* of PM was a consequence of a retrotransposition of the *CypA* sequence in the 3′ untranslated region (UTR) of the *TRIM5* gene, together with a single nucleotide polymorphism (SNP) at the exon 7 splice acceptor site. This SNP at the splice acceptor site leads to skipping exons 7 and 8 encoding the PRYSPRY domain and splicing to the inserted *CypA* gene. In addition to PM, it is reported so far that RM and CM also possess *TRIMCyp* in their genome (Brennan et al., 2008; Newman et al., 2008; Wilson et al., 2008). Interestingly, RM has geographic deviation in the frequency of *TRIMCyp*, depending on the country of origin (Wilson et al., 2008). It is reported that Indian RM possessed *TRIMCyp* more frequently than Chinese RM (Wilson et al., 2008; De Groot et al., 2011). We recently reported that CM also showed divergent frequency of *TRIMCyp* depending on their country of origin (Saito et al., 2012b). The frequency of *TRIMCyp* in Filipino CM was significantly higher than that in Malaysian and Indonesian CM. We demonstrated that wild-caught CM also had a geographic deviation in the frequency of *TRIMCyp* as seen in captive CM (Saito et al., 2012a). Consistently, Dietrich et al. (2011) reported that the frequency of *TRIMCyp* in Filipino CM was higher than those in Indonesian and Indochina CM. It was shown that Mauritian CM, a population thought to be derived from Indonesian CM, seemed not to possess *TRIMCyp*, probably due to the founder effects at the time of introduction by human (Dietrich et al., 2011; Berry et al., 2012). Since TRIM5α is expected to act as homomultimer (Mische et al., 2005; Perez-Caballero et al.,

2005), heterologous expression of *TRIM5*α in combination with *TRIM5* isoforms other than TRIM5α reportedly led to a dominant negative effect on the TRIM5α antiviral activity (Berthoux et al., 2005; Maegawa et al., 2008). Interestingly, it was reported that RM heterozygous for *TRIM5*α and *TRIMCyp* showed higher resistance to repeated intrarectal challenge of SIVsmE660 as compared to RM homozygous for *TRIM5*α or *TRIMCyp* (Reynolds et al., 2011). Since RM TRIMCyp could restrict SIVsm but not SIVmac (Kirmaier et al., 2010), it is reasonable to assume that the combination of *TRIM5*α and *TRIMCyp* may function more efficiently as antiviral factors against SIVsm. We will further discuss the impact of *TRIM5* polymorphism on the viral replication in the latter chapter. In summary, since *TRIM5* genotype would greatly influence the susceptibility to lentiviruses, the correlation between polymorphism of *TRIM5* gene in macaques and outcomes should be carefully evaluated.

UNIDENTIFIED RESTRICTION FACTORS

Viral infection usually stimulates cellular factors through pattern recognition receptors, such as Toll-like receptor (TLRs) and RIG-I-like receptors, expressed on many type of cells, leading to the induction of IFN production (Bowie and Unterholzner, 2008). In particular, type I IFN, which include IFN-α and IFN-β, puts a switch on the IFN-stimulated gene 15 (ISG15), leading to a cascade of antiviral status (Zhao et al., 2013). The expression levels of the restriction factors described above are reported to increase via IFN stimulation (Asaoka et al., 2005; Tanaka et al., 2006; Neil et al., 2007; Sakuma et al., 2007). Lately, Bitzegeio et al. (2013) have demonstrated that HIV-1-based chimeric viruses, engineered to overcome SAMHD1 or BST-2 as well as A3 and TRIM5 from PM, are still severely restricted in IFN-treated PM PBMCs. They have also demonstrated that the replication of SIVmac in IFN-treated human PBMCs is greatly suppressed, and *vice versa*. This finding strongly suggests the existence of unidentified, IFN-inducible restriction factors in each species. Therefore, it is also necessary to continue exploring such unidentified cellular factors.

CONSTRUCTION OF MACAQUE-TROPIC HIV-1

In virtue of the detailed understanding of the molecular relationship between antiviral host factors and viral antagonists (summarized in **Tables 1** and **2**), it became possible to create a macaque-tropic HIV-1 (HIV-1mt) with the ability to replicate in OWM cells. In 2006, two independent groups succeeded in the construction of an HIV-1mt that contains partial SIV-derived sequences on the HIV-1_{NL4-3} backbone. Hatziioannou et al. (2006) constructed HIV-1mt that contains the entire Gag-CA and *vif* from SIVmac in order to evade from TRIM5α- and A3G-mediated restriction, respectively. This HIV-1mt, which contains approximately 88% of HIV-1-derived sequence, was shown to efficiently replicate in RM PBLs. In parallel with that study, Kamada et al. (2006) constructed HIV-1mt named NL-DT5R in which the sequence of CypA binding loop [the loops of α-helices 4 and 5 (L4/5)] in Gag-CA and entire *vif* gene were replaced with those from SIVmac239. NL-DT5R, in which approximately 93% of its sequence was derived from HIV-1, was shown to replicate in a CM T cell line (HSC-F cells) as well as

Table 1 | Antiviral host factors and antagonism by lentiviral proteins.

Antiviral host factors	Antagonized by	NOT antagonized by
Human APOBEC3G	HIV-1 Vif	SIVagm Vif
	SIVmac Vif	
RM APOBEC3G	SIVmac Vif	HIV-1 Vif
	SIVagm Vif	
Human BST-2	HIV-1 Vpu	HIV-1 Nef
	HIV-2 Env	SIVmac Nef
RM BST-2	SIVgsn Vpu	HIV-1 Vpu*
	SIVden Vpu	
	SIVmac Nef	
Human SAMHD1	SIVdeb Vpr	HIV-1 Vpr
	SIVmus Vpr	SIVmac Vpr
	SIVmac Vpx	SIVrcm Vpx
	HIV-2 Vpx	SIVmnd Vpx
RM SAMHD1	SIVdeb Vpr	HIV-1 Vpr
	SIVmus Vpr	SIVmac Vpr
	SIVagm Vpr	SIVrcm Vpr
	SIVmac Vpx	
	HIV-2 Vpx**	
	SIVrcm Vpx	
	SIVmnd Vpx	

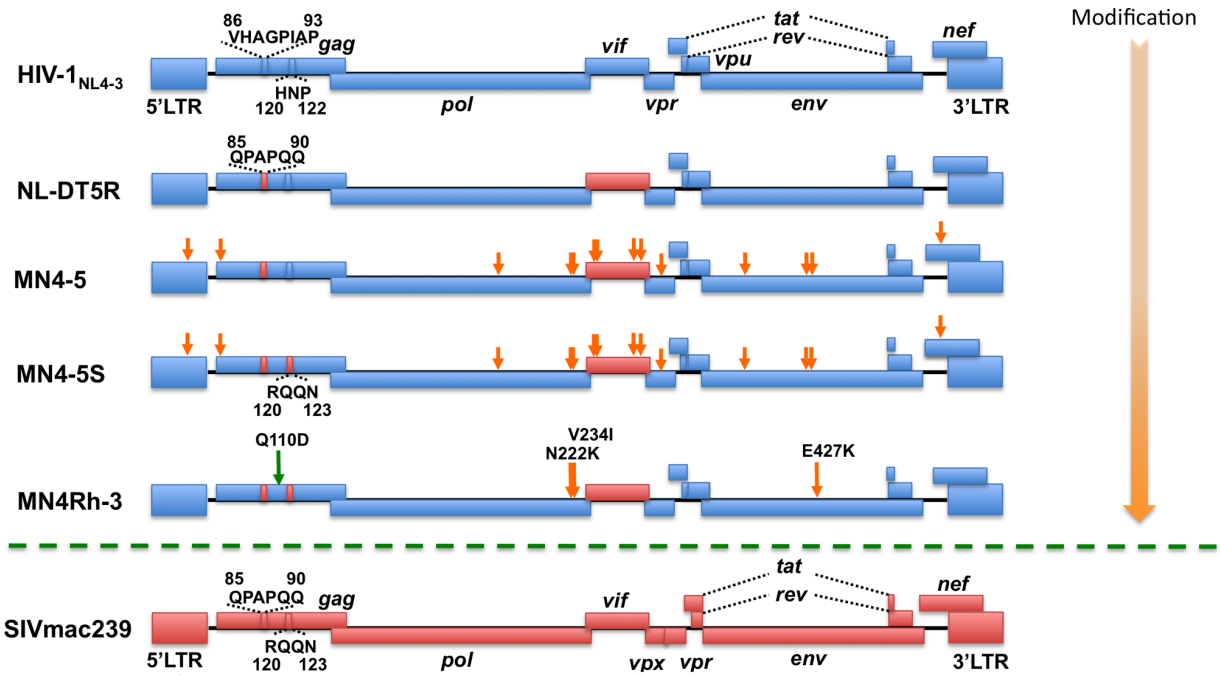
*Summary of findings about APOBEC3G–Vif interaction (Sheehy et al., 2002; Kao et al., 2003; Mariani et al., 2003; Zennou and Bieniasz, 2006; Virgen and Hatziioannou, 2007), BST-2–Vpu, Nef, and Env interaction (Neil et al., 2008; Van Damme et al., 2008; Jia et al., 2009; Le Tortorec and Neil, 2009; Sauter et al., 2009; Zhang et al., 2009; Serra-Moreno et al., 2011), and SAMHD1-Vpx and Vpr interaction (Hrecka et al., 2011; Laguette et al., 2011, 2012; Lim et al., 2012). *Vpus from some HIV-1 strains such as HIV-1_{DH12} are able to antagonize RM BST-2. **Vpxs from some HIV-2 strains are ineffective in antagonizing RM SAMHD1.*

Table 2 | Species-specific restriction of lentiviruses by primate TRIM5 proteins.

***TRIM5* alleles**	**Restrictive against:**		
	HIV-1	**HIV-1mt MN4Rh-3**	**SIVmac239**
Human TRIM5α	–	–	–
RM TRIM5α (TFP)	+	+	–
RM TRIM5α (Q)	+	+	–
CM TRIM5α	+	+	–
RM TRIMCyp	–	–	–
PM TRIMCyp	–	–	–
CM TRIMCyp (DK)	+	–	–
CM TRIMCyp (NE)	–	–	–

Summary of findings about interactions between each TRIM5 allele and lentiviruses (Stremlau et al., 2004; Newman et al., 2006; Liao et al., 2007; Brennan et al., 2008; Virgen et al., 2008; Dietrich et al., 2011; Saito et al., 2012b). "+" denotes restrictive, while "–" denotes not restrictive against each lentivirus, respectively.

$CD8^+$ cell-depleted PM PBMCs but hardly in $CD8^+$ cell-depleted RM PBMCs. Subsequently, Igarashi et al. (2007) investigated the replication capability of NL-DT5R in PM and found that this prototypic HIV-1mt was able to induce acute viremia up to around 1×10^4 copies/mL. Thereafter, in order to enhance the viral replication, we further modified the sequence of NL-DT5R-based HIV-1mt by 2 different approaches. First, we performed a long-term adaptation experiment in CM T cell lines to induce adaptive mutation in its genome. As a consequence of adaptation, several nucleotide substitutions were identified (see **Figure 1**, orange arrows in MN4-5 and MN4-5S). The functional significance of each mutation was molecularly evaluated (Nomaguchi et al., 2013a). Second, we introduced the α-helices 6 and 7 (L6/7) in addition to L4/5 of Gag-CA into MN4-5, resulting in MN4-5S. As shown in **Figure 2**, this substitution enhanced the viral replication *in vitro* (Kuroishi et al., 2009) and *in vivo* (Saito et al., 2011). We next constructed a new HIV-1mt named MN4Rh-3 carrying the Q110D substitution in Gag-CA. This HIV-1mt exhibited further enhanced growth property specifically in macaque cells but impaired replication in human cells (Nomaguchi et al., 2013b). We also examined the replicative property of MN4Rh-3 in CM (Saito et al., 2013). In accordance with *in vitro* data (Nomaguchi et al., 2013b), MN4Rh-3 induced higher viremia on average up to 50 times as compared to MN4-5S (**Figure 2**). Notably, *TRIMCyp* homozygotes were highly permissive to MN4Rh-3 infection, while the replication of MN4Rh-3 in *TRIM5* homozygotes was strongly suppressed. We also observed that CM heterologous for *TRIM5*α and *TRIMCyp* showed similar anti-HIV-1 activity with *TRIMCyp* homozygotes (Saito et al., 2013). These findings indicated that MN4Rh-3 enhanced the replicative capability in CM having *TRIMCyp*, but was still unable to overcome TRIM5α-mediated restriction. It should be noted that the sequence of most *TRIMCyp* encoded in CM are different from those in RM and PM. It was once thought that CM exclusively possessed TRIMCyp in which the amino acid residues at positions 369 (Cyp66) and 446 (Cyp143) were aspartic acid (D) and lysine (K) [denoted as the TRIMCyp (DK)], respectively, while PM and RM had TRIMCyp in which the amino acids at the corresponding positions were asparagine (N)



	Target region and the procedure of substitution	The aim of modification
Our current HIV-1mt	Gag-CA: From the loop between α-helices 4 and 5 of HIV-1_{NL4-3} with that of SIVmac239	To avoid TRIMCyp- and cyclophiln A-mediated restriction
	Gag-CA: From the loop between α-helices 6 and 7 of HIV-1_{NL4-3} with that of SIVmac239	To diminish macaque TRIM5-mediated restriction
	Vif: From the entire *vif* gene of HIV-1_{NL4-3} with that of SIVmac239	To counteract macaque APOBEC3-mediated restriction
Future HIV-1mt	Vpu: From the transmembrane domain of *vpu* gene of HIV-1_{NL4-3} with those of HIV-1_{DH12}, SIVgsn, and SIVden	To counteract macaque BST-2-mediated restriction

FIGURE 1 | Structure of HIV-1mt clones. Blue boxes denote HIV-1_{NL4-3}-derived sequences and red boxes denote SIVmac239-derived sequences, respectively. Orange arrows show adaptive mutations that enhance viral growth potential in macaque T cell lines. The green arrow indicates the CA-Q110D mutation. (Bottom) Summary of the modifications in our current and future HIV-1mts in order to evade from restriction factors in macaque cells.

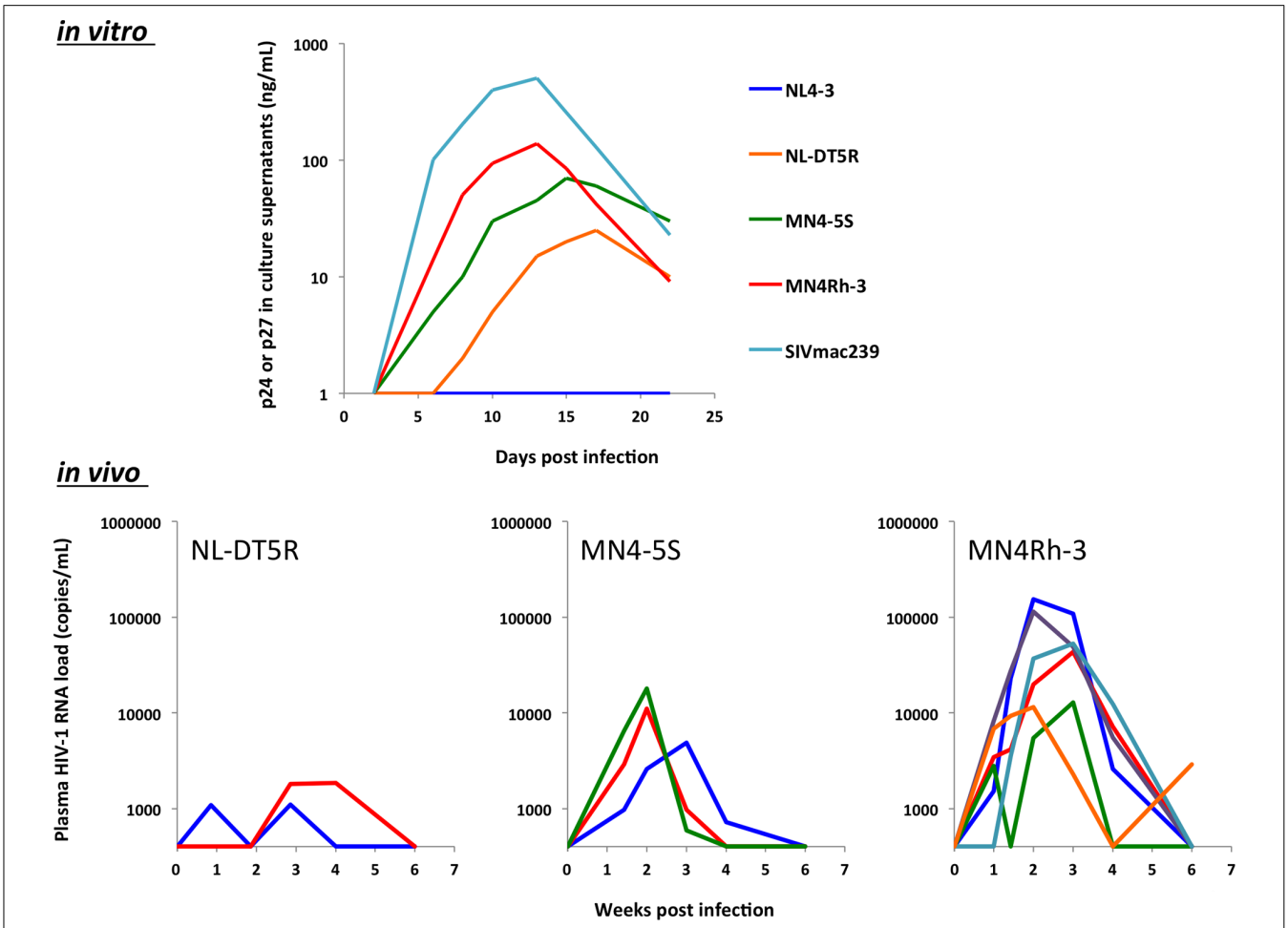


FIGURE 2 | Serial modifications of the viral genome lead to an enhanced viral replication *in vitro* and *in vivo*. (Top) $CD8^+$ T cell-depleted peripheral mononuclear blood cells from CMs homozygous for *TRIMCyp* were infected with each HIV-1mt. The representative result of viral replication kinetics was shown. (Bottom) CM having *TRIMCyp* were infected with each HIV-1mt intravenously. Plasma viral RNA loads in each monkey are shown.

and glutamic acid (E) [denoted as the TRIMCyp (NE); Brennan et al., 2008; Ylinen et al., 2010], respectively. However, others and we recently revealed that CM possessed TRIMCyp (NE) as well as TRIMCyp (DK) in spite of the low frequency of TRIMCyp (NE) haplotype in CM population (Dietrich et al., 2011; Saito et al., 2012a,b). Strikingly, others and we reported that TRIMCyp (DK) and TRIMCyp (NE) exhibit different anti-lentiviral activity. It is well established that the TRIMCyp (DK) efficiently restricts HIV-1 but weakly restricts HIV-2 (Saito et al., 2012b). On the other hand, the TRIMCyp (NE) fails to restrict HIV-1 but efficiently restrict HIV-2 (Wilson et al., 2008). It was also shown that both haplotypes hardly restricted SIVmac239 replication. These results indicate that the sequence variations in CypA greatly affect the spectrum of their anti-HIV-1 activity. However, how does TRIMCyp (DK) exert its anti-HIV-1 activity? Actually, TRIMCyp (DK) is expected to bind the L4/5 in Gag-CA. Moreover, the treatment of the target cells with cyclosporin A, an inhibitor against CypA, or the introduction of amino acid changes in this loop of the viral genome relieved the inhibitory effect by TRIMCyp (DK; Ylinen et al., 2010). Therefore, when we use CM homozygous for TRIMCyp (DK), it is necessary to modify the loop in order to evade restriction. In fact, we have used HIV-1mts in which the L4/5 in Gag-CA were replaced with the corresponding sequence of SIVmac239 (Kamada et al., 2006). In contrast, those research groups that used PM did not need to modify this region. Hatziioannou et al. (2009) have successfully constructed an HIV-1mt that induced persistent viremia in PM with modification of only *vif* and *env* gene. Similarly, Thippeshappa et al. (2011) also constructed an HIV-1mt named HSIV-*vif* that encoded *vif* gene from pathogenic PM-adapted SIVmne027. This HSIV-*vif* was shown to persistently replicate in PM but was unable to induce pathogenicity in animals. Overall, further understanding of the host–virus relationship would permit us to construct pathogenic HIV-1mt in future studies.

CONCLUSIONS AND FUTURE DIRECTIONS

Most HIV-1mts were constructed with the aim of evading from TRIM5 and APOBEC3-mediated restriction. In the future research, as discussed above, we should also focus on other factors such as BST-2 and SAMHD1. It will be promising to modify viral genome in order to overcome these restrictions. We expect that

such procedure will lead to the construction of a new HIV-1mt with the ability to infect various macaques persistently.

Also, as discussed in the "History of HIV-1 animal models" chapter, an R5-tropic virus would be promising to reproduce the transmission, latency, and pathogenicity of HIV-1 in macaques. In the future study, the construction of an R5-tropic virus on the HIV-1mt backbone would encourage us to examine the antiviral agents, vaccines, and microbicides in macaques. Moreover, HIV-1mt that robustly replicate and induce pathogenicity in monkeys will make feasible to investigate the role and mechanism of HIV-1 accessory genes in the HIV-1 lifecycle, persistence, and pathogenesis. In summary, although the road to the containment of HIV-1 epidemic may be long and steep, we have been moving forward slowly but steadily.

ACKNOWLEDGMENTS

The authors would like to give special thanks to Atsunori Higashino and Saori Suzuki for helpful suggestions. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

Agy, M. B., Frumkin, L. R., Corey, L., Coombs, R. W., Wolinsky, S. M., Koehler, J., et al. (1992). Infection of *Macaca nemestrina* by human immunodeficiency virus type-1. *Science* 257, 103–106. doi: 10.1126/science.1621083

Agy, M. B., Schmidt, A., Florey, M. J., Kennedy, B. J., Schaefer, G., Katze, M. G., et al. (1997). Serial in vivo passage of HIV-1 infection in *Macaca nemestrina*. *Virology* 238, 336–343. doi: 10.1006/viro.1997.8832

Akiyama, H., Ishimatsu, M., Miura, T., Hayami, M., and Ido, E. (2008). Construction and infection of a new simian/human immunodeficiency chimeric virus (SHIV) containing the integrase gene of the human immunodeficiency virus type 1 genome and analysis of its adaptation to monkey cells. *Microbes Infect.* 10, 531–539. doi: 10.1016/j.micinf.2008.02.001

Alexander, L., Illyinskii, P. O., Lang, S. M., Means, R. E., Lifson, J., Mansfield, K., et al. (2003). Determinants of increased replicative capacity of serially passaged simian immunodeficiency virus with nef deleted in rhesus monkeys. *J. Virol.* 77, 6823–6835. doi: 10.1128/JVI.77.12.6823-6835.2003

Alter, H. J., Eichberg, J. W., Masur, H., Saxinger, W. C., Gallo, R., Macher, A. M., et al. (1984). Transmission of HTLV-III infection from human plasma to chimpanzees: an animal model for AIDS. *Science* 226, 549–552. doi: 10.1126/science.6093251

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., et al. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 292, 69–74. doi: 10.1126/science.1058915

Ambrose, Z., Boltz, V., Palmer, S., Coffin, J. M., Hughes, S. H., and Kewalramani, V. N. (2004). In vitro characterization of a simian immunodeficiency virus-human immunodeficiency virus (HIV) chimera expressing HIV type 1 reverse transcriptase to study antiviral resistance in pigtail macaques. *J. Virol.* 78, 13553–13561. doi: 10.1128/JVI.78.24.13553-13561.2004

Asaoka, K., Ikeda, K., Hishinuma, T., Horie-Inoue, K., Takeda, S., and Inoue, S. (2005). A retrovirus restriction factor TRIM5alpha is transcriptionally regulated by interferons. *Biochem. Biophys. Res. Commun.* 338, 1950–1956. doi: 10.1016/j.bbrc.2005.10.173

Atchison, R. E., Gosling, J., Monteclaro, F. S., Franci, C., Digilio, L., Charo, I. F., et al. (1996). Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. *Science* 274, 1924–1926. doi: 10.1126/science.274.5294.1924

Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., et al. (2000). Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat. Med.* 6, 200–206. doi: 10.1038/72309

Baldauf, H. M., Pan, X., Erikson, E., Schmidt, S., Daddacha, W., Burggraf, M., et al. (2012). SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells. *Nat. Med.* 18, 1682–1687. doi: 10.1038/nm.2964

Barouch, D. H., Santra, S., Kuroda, M. J., Schmitz, J. E., Plishka, R., Buckler-White, A., et al. (2001). Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J. virol.* 75, 5151–5158. doi: 10.1128/JVI.75.11.5151-5158.2001

Belshan, M., Kimata, J. T., Brown, C., Cheng, X., Mcculley, A., Larsen, A., et al. (2012). Vpx is critical for SIVmne infection of pigtail macaques. *Retrovirology* 9, 32. doi: 10.1186/1742-4690-9-32

Berger, A., Sommer, A. F., Zwarg, J., Hamdorf, M., Welzel, K., Esly, N., et al. (2011). SAMHD1-deficient CD14+ cells from individuals with Aicardi–Goutieres syndrome are highly susceptible to HIV-1 infection. *PLoS Pathog.* 7:e1002425. doi: 10.1371/journal.ppat.1002425

Berges, B. K., and Rowan, M. R. (2011). The utility of the new generation of humanized mice to study HIV-1 infection: transmission, prevention, pathogenesis, and treatment. *Retrovirology* 8, 65. doi: 10.1186/1742-4690-8-65

Berry, N. J., Marzetta, F., Towers, G. J., and Rose, N. J. (2012). Diversity of TRIM5alpha and TRIMCyp sequences in cynomolgus macaques from different geographical origins. *Immunogenetics* 64, 267–278. doi: 10.1007/s00251-011-0585-x

Berthoux, L., Sebastian, S., Sayah, D. M., and Luban, J. (2005). Disruption of human TRIM5alpha antiviral activity by nonhuman primate orthologues. *J. Virol.* 79, 7883–7888. doi: 10.1128/JVI.79.12.7883-7888.2005

Besnier, C., Takeuchi, Y., and Towers, G. (2002). Restriction of lentivirus in monkeys. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11920–11925. doi: 10.1073/pnas.172384599

Bitzegeio, J., Sampias, M., Bieniasz, P. D., and Hatziioannou, T. (2013). Adaptation to the interferon-induced antiviral state by human and simian immunodeficiency viruses. *J. Virol.* 87, 3549–3560. doi: 10.1128/JVI.03219-12

Bomsel, M., Tudor, D., Drillet, A. S., Alfsen, A., Ganor, Y., Roger, M. G., et al. (2011). Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity* 34, 269–280. doi: 10.1016/j.immuni.2011.01.015

Bowie, A. G., and Unterholzner, L. (2008). Viral evasion and subversion of pattern-recognition receptor signaling. *Nat. Rev. Immunol.* 8, 911–922. doi: 10.1038/nri2436

Brennan, G., Kozyrev, Y., and Hu, S. L. (2008). TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3569–3574. doi: 10.1073/pnas.0709511105

Cafaro, A., Caputo, A., Fracasso, C., Maggiorella, M. T., Goletti, D., Baroncelli, S., et al. (1999). Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat. Med.* 5, 643–650. doi: 10.1038/9488

Cocka, L. J., and Bates, P. (2012). Identification of alternatively translated Tetherin isoforms with differing antiviral and signaling activities. *PLoS Pathog.* 8:e1002931. doi: 10.1371/journal.ppat.1002931

Cohen, J. (2007). Animal studies. NIH to end chimp breeding for research. *Science* 316, 1265. doi: 10.1126/science.316.5829.1265

Conticello, S. G., Harris, R. S., and Neuberger, M. S. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. *Curr. Biol.* 13, 2009–2013. doi: 10.1016/j.cub.2003.10.034

Courgnaud, V., Abela, B., Pourrut, X., Mpoudi-Ngole, E., Loul, S., Delaporte, E., et al. (2003). Identification of a new simian immunodeficiency virus lineage with a vpu gene present among different *cercopithecus* monkeys (*C. mona*, *C. cephus*, and *C. nictitans*) from Cameroon. *J. Virol.* 77, 12523–12534. doi: 10.1128/JVI.77.23.12523-12534.2003

Cowan, S., Hatziioannou, T., Cunningham, T., Muesing, M. A., Gottlinger, H. G., and Bieniasz, P. D. (2002). Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc. Nat. Acad. Sci. U.S.A.* 99, 11914–11919. doi: 10.1073/pnas.162299499

Crow, Y. J., and Rehwinkel, J. (2009). Aicardi–Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. *Hum. Mol. Genet.* 18, R130–R136. doi: 10.1093/hmg/ddp293

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C. (1992). Protective effects of

a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258, 1938–1941. doi: 10.1126/science.1470917

Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., et al. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 228, 1201–1204. doi: 10.1126/science.3159089

Dazza, M. C., Ekwalanga, M., Nende, M., Shamamba, K. B., Bitshi, P., Paraskevis, D., et al. (2005). Characterization of a novel vpu-harboring simian immunodeficiency virus from a Dent's Mona monkey (*Cercopithecus mona denti*). *J. Virol.* 79, 8560–8571. doi: 10.1128/JVI.79.13.8560-8571.2005

De Groot, N. G., Heijmans, C. M., Koopman, G., Verschoor, E. J., Bogers, W. M., and Bontrop, R. E. (2011). TRIM5 allelic polymorphism in macaque species/populations of different geographic origins: its impact on SIV vaccine studies. *Tissue Antigens* 78, 256–262. doi: 10.1111/j.1399-0039.2011.01768.x

Denton, P. W., Estes, J. D., Sun, Z., Othieno, F. A., Wei, B. L., Wege, A. K., et al. (2008). Antiretroviral preexposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. *PLoS Med.* 5:e16. doi: 10.1371/journal.pmed.0050016

Denton, P. W., Olesen, R., Choudhary, S. K., Archin, N. M., Wahl, A., Swanson, M. D., et al. (2012). Generation of HIV latency in humanized BLT mice. *J. Virol.* 86, 630–634. doi: 10.1128/JVI.06120-11

Descours, B., Cribier, A., Chable-Bessia, C., Ayinde, D., Rice, G., Crow, Y., et al. (2012). SAMHD1 restricts HIV-1 reverse transcription in quiescent CD4(+) T-cells. *Retrovirology* 9, 87. doi: 10.1186/1742-4690-9-87

De Silva, S., and Wu, L. (2011). TRIM5 acts as more than a retroviral restriction factor. *Viruses* 3, 1204–1209. doi: 10.3390/v3071204

Dietrich, E. A., Brennan, G., Ferguson, B., Wiseman, R. W., O'Connor, D., and Hu, S. L. (2011). Variable prevalence and functional diversity of the antiretroviral restriction factor TRIMCyp in *Macaca fascicularis*. *J. Virol.* 85, 9956–9963. doi: 10.1128/JVI.00097-11

Erikson, E., Adam, T., Schmidt, S., Lehmann-Koch, J., Over, B., Goffinet, C., et al. (2011). In vivo expression profile of the antiviral restriction factor and tumor-targeting antigen CD317/BST-2/HM1.24/tetherin in humans. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13688–13693. doi: 10.1073/pnas.1101684108

Feinberg, M. B., and Moore, J. P. (2002). AIDS vaccine models: challenging challenge viruses. *Nat. Med.* 8, 207–210. doi: 10.1038/nm0302-207

Filice, G., Cereda, P. M., and Varnier, O. E. (1988). Infection of rabbits with human immunodeficiency virus. *Nature* 335, 366–369. doi: 10.1038/335366a0

Fultz, P. N., Mcclure, H. M., Swenson, R. B., Mcgrath, C. R., Brodie, A., Getchell, J. P., et al. (1986). Persistent infection of chimpanzees with human T-lymphotropic virus type III/lymphadenopathy-associated virus: a potential model for acquired immunodeficiency syndrome. *J. Virol.* 58, 116–124.

Fultz, P. N., Siegel, R. L., Brodie, A., Mawle, A. C., Stricker, R. B., Swenson, R. B., et al. (1991). Prolonged CD4+ lymphocytopenia and thrombocytopenia in a chimpanzee persistently infected with human immunodeficiency virus type 1. *J. Infect. Dis.* 163, 441–447. doi: 10.1093/infdis/163.3.441

Fultz, P. N., Srinivasan, A., Greene, C. R., Butler, D., Swenson, R. B., and Mcclure, H. M. (1987). Superinfection of a chimpanzee with a second strain of human immunodeficiency virus. *J. Virol.* 61, 4026–4029.

Fultz, P. N., Wei, Q., and Yue, L. (1999). Rectal transmission of human immunodeficiency virus type 1 to chimpanzees. *J. Infect. Dis.* 179(Suppl. 3), S418–S421. doi: 10.1086/314796

Gabuzda, D. H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W. A., et al. (1992). Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. *J. Virol.* 66, 6489–6495. doi: 10.1186/1742-4690-9-93

Galao, R. P., Le Tortorec, A., Pickering, S., Kueck, T., and Neil, S. J. (2012). Innate sensing of HIV-1 assembly by Tetherin induces NFkappaB-dependent proinflammatory responses. *Cell Host Microbe* 12, 633–644. doi: 10.1016/j.chom.2012.10.007

Gardner, M. B., and Luciw, P. A. (1989). Animal models of AIDS. *FASEB J.* 3, 2593–2606. doi: 10.1038/nrmicro2911

Gautam, R., Nishimura, Y., Lee, W. R., Donau, O., Buckler-White, A., Shingai, M., et al. (2012). Pathogenicity and mucosal transmissibility of the R5-tropic simian/human immunodeficiency virus SHIV(AD8) in rhesus macaques: implications for use in vaccine studies. *J. Virol.* 86, 8516–8526. doi: 10.1128/JVI.00644-12

Gibbs, J. S., Lackner, A. A., Lang, S. M., Simon, M. A., Sehgal, P. K., Daniel, M. D., et al. (1995). Progression to AIDS in the absence of a gene for vpr or vpx. *J. Virol.* 69, 2378–2383.

Goila-Gaur, R., and Strebel, K. (2008). HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology* 5:51. doi: 10.1186/1742-4690-5-51

Goujon, C., Arfi, V., Pertel, T., Luban, J., Lienard, J., Rigal, D., et al. (2008). Characterization of simian immunodeficiency virus SIVSM/human immunodeficiency virus type 2 Vpx function in human myeloid cells. *J. Virol.* 82, 12335–12345. doi: 10.1128/JVI.01181-08

Goujon, C., Riviere, L., Jarrosson-Wuilleme, L., Bernaud, J., Rigal, D., Darlix, J. L., et al. (2007). SIVSM/HIV-2 Vpx proteins promote retroviral escape from a proteasome-dependent restriction pathway present in human dendritic cells. *Retrovirology* 4, 2. doi: 10.1186/1742-4690-4-2

Gramberg, T., Kahle, T., Bloch, N., Wittmann, S., Müllers, E., Daddacha, W., et al. (2013). Restriction of diverse retroviruses by SAMHD1. *Retrovirology* 10, 26. doi: 10.1186/1742-4690-10-26

Gupta, R. K., Mlcochova, P., Pelchen-Matthews, A., Petit, S. J., Mattiuzzo, G., Pillay, D., et al. (2009). Simian immunodeficiency virus envelope glycoprotein counteracts tetherin/BST-2/CD317 by intracellular sequestration. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20889–20894. doi: 10.1073/pnas.0907075106

Harouse, J. M. (1999). Distinct Pathogenic Sequela in Rhesus Macaques Infected with CCR5 or CXCR4 Utilizing SHIVs. *Science* 284, 816–819. doi: 10.1126/science.284.5415.816

Hatziioannou, T., Ambrose, Z., Chung, N. P., Piatak, M. Jr., Yuan, F., Trubey, C. M., et al. (2009). A macaque model of HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4425–4429. doi: 10.1073/pnas.0812587106

Hatziioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., and Bieniasz, P. D. (2004). Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10774–10779. doi: 10.1073/pnas.0402361101

Hatziioannou, T., Princiotta, M., Piatak, M. Jr., Yuan, F., Zhang, F., Lifson, J. D., et al. (2006). Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* 314, 95. doi: 10.1126/science.1130994

Hirsch, V. M., Sharkey, M. E., Brown, C. R., Brichacek, B., Goldstein, S., Wakefield, J., et al. (1998). Vpx is required for dissemination and pathogenesis of SIV(SM) PBj: evidence of macrophage-dependent viral amplification. *Nat. Med.* 4, 1401–1408. doi: 10.1038/3992

Ho, S. H., Shek, L., Gettie, A., Blanchard, J., and Cheng-Mayer, C. (2005). V3 loop-determined coreceptor preference dictates the dynamics of CD4+-T-cell loss in simian-human immunodeficiency virus-infected macaques. *J. Virol.* 79, 12296–12303. doi: 10.1128/JVI.79.19.12296-12303.2005

Hofmann, H., Logue, E. C., Bloch, N., Daddacha, W., Polsky, S. B., Schultz, M. L., et al. (2012). The Vpx lentiviral accessory protein targets SAMHD1 for degradation in the nucleus. *J. Virol.* 86, 12552–12560. doi: 10.1128/JVI.01657-12

Hofmann, W., Schubert, D., LaBonte, J., Munson, L., Gibson, S., Scammell, J., et al. (1999). Species-specific, postentry barriers to primate immunodeficiency virus infection. *J. Virol.* 73, 10020–10028. doi: 10.1038/nature02343

Hrecka, K., Hao, C., Gierszewska, M., Swanson, S. K., Kesik-Brodacka, M., Srivastava, S., et al. (2011). Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 474, 658–661. doi: 10.1038/nature10195

Hultquist, J. F., Binka, M., Larue, R. S., Simon, V., and Harris, R. S. (2012). Vif proteins of human and simian immunodeficiency viruses require cellular CBFbeta to degrade APOBEC3 restriction factors. *J. Virol.* 86, 2874–2877. doi: 10.1128/JVI.06950-11

Humes, D., Emery, S., Laws, E., and Overbaugh, J. (2012). A species-specific amino acid difference in the macaque CD4 receptor restricts replication by global circulating HIV-1 variants representing viruses from recent infection. *J. Virol.* 86, 12472–12483. doi: 10.1128/JVI.02176-12

Humes, D., and Overbaugh, J. (2011). Adaptation of subtype a human immunodeficiency virus type 1 envelope to pig-tailed macaque cells. *J. Virol.* 85, 4409–4420. doi: 10.1128/JVI.02244-10

Igarashi, T., Ami, Y., Yamamoto, H., Shibata, R., Kuwata, T., Mukai, R., et al. (1997). Protection of monkeys vaccinated with vpr- and/or nef-defective simian immunodeficiency virus strain mac/human immunodeficiency virus type 1 chimeric viruses:

a potential candidate live-attenuated human AIDS vaccine. *J. Gen. Virol.* 78, 985–989. doi: 10.1186/1742-4690-4-66

Igarashi, T., Iyengar, R., Byrum, R. A., Buckler-White, A., Dewar, R. L., Buckler, C. E., et al. (2007). Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques. *J. Virol.* 81, 11549–11552. doi: 10.1128/JVI.00960-07

Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., et al. (2002). NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100, 3175–3182. doi: 10.1182/blood-2001-12-0207

Jager, S., Kim, D. Y., Hultquist, J. F., Shindo, K., Larue, R. S., Kwon, E., et al. (2012). Vif hijacks CBF-beta to degrade APOBEC3G and promote HIV-1 infection. *Nature* 481, 371–375. doi: 10.1038/nature10693

Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., et al. (2002). An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* 79, 285–296. doi: 10.1006/geno.2002.6718

Jia, B., Serra-Moreno, R., Neidermyer, W., Rahmberg, A., Mackey, J., Fofana, I. B., et al. (2009). Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. *PLoS Pathog.* 5:e1000429. doi: 10.1371/journal.ppat.1000429

Johnson, B. K., Stone, G. A., Godec, M. S., Asher, D. M., Gajdusek, D. C., and Gibbs, C. J. Jr. (1993). Long-term observations of human immunodeficiency virus-infected chimpanzees. *AIDS Res. Hum. Retroviruses* 9, 375–378. doi: 10.1089/aid.1993.9.375

Kamada, K., Igarashi, T., Martin, M. A., Khamsri, B., Hatcho, K., Yamashita, T., et al. (2006). Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16959–16964. doi: 10.1073/pnas.0608289103

Kao, S., Khan, M. A., Miyagi, E., Plishka, R., Buckler-White, A., and Strebel, K. (2003). The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *J. Virol.* 77, 11398–11407. doi: 10.1128/JVI.77.21.11398-11407.2003

Kaushik, R., Zhu, X., Stranska, R., Wu, Y., and Stevenson, M. (2009). A cellular restriction dictates the permissivity of non-dividing monocytes/macrophages to lentivirus and gammaretrovirus infection. *Cell Host Microbe* 6, 68–80. doi: 10.1016/j.chom.2009.05.022

Keckesova, Z., Ylinen, L. M., and Towers, G. J. (2004). The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10780–10785. doi: 10.1073/pnas.0402474101

Kestler, H., Kodama, T., Ringler, D., Marthas, M., Pedersen, N., Lackner, A., et al. (1990). Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. *Science* 248, 1109–1112. doi: 10.1126/science.2160735

Kestler, H. W. III., Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., et al. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* 65, 651–662. doi: 10.1016/0092-8674(91)90097-I

Kidd, J. M., Newman, T. L., Tuzun, E., Kaul, R., and Eichler, E. E. (2007). Population stratification of a common APOBEC gene deletion polymorphism. *PLoS Genet.* 3:e63. doi: 10.1371/journal.pgen.0030063

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., et al. (2007). CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* 13, 46–53. doi: 10.1038/nm1520

Kirmaier, A., Wu, F., Newman, R. M., Hall, L. R., Morgan, J. S., O'connor, S., et al. (2010). TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. *PLoS Biol.* 8:e1000462. doi: 10.1371/journal.pbio.1000462

Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A., and Orenstein, J. M. (1990). The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J. Virol.* 64, 621–629. doi: 10.1016/S1286-4579(03)00191-6

Kono, K., Song, H., Shingai, Y., Shioda, T., and Nakayama, E. E. (2008). Comparison of anti-viral activity of rhesus monkey and cynomolgus monkey TRIM5alphas against human immunodeficiency virus type 2 infection. *Virology* 373, 447–456. doi: 10.1016/j.virol.2007.12.022

Kraus, G., Werner, A., Baier, M., Binniger, D., Ferdinand, F. J., Norley, S., et al. (1989). Isolation of human immunodeficiency virus-related simian immunodeficiency viruses from African green monkeys. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2892–2896. doi: 10.1073/pnas.86.8.2892

Kulaga, H., Folks, T., Rutledge, R., Truckenmiller, M. E., Gugel, E., and Kindt, T. J. (1989). Infection of rabbits with human immunodeficiency virus 1. A small animal model for acquired immunodeficiency syndrome. *J. Exp. Med.* 169, 321–326. doi: 10.1084/jem.169.1.321

Kupzig, S., Korolchuk, V., Rollason, R., Sugden, A., Wilde, A., and Banting, G. (2003). Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. *Traffic* 4, 694–709. doi: 10.1034/j.1600-0854.2003.00129.x

Kuroishi, A., Saito, A., Shingai, Y., Shioda, T., Nomaguchi, M., Adachi, A., et al. (2009). Modification of a loop sequence between alpha-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) vif and CA alpha-helices 4 and 5 loop improves replication in cynomolgus monkey cells. *Retrovirology* 6, 70. doi: 10.1186/1742-4690-6-70

Kwak, Y. T., Ivanov, D., Guo, J., Nee, E., and Gaynor, R. B. (1999). Role of the human and murine cyclin T proteins in regulating HIV-1 tat-activation. *J. Mol. Biol.* 288, 57–69. doi: 10.1006/jmbi.1999.2664

LaBonte, J. A., Babcock, G. J., Patel, T., and Sodroski, J. (2002). Blockade of HIV-1 infection of New World monkey cells occurs primarily at the stage of virus entry. *J. Exp. Med.* 196, 431–445. doi: 10.1084/jem.20020468

Laguette, N., Rahm, N., Sobhian, B., Chable-Bessia, C., Munch, J., Snoeck, J., et al. (2012). Evolutionary and functional analyses of the interaction between the myeloid restriction factor SAMHD1 and the lentiviral Vpx protein. *Cell Host Microbe* 11, 205–217. doi: 10.1016/j.chom.2012.01.007

Laguette, N., Sobhian, B., Casartelli, N., Ringeard, M., Chable-Bessia, C., Segeral, E., et al. (2011). SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 474, 654–657. doi: 10.1038/nature10117

Lahouassa, H., Daddacha, W., Hofmann, H., Ayinde, D., Logue, E. C., Dragin, L., et al. (2012). SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat. Immunol.* 13, 223–228. doi: 10.1038/ni.2236

Le Tortorec, A., and Neil, S. J. (2009). Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *J. Virol.* 83, 11966–11978. doi: 10.1128/JVI.01515-09

Letvin, N. L., Daniel, M. D., Sehgal, P. K., Desrosiers, R. C., Hunt, R. D., Waldron, L. M., et al. (1985). Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* 230, 71–73. doi: 10.1126/science.2412295

Letvin, N. L., Montefiori, D. C., Yasutomi, Y., Perry, H. C., Davies, M. E., Lekutis, C., et al. (1997). Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc. Natl. Acad. Sci. U.S.A.* 94, 9378–9383. doi: 10.1073/pnas.94.17.9378

Liao, C. H., Kuang, Y. Q., Liu, H. L., Zheng, Y. T., and Su, B. (2007). A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. *AIDS* 21(Suppl. 8), S19–S26. doi: 10.1097/01.aids.0000304692.09143.1b

Liddament, M. T., Brown, W. L., Schumacher, A. J., and Harris, R. S. (2004). APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Curr. Biol.* 14, 1385–1391. doi: 10.1016/j.cub.2004.06.050

Lim, E. S., Fregoso, O. I., Mccoy, C. O., Matsen, F. A., Malik, H. S., and Emerman, M. (2012). The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. *Cell Host Microbe* 11, 194–204. doi: 10.1016/j.chom.2012.01.004

Lusso, P., Markham, P. D., Ranki, A., Earl, P., Moss, B., Dorner, F., et al. (1988). Cell-mediated immune response toward viral envelope and core antigens in gibbon apes (*Hylobates lar*) chronically infected with

human immunodeficiency virus-1. *J. Immunol.* 141, 2467–2473. doi: 10.1126/science.1621083

Madani, N., and Kabat, D. (1998). An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J. Virol.* 72, 10251–10255. doi: 10.1038/nature00939

Maegawa, H., Nakayama, E. E., Kuroishi, A., and Shioda, T. (2008). Silencing of tripartite motif protein (TRIM) 5α mediated anti-HIV-1 activity by truncated mutant of TRIM5α. *J. Virol. Methods* 151, 249–256. doi: 10.1016/j.jviromet.2008.04.012

Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., et al. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114, 21–31. doi: 10.1016/S0092-8674(03)00515-4

Marsden, M. D., Kovochich, M., Suree, N., Shimizu, S., Mehta, R., Cortado, R., et al. (2012). HIV latency in the humanized BLT mouse. *J. Virol.* 86, 339–347. doi: 10.1128/JVI.06366-11

Matsuda, K., Inaba, K., Fukazawa, Y., Matsuyama, M., Ibuki, K., Horiike, M., et al. (2010). In vivo analysis of a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a derivative of SHIV-89.6. *Virology* 399, 134–143. doi: 10.1016/j.virol.2010.01.008

McCune, J. M. (2001). The dynamics of CD4+ T-cell depletion in HIV disease. *Nature* 410, 974–979. doi: 10.1038/35073648

McNatt, M. W., Zang, T., Hatziioannou, T., Bartlett, M., Fofana, I. B., Johnson, W. E., et al. (2009). Species-specific activity of HIV-1 Vpu and positive selection of tetherin transmembrane domain variants. *PLoS Pathog.* 5:e1000300. doi: 10.1371/journal.ppat.1000300

Mehle, A., Strack, B., Ancuta, P., Zhang, C., Mcpike, M., and Gabuzda, D. (2004). Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J. Biol. Chem.* 279, 7792–7798. doi: 10.1074/jbc.M313093200

Melkus, M. W., Estes, J. D., Padgett-Thomas, A., Gatlin, J., Denton, P. W., Othieno, F. A., et al. (2006). Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat. Med.* 12, 1316–1322. doi: 10.1038/nm1431

Mercenne, G., Bernacchi, S., Richer, D., Bec, G., Henriet, S., Paillart, J. C., et al. (2010). HIV-1 Vif binds to APOBEC3G mRNA and inhibits its translation. *Nucleic Acids Res.* 38, 633–646. doi: 10.1093/nar/gkp1009

Mische, C. C., Javanbakht, H., Song, B., Diaz-Griffero, F., Stremlau, M., Strack, B., et al. (2005). Retroviral restriction factor TRIM5alpha is a trimer. *J. Virol.* 79, 14446–14450. doi: 10.1128/JVI.79.22.14446-14450.2005

Miyagi, E., Brown, C. R., Opi, S., Khan, M., Goila-Gaur, R., Kao, S., et al. (2010). Stably expressed APOBEC3F has negligible antiviral activity. *J. Virol.* 84, 11067–11075. doi: 10.1128/JVI.01249-10

Moldt, B., Rakasz, E. G., Schultz, N., Chan-Hui, P. Y., Swiderek, K., Weisgrau, K. L., et al. (2012). Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 109, 18921–18925. doi: 10.1073/pnas.1214785109

Mosier, D. E., Gulizia, R. J., Baird, S. M., and Wilson, D. B. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* 335, 256–259. doi: 10.1038/335256a0

Munk, C., Brandt, S. M., Lucero, G., and Landau, N. R. (2002). A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13843–13848. doi: 10.1073/pnas.212400099

Naidu, Y. M., Kestler, H. W. III., Li, Y., Butler, C. V., Silva, D. P., Schmidt, D. K., et al. (1988). Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. *J. Virol.* 62, 4691–4696. doi: 10.1126/science.2160735

Nakayama, E. E., and Shioda, T. (2010). Anti-retroviral activity of TRIM5 alpha. *Rev. Med. Virol.* 20, 77–92. doi: 10.1002/rmv.637

Nara, P. L., Robey, W. G., Arthur, L. O., Asher, D. M., Wolff, A. V., Gibbs, C. J. Jr., et al. (1987). Persistent infection of chimpanzees with human immunodeficiency virus: serological responses and properties of reisolated viruses. *J. Virol.* 61, 3173–3180. doi: 10.1007/978-1-4684-5976-0_4

Neil, S. J., Sandrin, V., Sundquist, W. I., and Bieniasz, P. D. (2007). An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. *Cell Host Microbe* 2, 193–203. doi: 10.1016/j.chom.2007.08.001

Neil, S. J., Zang, T., and Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425–430. doi: 10.1038/nature06553

Newman, E. N., Holmes, R. K., Craig, H. M., Klein, K. C., Lingappa, J. R., Malim, M. H., et al. (2005). Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. *Curr. Biol.* 15, 166–170. doi: 10.1016/j.cub.2004.12.068

Newman, R. M., Hall, L., Connole, M., Chen, G. L., Sato, S., Yuste, E., et al. (2006). Balancing selection and the evolution of functional polymorphism in Old World monkey TRIM5alpha. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19134–19139. doi: 10.1073/pnas.0605838103

Newman, R. M., Hall, L., Kirmaier, A., Pozzi, L. A., Pery, E., Farzan, M., et al. (2008). Evolution of a TRIM5-CypA splice isoform in old world monkeys. *PLoS Pathog.* 4:e1000003. doi: 10.1371/journal.ppat.1000003

Nishimura, Y., Igarashi, T., Donau, O. K., Buckler-White, A., Buckler, C., Lafont, B. A., et al. (2004). Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12324–12329. doi: 10.1073/pnas.0404620101

Nishimura, Y., Igarashi, T., Haigwood, N., Sadjadpour, R., Plishka, R. J., Buckler-White, A., et al. (2002). Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. *J. Virol.* 76, 2123–2130. doi: 10.1128/jvi.76.5.2123-2130.2002

Nomaguchi, M., Doi, N., Fujiwara, S., Saito, A., Akari, H., Nakayama, E. E., et al. (2013a). Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells. *Microbes Infect.* 15, 319–328. doi: 10.1016/j.micinf.2013.01.005

Nomaguchi, M., Yokoyama, M., Kono, K., Nakayama, E. E., Shioda, T., Saito, A., et al. (2013b). Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells. *Microbes Infect.* 15, 56–65. doi: 10.1016/j.micinf.2012.10.013

North, T. W., Van Rompay, K. K., Higgins, J., Matthews, T. B., Wadford, D. A., Pedersen, N. C., et al. (2005). Suppression of virus load by highly active antiretroviral therapy in rhesus macaques infected with a recombinant simian immunodeficiency virus containing reverse transcriptase from human immunodeficiency virus type 1. *J. Virol.* 79, 7349–7354. doi: 10.1128/JVI.79.12.7349-7354.2005

Novembre, F. J., Saucier, M., Anderson, D. C., Klumpp, S. A., O'Neil, S. P., Brown, C. R. III., et al. (1997). Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. *J. Virol.* 71, 4086–4091. doi: 10.1089/aid.2007.0182

Ohta, Y., Masuda, T., Tsujimoto, H., Ishikawa, K., Kodama, T., Morikawa, S., et al. (1988). Isolation of simian immunodeficiency virus from African green monkeys and seroepidemiologic survey of the virus in various non-human primates. *Int. J. Cancer* 41, 115–122. doi: 10.1002/ijc.2910410121

O'Neil, S. P., Novembre, F. J., Hill, A. B., Suwyn, C., Hart, C. E., Evans-Strickfaden, T., et al. (2000). Progressive infection in a subset of HIV-1-positive chimpanzees. *J. Infect. Dis.* 182, 1051–1062. doi: 10.1086/315823

Perez-Caballero, D., Hatziioannou, T., Yang, A., Cowan, S., and Bieniasz, P. D. (2005). Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *J. Virol.* 79, 8969–8978. doi: 10.1128/JVI.79.14.8969-8978.2005

Perron, M. J., Stremlau, M., Song, B., Ulm, W., Mulligan, R. C., and Sodroski, J. (2004). TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11827–11832. doi: 10.1073/pnas.0403364101

Pertel, T., Hausmann, S., Morger, D., Zuger, S., Guerra, J., Lascano, J., et al. (2011). TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472, 361–365. doi: 10.1038/nature09976

Powell, R. D., Holland, P. J., Hollis, T., and Perrino, F. W. (2011). Aicardi–Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase. *J. Biol. Chem.* 286, 43596–43600. doi: 10.1074/jbc.C111.317628

Prince, A. M., Horowitz, B., Baker, L., Shulman, R. W., Ralph, H.,

Valinsky, J., et al. (1988). Failure of a human immunodeficiency virus (HIV) immune globulin to protect chimpanzees against experimental challenge with HIV. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6944–6948. doi: 10.1073/pnas.85.18.6944

Reimann, K. A., Li, J. T., Veazey, R., Halloran, M., Park, I. W., Karlsson, G. B., et al. (1996). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70, 6922–6928. doi: 10.1086/512243

Reina, S., Markham, P., Gard, E., Rayed, F., Reitz, M., Gallo, R. C., et al. (1993). Serological, biological, and molecular characterization of New Zealand white rabbits infected by intraperitoneal inoculation with cell-free human immunodeficiency virus. *J. Virol.* 67, 5367–5374. doi: 10.1089/aid.1994.10.285

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., et al. (2001). The tripartite motif family identifies cell compartments. *EMBO J.* 20, 2140–2151. doi: 10.1093/emboj/20.9.2140

Reynolds, M. R., Sacha, J. B., Weiler, A. M., Borchardt, G. J., Glidden, C. E., Sheppard, N. C., et al. (2011). The TRIM5{alpha} genotype of rhesus macaques affects acquisition of simian immunodeficiency virus SIVsmE660 infection after repeated limiting-dose intrarectal challenge. *J. Virol.* 85, 9637–9640. doi: 10.1128/JVI.05074-11

Rice, G. I., Bond, J., Asipu, A., Brunette, R. L., Manfield, I. W., Carr, I. M., et al. (2009). Mutations involved in Aicardi–Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response. *Nat. Genet.* 41, 829–832. doi: 10.1038/ng.373

Saito, A., Kawamoto, Y., Higashino, A., Yoshida, T., Ikoma, T., Suzaki, Y., et al. (2012a). Allele frequency of antiretroviral host factor TRIMCyp in wild-caught cynomolgus macaques (*Macaca fascicularis*). *Front. Microbiol.* 3:314. doi: 10.3389/fmicb.2012.00314

Saito, A., Kono, K., Nomaguchi, M., Yasutomi, Y., Adachi, A., Shioda, T., et al. (2012b). Geographical, genetic and functional diversity of antiretroviral host factor TRIMCyp in cynomolgus macaque (*Macaca fascicularis*). *J. Gen. Virol.* 93, 594–602. doi: 10.1099/vir.0.038075-0

Saito, A., Nomaguchi, M., Iijima, S., Kuroishi, A., Yoshida, T., Lee, Y. J., et al. (2011). Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications. *Microbes Infect.* 13, 58–64. doi: 10.1016/j.micinf.2010.10.001

Saito, A., Nomaguchi, M., Kono, K., Iwatani, Y., Yokoyama, M., Yasutomi, Y., et al. (2013). TRIM5 genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceptibility to monkey-tropic human immunodeficiency virus type 1. *J. Gen. Virol.* 94, 1318–1324. doi: 10.1099/vir.0.050252-0

Sakai, H., Shibata, R., Sakuragi, J., Sakuragi, S., Kawamura, M., and Adachi, A. (1993). Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. *J. Virol.* 67, 1663–1666. doi: 10.1128/JVI.75.16.7252-7265.2001

Sakai, H., Tokunaga, K., Kawamura, M., and Adachi, A. (1995). Function of human immunodeficiency virus type 1 Vpu protein in various cell types. *J. Gen. Virol.* 76(Pt 11), 2717–2722. doi: 10.1099/0022-1317-76-11-2717

Sakuma, R., Mael, A. A., and Ikeda, Y. (2007). Alpha interferon enhances TRIM5alpha-mediated antiviral activities in human and rhesus monkey cells. *J. Virol.* 81, 10201–10206. doi: 10.1128/JVI.00419-07

Sato, K., Misawa, N., Fukuhara, M., Iwami, S., An, D. S., Ito, M., et al. (2012). Vpu augments the initial burst phase of HIV-1 propagation and downregulates BST2 and CD4 in humanized mice. *J. Virol.* 86, 5000–5013. doi: 10.1128/JVI.07062-11

Sauter, D., Schindler, M., Specht, A., Landford, W. N., Munch, J., Kim, K. A., et al. (2009). Tetherin-driven adaptation of Vpu and Nef function and the evolution of pandemic and nonpandemic HIV-1 strains. *Cell Host Microbe* 6, 409–421. doi: 10.1016/j.chom.2009.10.004

Sayah, D. M., Sokolskaja, E., Berthoux, L., and Luban, J. (2004). Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 430, 569–573. doi: 10.1038/nature02777

Schmitt, K., Guo, K., Algaier, M., Ruiz, A., Cheng, F., Qiu, J., et al. (2011). Differential virus restriction patterns of rhesus macaque and human APOBEC3A: implications for lentivirus evolution. *Virology* 419, 24–42. doi: 10.1016/j.virol.2011.07.017

Schrofelbauer, B., Senger, T., Manning, G., and Landau, N. R. (2006). Mutational alteration of human immunodeficiency virus type 1 Vif allows for functional interaction with nonhuman primate APOBEC3G. *J. Virol.* 80, 5984–5991. doi: 10.1128/JVI.00388-06

Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., De Goede, R. E., Van Steenwijk, R. P., et al. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J. Virol.* 66, 1354–1360. doi: 10.1038/nm0398-346

Serra-Moreno, R., Jia, B., Breed, M., Alvarez, X., and Evans, D. T. (2011). Compensatory changes in the cytoplasmic tail of gp41 confer resistance to tetherin/BST-2 in a pathogenic nef-deleted SIV. *Cell Host Microbe* 9, 46–57. doi: 10.1016/j.chom.2010.12.005

Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418, 646–650. doi: 10.1038/nature00939

Shibata, R., and Adachi, A. (1992). SIV/HIV recombinants and their use in studying biological properties. *AIDS Res. Hum. Retroviruses* 8, 403–409. doi: 10.1089/aid.1992.8.403

Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., et al. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat. Med.* 5, 204–210. doi: 10.1038/5568

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., and Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* 65, 3514–3520. doi: 10.1128/JVI.77.1.726-731.2003

Shibata, R., Sakai, H., Kawamura, M., Tokunaga, K., and Adachi, A. (1995). Early replication block of human immunodeficiency virus type 1 in monkey cells. *J. Gen. Virol.* 76, 2723–2730. doi: 10.1099/0022-1317-76-11-2723

Shingai, M., Yoshida, T., Martin, M. A., and Strebel, K. (2011). Some human immunodeficiency virus type 1 Vpu proteins are able to antagonize macaque BST-2 in vitro and in vivo: Vpu-negative simian-human immunodeficiency viruses are attenuated in vivo. *J. Virol.* 85, 9708–9715. doi: 10.1128/JVI.00626-11

Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Chaleff, S., et al. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J. Immunol.* 174, 6477–6489. doi: 10.3410/f.1003045.323931

Simon, J. H., Gaddis, N. C., Fouchier, R. A., and Malim, M. H. (1998). Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nat. Med.* 4, 1397–1400. doi: 10.1038/3987

Speck, R. F., Penn, M. L., Wimmer, J., Esser, U., Hague, B. F., Kindt, T. J., et al. (1998). Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. *J. Virol.* 72, 5728–5734. doi: 10.1186/1742-4690-4-53

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848–853. doi: 10.1038/nature02343

Tanaka, Y., Marusawa, H., Seno, H., Matsumoto, Y., Ueda, Y., Kodama, Y., et al. (2006). Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. *Biochem. Biophys. Res. Commun.* 341, 314–319. doi: 10.1016/j.bbrc.2005.12.192

Tervo, H. M., and Keppler, O. T. (2010). High natural permissivity of primary rabbit cells for HIV-1, with a virion infectivity defect in macrophages as the final replication barrier. *J. Virol.* 84, 12300–12314. doi: 10.1128/JVI.01607-10

Thippeshappa, R., Polacino, P., Yu Kimata, M. T., Siwak, E. B., Anderson, D., Wang, W., et al. (2011). Vif substitution enables persistent infection of pig-tailed macaques by human immunodeficiency virus type 1. *J. Virol.* 85, 3767–3779. doi: 10.1128/JVI.02438-10

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., et al. (2004). Development of a human adaptive immune system in cord blood cell-transplanted

mice. *Science* 304, 104–107. doi: 10.1126/science.1093933

Uberla, K., Stahl-Hennig, C., Bottiger, D., Matz-Rensing, K., Kaup, F. J., Li, J., et al. (1995). Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8210–8214. doi: 10.1073/pnas.92.18.8210

Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., et al. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 3, 245–252. doi: 10.1016/j.chom.2008.03.001

Virgen, C. A., and Hatziioannou, T. (2007). Antiretroviral activity and Vif sensitivity of rhesus macaque APOBEC3 proteins. *J. Virol.* 81, 13932–13937. doi: 10.1128/JVI.01760-07

Virgen, C. A., Kratovac, Z., Bieniasz, P. D., and Hatziioannou, T. (2008). Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3563–3568. doi: 10.1073/pnas.0709258105

Wiegand, H. L., Doehle, B. P., Bogerd, H. P., and Cullen, B. R. (2004). A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J.* 23, 2451–2458. doi: 10.1038/sj.emboj.7600246

Wilson, S. J., Webb, B. L., Ylinen, L. M., Verschoor, E., Heeney, J. L., and Towers, G. J. (2008). Independent evolution of an antiviral TRIMCyp in rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3557–3562. doi: 10.1073/pnas.0709003105

Wyand, M. S., Manson, K., Montefiori, D. C., Lifson, J. D., Johnson, R. P., and Desrosiers, R. C. (1999). Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* 73, 8356–8363. doi: 10.1371/journal.pone.0023092

Wyand, M. S., Manson, K. H., Garcia-Moll, M., Montefiori, D., and Desrosiers, R. C. (1996). Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* 70, 3724–3733. doi: 10.1038/nm0197-32

Yap, M. W., Nisole, S., Lynch, C., and Stoye, J. P. (2004). Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc. Nat. Acad. Sci. U.S.A.* 101, 10786–10791. doi: 10.1073/pnas.0402876101

Ylinen, L. M., Price, A. J., Rasaiyaah, J., Hue, S., Rose, N. J., Marzetta, F., et al. (2010). Conformational adaptation of Asian macaque TRIMCyp directs lineage specific antiviral activity. *PLoS Pathog.* 6:e1001062. doi: 10.1371/journal.ppat.1001062

Zennou, V., and Bieniasz, P. D. (2006). Comparative analysis of the antiretroviral activity of APOBEC3G and APOBEC3F from primates. *Virology* 349, 31–40. doi: 10.1016/j.virol.2005.12.035

Zhang, F., Wilson, S. J., Landford, W. C., Virgen, B., Gregory, D., Johnson, M. C., et al. (2009). Nef proteins from simian immunodeficiency viruses are tetherin antagonists. *Cell Host Microbe* 6, 54–67. doi: 10.1016/j.chom.2009.05.008

Zhao, C., Collins, M. N., Hsiang, T. Y., and Krug, R. M. (2013). Interferon-induced ISG15 pathway: an ongoing virus-host battle. *Trends Microbiol.* 21, 181–186. doi: 10.1016/j.tim.2013.01.005

Zheng, Y. H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., and Peterlin, B. M. (2004). Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J. Virol.* 78, 6073–6076. doi: 10.1128/JVI.78.11.6073-6076.2004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 April 2013; paper pending published: 14 May 2013; accepted: 20 June 2013; published online: 09 July 2013.
Citation: Saito A and Akari H (2013) Macaque-tropic human immunodeficiency virus type 1: breaking out of the host restriction factors. Front. Microbiol. ***4****:187. doi: 10.3389/fmicb.2013.00187*
This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 22 July 2013
doi: 10.3389/fmicb.2013.00204

Genetic similarity of circulating and small intestinal virus at the end stage of acute pathogenic simian-human immunodeficiency virus infection

Megumi Matsuyama-Murata, Katsuhisa Inaba, Reii Horiuchi, Yoshinori Fukazawa, Kentaro Ibuki, Masanori Hayami and Tomoyuki Miura*

Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, Kyoto, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan
Akatsuki Saito, Kyoto University, Japan

Correspondence:
Tomoyuki Miura, Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoinkawaramachi, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: tmiura@virus.kyoto-u.ac.jp

To understand the pathogenicity of acquired immune deficiency syndrome (AIDS), it is important to clarify where, when and how the virus replicates in the body of infected individuals. To identify the major virus replication site at the end stage of SHIV infection, we investigated the systemic tissues of SHIV-infected monkeys that developed AIDS-like disease. We quantified proviral DNA, and compared the mutation patterns of the viruses in various systemic tissues and in peripheral blood through phylogenetic analysis of the full genome sequence. We found that the amounts of proviral DNA detected in internal tissues were higher than those in peripheral blood mononuclear cells. In the sequence and phylogenetic tree analyses, the mutation patterns of the viruses in each tissue were generally different. However, the mutation pattern of the viruses in the jejunum and mesenteric lymph node were most similar to that of plasma viral RNA among the tissues examined in all three monkeys. In two of the three monkeys, which were euthanized earlier, viruses in the jejunum and mesenteric lymph node occupied the root position of the phylogenetic tree. Furthermore, in these tissues, more than 50% of SHIV-expressing cells were identified as macrophages based on co-expression of CD68. These results suggest that macrophages of the small intestine and/or mesenteric lymph node are the major virus production site at the end stage of SHIV infection of macaques.

Keywords: AIDS, HIV, SIV, SHIV, rhesus, phylogenesis, evolution, immunohistochemistry

INTRODUCTION

To understand the pathogenicity of AIDS, it is important to clarify where, when and how HIV replicates in the body of infected individuals. However, there are limitations to analysis of systemic human tissues. Animal models can substitute for humans; i.e., the simian immunodeficiency virus (SIV)-macaque model. SIV-infected macaques develop AIDS-like disease at an interval from several months to years (Desrosiers, 1990). However, SIV is closely related to HIV-2, but not HIV-1; in particular, the immune response of SIV *env* is thought to be different from the immune response of HIV-1 (Overbaugh et al., 1991).

Another animal model is that comprising simian-human immunodeficiency virus (SHIV) and the macaque. SHIVs are chimeric viruses that contain HIV-1 *env*, *rev*, *tat*, and *vpu* genes on a background of SIVmac, and some strains of SHIV cause AIDS-like disease (Reimann et al., 1996, 1999; Joag et al., 1997; Harouse et al., 1998). One such virus is SHIV-KS661, which is characterized by the profound depletion of CD4+ T cells and maintenance of high viral loads (Fukazawa et al., 2008). The SHIV-macaque model has two main merits. First, SHIVs contain HIV-1 *env, rev, tat*, and *vpu* genes (Kuwata et al., 1995), and it is especially important that the *env* gene is from HIV-1. SHIVs are reported to include both CXCR4-tropic and CCR5-tropic strains; CXCR4-SHIV targets the peripheral blood and thymus, whereas CCR5-SHIV targets gut-associated lymphoid tissues (Harouse et al., 1999; Ho et al., 2005), although most known SIV strains are CCR5-tropic. Therefore, this would be a useful approach to understanding the cell tropism and pathogenesis of the *env* gene of HIV-1. The second advantage of using the SHIV-macaque model is that it is easy to evaluate pathogenesis because the disease progresses more quickly in CXCR4-SHIV-infected macaques compared to SIV-infected macaques. SHIV-infected macaques develop CD4+ T cell depletion within several weeks, and acute AIDS-like disease from several weeks to months (Joag et al., 1996; Reimann et al., 1996).

Previous studies using the SIV-macaque model of AIDS have demonstrated that SIV replicates rapidly around the intestine during primary infection, within a couple of weeks post-infection (Veazey et al., 1998; Li et al., 2005; Mattapallil et al., 2005). Moreover, CD4+ T cell depletion upon primary infection with HIV-1 has been reported to occur in the gastrointestinal tract (Meng et al., 2000; Brenchley et al., 2004; Mehandru et al., 2004). However, the dynamic states of the virus during other phases, especially the AIDS stage, have not been defined.

During primary infection of SIV or SHIV, the virus-producing site has been defined by quantification of proviral DNA and viral RNA using quantitative PCR and plaque assay in systemic tissues (Couedel-Courteille et al., 1999, 2003; Miyake et al., 2004). However, because the viruses were detected in systemic tissues at high levels during the AIDS stage, it was difficult to

identify the site of virus production by only quantification of proviral DNA and viral RNA. Therefore, we focused on the nucleotide mutations of virus in systemic tissues and in peripheral blood, and examined the viral dynamics in SHIV-infected macaques at the end stage. Previous studies have analyzed the viral sequences in several tissues of infected individuals and suggested that HIV and SIV evolve differentially in those tissues (Kodama et al., 1993; Wong et al., 1997; Oue et al., 2013). However, they did not define the major site of virus production because analyzed tissues were limited. Based on previous reports, we hypothesized that the site of virus replication might be determined by phylogenetic analyses. In this study, direct sequencing of the full SHIV genome was performed to detect mutations, and a consensus sequence was determined for each tissue and for peripheral blood. Then, phylogenetic analyses were performed to compare the virus sequence patterns among tissues. Furthermore, we conducted an immunohistochemical analysis of virus-producing cells. Based on these analyses, we identified the major virus replication site at the end stage of SHIV infection.

MATERIALS AND METHODS

VIRUSES

We used the molecular clone viruses, SHIV-KS661 (GenBank accession no. AF217181) and SHIV-KS705. SHIV-KS661 was constructed from the consensus sequence of SHIV-C2/1. SHIV-C2/1 was generated by *in vivo* passage in cynomolgus monkey (*Macaca fascicularis*) of SHIV-89.6 (containing *env*, *tat*, *rev* and *vpu* gene of HIV-1) (Shinohara et al., 1999).

SHIV-KS661 causes rapid and profound depletion of CD4+ T cells. In this study, the SHIV-KS661 virus stock was prepared by two methods. In method 1, SHIV-KS661 was prepared from culture supernatants of COS-1 cells by direct transfection with a molecular clone. In method 2, it was prepared from the supernatants of a human cell line, CEMX174 by infection with the virus of method 1. SHIV-KS705 is a chimeric virus of SHIV-KS661 and SHIV-#64, and contains the *env* gene of SHIV-KS661 (SphI ∼ XhoI fragment) in a SHIV-#64 background. SHIV-#64 was a molecular clone derived from SHIV-89.6P, which was generated by *in vivo* passage of SHIV-89.6 in rhesus monkeys (*Macaca mulatta*) (Kozyrev et al., 2001). The SHIV-KS705 stock was prepared from the supernatant of culture of a human cell line, M8166 by infection with the virus of method 1. There was no serious influence of the virus preparation on the results.

MONKEYS AND INOCULATION

Three Indian rhesus macaques were used. Throughout the experimental period, the monkeys were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates at the Institute for Virus Research, Kyoto University. Monkey MM273 was inoculated intravenously with 1.2×10^4 $TCID_{50}$ SHIV-KS661; MM376 was inoculated intrarectally with 2000 $TCID_{50}$ SHIV-KS661, and MM340 was inoculated intravenously with 10^4 $TCID_{50}$ SHIV-KS705. All three monkeys (MM340, MM376 and MM273) developed AIDS-like disease and were euthanized at 22, 33, and 90 weeks post-inoculation (wpi), respectively.

SAMPLE COLLECTION

Blood was collected under ketamine anesthesia. After the separation of plasma, peripheral blood mononuclear cells (PBMC) were separated from anticoagulated blood with Lymphocyte Separation Solution (Nacalai Tesque, Kyoto, Japan) by density gradient centrifugation. Plasma and PBMC were frozen at −80°C until analysis. Intravenous pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) (40 mg/kg) was administered for deeper anesthesia. Following thoracotomy, the right atrium was incised, and 1000 ml of sterile heparinized saline (5 U/ml) was infused into the left ventricle using an 18-gauge needle attached to infusion tubing. Following perfusion, systemic organs were obtained. Organ samples were frozen at −80°C until used for quantification of proviral DNA.

QUANTIFICATION OF PLASMA VIRAL RNA

Virion-associated SHIV RNA loads in plasma were measured by real-time reverse transcription (RT)-PCR assay (Suryanarayana et al., 1998; Motohara et al., 2006). Briefly, total RNA was prepared from plasma (140 μl) of each monkey using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany). RT reactions and PCR were performed using the Platinum® qRT-PCR ThermoScript™ One-Step System (Invitrogen, Carlsbad, CA) for the SIV *gag* region using the following primers: SIV2-696F (5′-GGA AAT TAC CCA GTA CAA CAA ATA GG-3′) and SIV2-784R (5′-TCT ATC AAT TTT ACC CAG GCA TTT A-3′). A labeled probe, SIV2-731T (5′-Fam-TGT CCA CCT GCC ATT AAG CCC G-Tamra-3′), was used for detection of the PCR products. These reactions were performed using a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analysed using the manufacturer's software. A standard curve was generated from dilutions whose copy numbers were known, and the RNA in the plasma samples was quantified based on this standard curve.

QUANTIFICATION OF PROVIRAL DNA

The proviral DNA loads in tissues were determined by quantitative PCR, as described previously (Motohara et al., 2006). DNA samples were extracted directly from frozen tissues using a DNeasy® Tissue Kit (Qiagen). PCR was performed with Platinum® Quantitative PCR SuperMix-UDG (Invitrogen) using the same primer set and probes that were used for RT-PCR. A standard curve was generated from a plasmid DNA sample containing the full genome of SHIV-KS661, which was quantified using a UV-spectrophometer. Tissue DNA samples were also quantified using a UV-spectrophometer to use 1 μg for each reaction and detection limit of this assay was 10 copies/μg.

FLOW CYTOMETRY

The frequency of CD4-positive T cells in peripheral blood was examined by flow cytometry. Lymphocytes were treated with anti-CD3 (FN-8-FITC; Biosource, Camarillo, CA) and anti-CD4 (Nu-TH/I-PE; Nichirei, Tokyo, Japan) monoclonal antibodies and examined using a FACScan analyser (Becton Dickinson Biosciences). The absolute number of lymphocytes in the blood was determined using an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

SEQUENCE ANALYSIS

Total DNA was extracted from the cell pellet with a DNeasy® Tissue Kit. Total RNA was extracted from the cell pellet with a RNeasy Protect Kit (Qiagen). RNA extraction was performed in the condition adding DNase I to exclude the possibility of contamination of proviral DNA into RNA. Virion RNA was prepared from plasma using a QIAamp Viral RNA Kit (Qiagen). Seventy-four consensus primers were synthesized for every 250–300 bp throughout the genome of SHIV for full genome sequencing of both the forward and reverse strands. Using these primers with RNA samples, RT-PCR was performed using the One-step RT-PCR Kit (Qiagen). For DNA samples, PCR was performed using Platinum® PCR SuperMix (Invitrogen). The PCR products were purified using QIAquick Spin (Qiagen) and direct sequencing was carried out using the ABI PRISM® Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with an automated sequencer (ABI PRISM 310 genetic analyser; PerkinElmer, Emeryville, CA).

PHYLOGENETIC ANALYSIS

Sequence alignments were performed using the ClustalW program (Thompson et al., 1997). Final tree topologies were visualized with Tree View.

IMMUNOHISTOCHEMICAL ANALYSIS

The tissue samples obtained from sacrificed monkeys were fixed in 4% paraformaldehyde (PFA)-PBS (1× phosphate-buffered saline) at 4°C overnight, and embedded in paraffin wax. For immunohistochemistry, 4-μm sections were rehydrated and processed for 10 min in an autoclave in 10 mM citrate buffer (pH 6.0) to unmask the antigens. The samples were treated sequentially with TBS (Tris-buffered saline)-0.05% Tween 20 and 3% aqueous hydrogen peroxide. Anti-SIV Nef mouse monoclonal antibody (FIT Biotech, Tampere, Finland) reactions were conducted at 4°C overnight. After washing with TBS-0.05% Tween 20, sections were incubated at room temperature for 1 h with Envision+ Kit, a horseradish peroxidase-labeled anti-mouse immunoglobulin polymer (DAKO Corp., Carpinteria, CA). Subsequently, the reaction was visualized using diaminobenzidine substrate (DAKO) as a chromogen at room temperature for 5 min. Samples were then rinsed in distilled water and counterstained with hematoxylin.

To detach the primary antibody from enzymatic-immunostained sections, those sections were washed three times in 0.2 M glycine (pH 2.2) buffer for 2 h each at room temperature. The sections were processed for 10 min in an autoclave in 10 mM citrate buffer (pH 6.0), and were then incubated with anti-human CD3 rabbit polyclonal antibody (DAKO) and anti-human CD68 mouse monoclonal antibody (DAKO) at room temperature. Anti-rabbit IgG antibody-labeled Alexa 488 and anti-mouse IgG antibody-labeled Alexa 594 were used as secondary antibodies. Multiple staining samples were observed with a Leica confocal microscope.

RESULTS

INFECTION OF MACAQUE MONKEYS WITH SHIV-KS661 OR SHIV-KS705

Two of three macaque monkeys, MM273 and MM376, were inoculated intravenously and intrarectally with SHIV-KS661, respectively. The other macaque monkey, MM340, was inoculated intravenously with SHIV-KS705. In all monkeys, plasma viral RNA increased rapidly after inoculation, and reached peak levels of $\sim 10^8$ copies/ml at 2 weeks post-inoculation (wpi). After that, plasma viral RNA decreased to $\sim 10^6$ to 10^7 copies/ml and was maintained at $>10^6$ copies/ml (**Figure 1A**). The number of CD4+ T cells declined to ~200 cells/μl at 3 weeks after inoculation, and remained at zero level throughout the infection in MM376 and MM340, whereas in MM273, the number of CD4+ T cells recovered to ~300 cells/μl from 12 to 20 wpi, but after 46 wpi, it maintained a zero level (**Figure 1B**). All monkeys showed acute diarrhea and had lost weight at the time of euthanasia. MM340, MM376, and MM273 were euthanized at 22, 33, and 90 wpi, respectively.

QUANTIFICATION OF PROVIRAL DNA IN SYSTEMIC TISSUES

Proviral DNA in systemic tissues was quantified by quantitative PCR (**Figure 2**). In all three monkeys, the proviral DNA was detected at high levels in the intestinal tract (jejunum and rectum), as well as lymph nodes (axillary LN, inguinal LN, mesenteric LN and colon LN); the proviral DNA level in lung

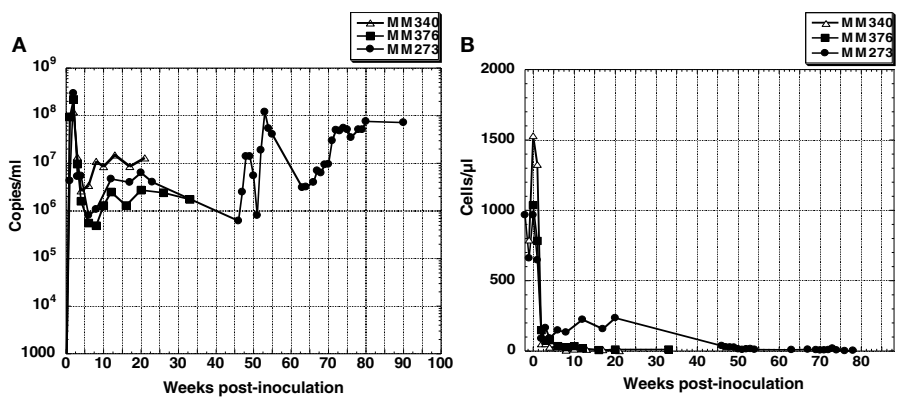


FIGURE 1 | (A) Plasma viral RNA load and **(B)** CD4+ T cell count in peripheral blood of three SHIV-inoculated monkeys. Monkey MM340 inoculated with SHIV-KS705 was euthanized at 22 wpi; MM376 and MM273 inoculated with SHIV-KS661 were euthanized at 33 wpi and 90 wpi, respectively.

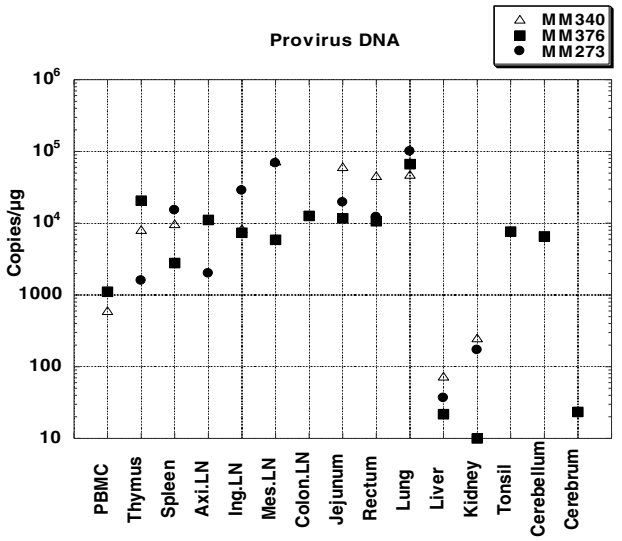


FIGURE 2 | Proviral DNA loads in systemic tissues of three SHIV-inoculated monkeys. Viral loads were determined by quantitative PCR and are expressed as viral DNA copy numbers per microgram of total DNA extracted from the tissue homogenates. PBMC was not collected from MM273. Axillary LN was not collected from MM340. Colon LN, cerebellum, cerebrum, and tonsil were collected only from MM376.

was especially high (10^5 copies/μg). The level of proviral DNA in PBMC was 10^3 copies/μg, which was lower than that in other tissues. In monkey MM376, high titers of proviral DNA were detected in the cerebellum. These results showed that the virus infected various systemic tissues at higher levels than that in PBMC during the AIDS stage.

SEQUENCE ANALYSIS

To compare the genotypes of viruses in systemic tissues and in peripheral blood, direct sequencing was performed, covering the whole genome of the viral RNA extracted from plasma and proviral DNA extracted from various tissue samples. In systemic tissue samples from MM340, 16 mutations and one nucleotide deletion were detected in the whole genome of SHIV-KS705 (**Table 1**). Except for one mutation in the *env* gene (8816th nucleotide), all of the mutations were associated with amino acid substitutions. Most mutations were detected in the spleen. For MM376, 30 mutations were detected in the whole genome of SHIV-KS661 (**Table 2**). Most mutations were accumulated in the rectum. MM340 and MM376, which were euthanized earlier, showed more mutations in systemic tissues than in peripheral blood such as plasma and PBMC, and these occurred in the region of the envelope gene. For MM273, 90 mutations were observed in the whole genome of SHIV-KS661. Almost all mutations were associated with amino acid substitutions. In axillary LN, few mutations were observed in the region of *env*-gp120. Also in MM273, the number of mutations observed in peripheral blood was the same as that in the various tissues. To compare the mutation patterns of produced viruses with those of proviruses, viral RNA in tissues of MM273 was sequenced. In the jejunum and mesenteric LN, the regions of mutations in virus RNA and provirus DNA were almost identical (**Table 3**). This suggested that the viruses produced in tissues are the same as the provirus DNA in the infected cells. In all monkeys, the mutation patterns of viruses differed among tissues; in the monkey that was euthanized earlier, the mutations were limited, whereas more mutations had accumulated in the monkey euthanized later.

Moreover, in this study, two mutations of *gag*-matrix (569th and 638th nucleotide) were detected in all three macaques, while a single mutation of *nef* (9359th nucleotide) was detected in two of three macaques (**Tables 1–3**).

PHYLOGENETIC ANALYSIS

To clarify the relationships among the genotype of viruses in systemic tissues, phylogenetic analyses were performed. In MM340, the mutations of viruses in systemic tissues and peripheral blood exhibited different patterns, but similarities were noted among viruses in plasma, PBMC, mesenteric LN, jejunum and lung (**Figure 3A**). In MM376, the mutations of viruses in each tissue and peripheral blood also exhibited different patterns. Similarities were noted among viruses in plasma, jejunum, mesenteric LN, and axillary LN (**Figure 3B**). In MM273, the viruses accumulated many more mutations in systemic tissues. The mutation patterns among viruses in plasma, rectum, jejunum and mesenteric LN showed greater relatedness than those in other tissues (**Figure 3C**). In all monkeys, the genotypes of viruses related to the small intestine, such as the jejunum and mesenteric LN, were similar to those of viruses in peripheral blood. In the monkeys that were euthanized earlier (MM340 and MM376), viruses from the jejunum, mesenteric LN and peripheral blood occupied the root position of the phylogenetic tree; whereas in the monkey that maintained a high viral load and was euthanized later (MM273), many mutations accumulated in each tissue independently, but the jejunum, mesenteric LN and peripheral blood formed a cluster. Additionally, virus RNA in the jejunum and mesenteric LN also showed greater similarities with provirus DNA in those tissues (**Table 3**). Also, in spleen, the mutation pattern of viral RNA was almost identical to that of the provirus DNA (data not shown).

IMMUNOHISTOCHEMICAL ANALYSIS

To identify the type of cells in which viruses replicate in the jejunum and mesenteric LN, an immunohistochemical analysis of CD3+ T cells and CD68+ macrophages in jejunum and mesenteric LN was performed. In all monkeys, >50% of SIV *nef*-expressing cells were judged macrophages according to co-localization of CD68 in jejunum (**Figure 4**) and mesenteric LN (data not shown). SIV *nef*-expressing CD3+ T cells were rare, and the remaining *nef*-expressing cells were negative for both CD3 and CD68.

DISCUSSION

In this study, we analysed mutations across the whole SHIV genome in systemic tissues, including the intestine, of SHIV-KS661- or -KS705-infected macaques. Although this study has a limitation as we could analyzed only three macaques, including different viruses and different inoculation route, we think common tendency observed in the three macaques is significant.

Table 1 | Consensus nucleotide mutations in MM340.

Nucleotide position	KS705	Plasma	PBMC	Thymus	Spleen	Ing.LN	Mes.LN	Rectum	Jejunum	Lung	Amino acid change	Genomic region
312	T	C	C	C	C	C	C	C	C	C		PBS
323	T				A	A						
543	T			C							V/A	
569	A	G	G	G	G	G	G	G	G	G	K/E	gag-MA
638	G	A	A	A	A	A	A	A	A	A	A/V	
2948	C	T	T		T	T	T		T		L/S	RT
5165	C	T			T		T		T		P/S	vif
5190	G										R/K	
6838	T				G						I/R	
6859	A							G			E/G	env gp120
6906	A			G							S/G	
7272	A			G	G	G					I/V	
8624	C				A	A					C/stop	env gp41
											P/T	rev
8803	A			G							E/G	env gp41
											R/G	nef
8816	C	T	T		T	T	T	T	T		A/A	env
											P/L	nef
9359	A	G		G			G	G	G		Y/C	U3
9644 ~ 9		1bp-del		1bp-del					1bp-del			

Previous studies indicated that HIV evolves differentially in various tissues (Delassus et al., 1992; Wong et al., 1997; Buckner et al., 2002; Ritola et al., 2004; Ndolo et al., 2005). Viruses produced from each tissue flow into the blood. Therefore, the majority of the circulating virus population is derived from the major production site. We demonstrated similarities among the mutation patterns of viruses from plasma, jejunum and mesenteric lymph node. This suggests that the major site of virus production at the end stage of SHIV infection of macaques is the small intestine and/or mesenteric LN. Moreover, previous whole-body positron emission tomography (PET) analyses of HIV-1-infected patients and SIV-infected macaques also suggested that the intestine was the target organ at the end stage of HIV-1/SIV infection (Scharko et al., 1996, 2003). Therefore, our finding that the small intestine is the major production site is considered to be a general phenomenon of primate lentivirus infection, and not specific to SHIV.

Concerning primary infection with HIV and SIV, which are transmitted using mainly CCR5 as their co-receptor, some reports suggest affinity for the small intestine. It has been suggested that CD4 lymphocytes of the intestinal tract are depleted selectively during early HIV infection (Clayton et al., 1997). Veazey *et al.* demonstrated that SIV eliminated intestinal CD4+ T cells selectively (Veazey et al., 1998). Other studies demonstrated that SIV replicated exclusively in memory CD4+ T cells and directly destroyed memory CD4+ T cells in the small intestine (Li et al., 2005; Mattapallil et al., 2005). Mesenteric LN harbored viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy (Horiike et al., 2012). Another study demonstrated that CXCR4-tropic SHIV decreased peripheral CD4+ T cells, and that CCR5-tropic SHIV decreased intestinal CD4+ T cells (Harouse et al., 1999; Ho et al., 2005). SHIV-KS661 and -KS705 have essentially the same *env* gene and use predominantly CXCR4 as a co-receptor for virus entry (Matsuda et al., 2010; Fujita et al., 2013). However, SHIV-KS661 decreased CD4+ T cells in systemic and mucosal immune tissues during primary infection (Miyake et al., 2006). Macrophage-tropic SHIV use CXCR4, not CCR5, for infections of rhesus macaque peripheral blood mononuclear cells and alveolar macrophages (Igarashi et al., 2003). In this study, we suggested that the small intestine and/or mesenteric LN were the major virus production site at the end stage of SHIV-KS661 and –KS705 infection, regardless of the infection route. These findings suggest that the small intestine is the critical target organ of not only CCR5-tropic HIV/SIV infection, but also CXCR4-tropic SHIV infection.

Phylogenetic analysis suggested that the phylogenetic trees differed among the three macaques. In MM340 and MM376, viruses in the jejunum and mesenteric LN, together with viruses

Table 2 | Consensus nucleotide mutations in MM376.

Nucleotide position	KS661 original	Plasma	PBMC	Thymus	Spleen	Axi.LN	Ing.LN	Mes.LN	Colon. LN	Jejunum	Rectum	Lung	Cerebellum	Tonsil	Amino acid change	Genomic region
135	T											C				LTR
543	T											C			V/A	
569	A	G	G	G	G	G		G	G	G	G		G	G	K/E	
573	C										T				A/V	
638	G	A	A	A	A	A	A	A	A	A	A	A	A	A	V/I	gag-MA
737	G														V/M	
752	G				A										E/K	
1048	A														V/V	
4644	A												G		Q/Q	pol
5129	A	G			G	G		G	G	G	G	G	G		N/D	
5249	T				C					C	C	C	C		Y/H	vif
5250	A						G		G					G	Y/C	
6186	C				T	T	T	T	T		T	T		T	P/L	vpu
6804	A				G	G			G		G		G		I/V	
6841	G													A	R/K	
6859	A			G											E/G	env gp120
6922	G										A				R/K	
7648	C				A		A	A			A		A	A	T/K	
8095	G										C	C			R/T	
8109	A				C									C	M/L	
8238	G	A	A		A	A	A	A	A	A	A	A		A	D/N	env gp41
8326	A										C	C			K/T	
8576	A										G			G	Q/R	tat
															S/G	rev
															S/S	env gp41
8598	A	G			G	G	G	G		G		G			Y/C	rev
															I/V	env gp41
8624	C				A							A			P/T	rev
															C/stop	env gp41
9011	G											A			R/K	nef
9263	C			T							T				S/L	
9275	T										C				I/T	
9281	A	T	T		T	T	T	T	T	T		T	T	T	T/F	nef/U3
9359	A	G	G	G		G	G	G	G	G	G	G	G		Y/C	

Table 3 | Consensus nucleotide mutations in MM273.

Nucleotide position	KS661 original	Plasma	Thymus	Spleen	Axi.LN	Ing.LN	Mes.LN	Mes.LN RNA	Jejunum	Jejunum RNA	Rectum	Lung	Amino acid change	Genomic region
107	G										A	A		LTR
135	T	C		C			C	C	C	C	C	C		
543	T	C	C	C	C	C	C	C	C	C	C	C	V/A	
569	A	G	G	G	G	G	G	G	G	G	G	G	K/E	
572	G	A	A	A	A	A	A	A	A	A	A	A	A/I	gag-MA
638	G		A	A	A	A	A	A	A	A	A	A	V/I	
969	T	C	G	G		C	C	C	C	C		C	V/A	
1016	A	G					G	G	G	G			I/V	gag-CA
1532	C	A	A	A	A	A	A	A	A	A	A	A	L/I	
2453	A	G	G	G	G	G	G	G	G	G	G	G	K/R	pol-RT
4172	C	T	T	T	T	T	T	T	T	T	T		A/V	
4300	C		G	G		G	G						T/A	
4478	A		T			T							H/L	pol-INT
4664	A					T							S/L	
5057	A	G	G	G	G	G	G	G	G	G	G	G	K/E	
5072	A	G	G	G	G	G	G	G	G	G		G	T/A	
5238	G		A	A				A				A	R/K	vif
5249	T	C		C			C	C	C	C	C	C	Y/H	
5250	A		G	G	G	G							Y/C	
5918	G	A	A	A	A	A	A	A	A	A	A	A		vpr
6040	C											T		
6126	A			C	C			C				C	Q/P	tat
6146	G	A	A	A	A	A	A	A	A	A	A	A		
6328	A								G					
6363	C	A				A	A	A	A	A	A		A/E	vpu
6394	C				T									
6553	A		G	G	G							G	H/R	
6766	T	C				C	C	C					L/P	
6772	G	A	A	A							A		S/N	
6788	G					A							M/I	
6843	A	G	G	G		G	G	G	G	G	G	G	N/D	
6846	A		G	G		G						G	K/E	
6850	T	C					C	C	C	C	C		V/A	
6877	G					T							R/I	
6880	T		A										L/Q	

(Continued)

Table 3 | Continued

Nucleotide position	KS661 original	Plasma	Thymus	Spleen	Axi.LN	Ing.LN	Mes.LN	Mes.LN RNA	Jejunum	Jejunum RNA	Rectum	Lung	Amino acid change	Genomic region
6890	A					G								
6893	A	G	G				G		G	G	G			
6897	A	G				G	G	G	G		G		K/E	
6907	G	A				A	A	A	A	A	A		S/N	
6936	A			G		G	G					G	N/D	
7003	T	C	C	C		C	C	C	C	C	C	C	V/A	
7182	G	A	A	A		A	A	A	A	A	A	A	D/N	
7251	A		T	T								T	N/Y	
7269	T					C							S/P	env-gp120
7284	A					G							R/G	
7306	A	G	G	G		G	G	G	G	G			N/S	
7385	A											T		
7402	T		A	A		A							F/Y	
7464	A	C	C	C			C	C	C	C	C	C	S/R	
7465	G	A	A	A		A	A	A	A	A	A	A	S/Q	
7533	G	A	A	A		A	A	A	A	A	A	A	A/T	
7554	G	A	A	A			A	A	A	A	A		E/K	
7647	A	G	G	G		G	G	G	G	G	G	G	T/A	
7707	A			G					G		G		N/R	
7711	G	T		A		T	A	T			A		S/IorN	
7725	A	C	C	C	C	C	C	C	C	C	C	C	T/P	
7831	G								A	A			R/K	
7847	A	G	G	G	G	G	G	G	G	G	G	G		
8109	A					T							M/V	
8134	A	G	G	G	G	G	G	G	G	G	G	G	K/R	
8238	G	A	A	A	A	T	A	A		A		A	D/N	
8352	G											A	D/N	
8431	C					T							A/V	
8453	A	G	G	G	G	G	G	G	G	G	G	G		
8492	C	T			T		T	T	T	T	T			
8493	C	T	T	T	T	T	T	T	T	T	T	T	L/F	
8587	C	T	T	T	T	T	T	T	T	T	T	T	S/F	env-gp41
8605	C	T	T		T	T	T	T	T	T	T	T	A/V	
8613	C		T	T		T	T					T	R/W	
8734	A	T	T	T	T	T	T	T	T	T	T	T	Q/L	
8743	G		A										S/N	
8759	T	G	G	G	G	G	G	G	G	G	G	G	N/K	
8780	A	G		G	G	G	G	G	G	G	G	G		

(Continued)

Table 3 | Continued

Nucleotide position	KS661 original	Plasma	Thymus	Spleen	Axi.LN	Ing.LN	Mes.LN	Mes.LN RNA	Jejunum	Jejunum RNA	Rectum	Lung	Amino acid change	Genomic region
8792	C		T										A/V	
8803	A											G	R/G	
9011	G	A	A	A	A	A	A	A	A	A	A	A	R/K	
9080	T	C	C	C	C	C	C	C	C	C	C	C	V/A	
9082	T	C	C	C	C	C	C	C	C	C	C	C	S/P	
9100	C	A	A	A	A	A	A	A	A	A	A	A	P/T	
9116	G		C										S/T	
9226	G	A				A	A	A	A	A	A		E/K	
9229	G	A	A		A	A	A	A	A	A	A	A	E/K	nef
9322	A	G					G	G	G	G	G		N/D	
9341	A	G	G	G	G	G	G	G	G	G	G	G		
9380	C			G	G	G							T/S	
9430	C	T	T	T			T	T	T	T	T		P/S	
9460	G	A	A	A	A	A	A	A	A	A	A	A	V/I	
9518	G	A	A	A	A	A	A	A	A	A	A	A	R/K	
9558	G				A									
9661	A	G	G	G	G	G	G	G	G	G	G	G	I/V	
6220–6228		AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins	AAT-ins		vpu
6898–6901		AAA-del					AAA-del	AAA-del	AAA-del	AAA-del	AAA-del			env-gp120(V2)
6757–6775				18bp-del										env-gp120(V1)
6757–6778			21bp-del											env-gp120(V1)
6757–6779												23bp-del		env-gp120(V1)
6908–6916			9bp-del									9bp-del		env-gp120(V2)
6911–6920				9bp-del										env-gp120(V2)
8799–8831		33bp-del		33bp-del		33bp-del	33bp-del	33bp-del	33bp-del	33bp-del	33bp-del			nef
8839–8886			48bp-del											nef

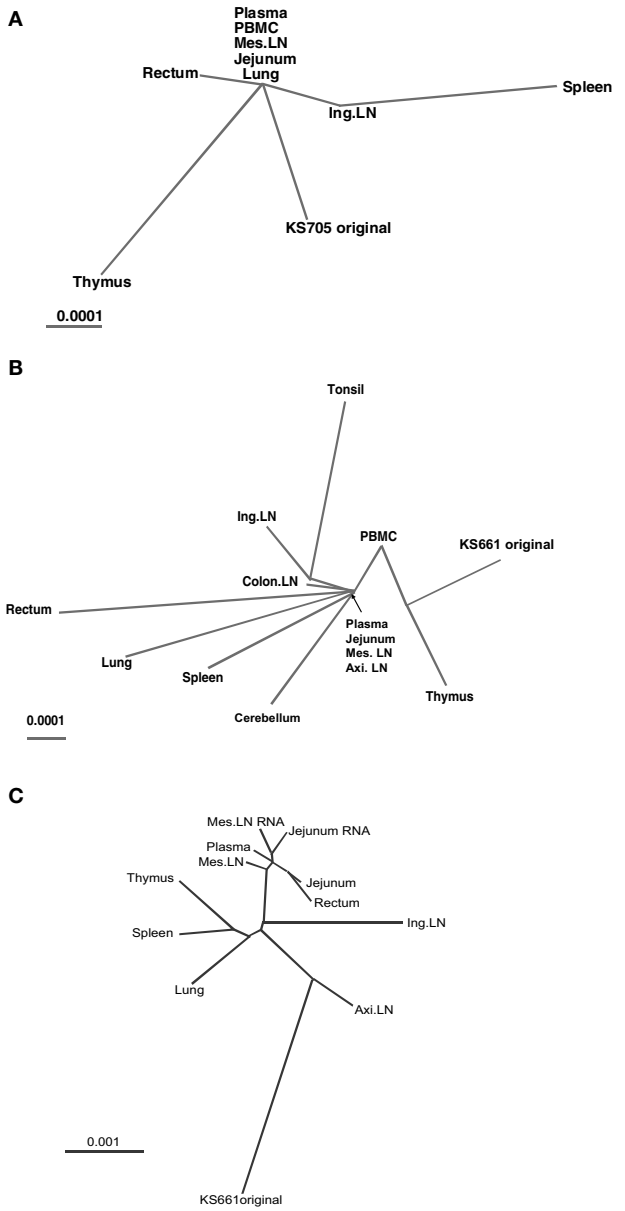


FIGURE 3 | Unrooted phylogenetic trees of consensus sequences. Branch length is drawn to scale. **(A)** Phylogenetic tree demonstrating the relationships among consensus sequences from seven tissues and peripheral blood of monkey MM340. **(B)** Phylogenetic tree demonstrating the relationships among consensus sequences from 11 tissues and peripheral blood of MM376. **(C)** Phylogenetic tree demonstrating the relationships among consensus sequences from eight tissues and peripheral blood of MM273.

in plasma, occupied the root position of the phylogenetic tree, whereas in MM273, the viral sequences from plasma, jejunum, mesenteric LN and rectum did not occupy the root position, but clustered separately from other tissues. In a phylogenetic tree, viruses in the root position have a consensus mutation pattern. Moreover, viruses in circulating blood tend to have the consensus mutation pattern either because they flow from tissues to the same degree or no particular virus production site is predominant. Therefore, in MM340 and MM376, viruses of the jejunum and mesenteric LN have mutation patterns similar to those in plasma, suggesting that circulating viruses accumulated in and were produced from the small intestine and/or mesenteric LN. On the other hand, in MM273, which retained a high viral load and was euthanized later, the viral sequences from plasma, jejunum, mesenteric LN and rectum did not occupy the root position, but clustered separately from other tissues. This suggests that the viruses in the intestine and/or mesenteric LN were produced mainly in MM273, and that viral production levels from other tissues were relatively low.

Next, we investigated the phenotype of SHIV-producing cells in the small intestine at the end stage of infection. Some studies have suggested that the target cells during the acute phase are CCR5+ memory CD4+ T cells (Li et al., 2005; Mattapallil et al., 2005). However, CD4+ T cells are unlikely to be the target cell since SIV- and SHIV-infected macaques resulted in CD4+ T cell depletion at the end stage of infection (Veazey et al., 2000). Generally, macrophages are regarded as a reservoir because their half-life when infected is substantially longer than that of T cells. As a result, macrophages continue to accumulate HIV-1 and show replication for an extended period, even in patients receiving combined antiretroviral therapy (Sharova et al., 2005). Also, in SHIV-infected macaques, after depletion of CD4+ T cells, macrophages were identified as the virus producing cells (Igarashi et al., 2001). In macaques inoculated with rapid-progressing SIVsmm, the majority of SIV-positive cells in the lymph nodes and gastrointestinal tract were macrophages (Brown et al., 2007). As already reported, macrophages have the potential to produce viruses at the end stage of infection. Our immunohistochemical study also demonstrated that >50% of the SIV *nef*-positive cells were co-localized with CD68-positive cells in the jejunum and mesenteric LN of the three macaques. Because CD68 is expressed in activated cells by phagocytosis and localizes on the membrane of lysosomes, CD68-positive cells can be regarded as macrophages. So, our results are consistent with those findings. Taken together, our data suggest that circulating viruses accumulate and are produced in CD68-positive cells; i.e., macrophages, in the small intestine and/or mesenteric LN. Sequence analyses of virus DNA and RNA extracted from CD68-positive cells isolated from jejunum and mesenteric LN should be investigated to determine which tissue is the real major source of plasma virus in future.

Finally, we detected two mutations of *gag-matrix* (569, 638) in all three macaques, and one mutation of *nef* (9359) in two of three macaques. The fixation of randomly occurring mutants and the emergence of particular variants are caused by selective pressure and the rate of virus turnover (Coffin, 1992). Because common mutations are often detected in SHIV-KS661-infected macaques, these mutations may be important for viral replication *in vivo* at the end stage of SHIV infection. These mutations do not necessarily lead to increased pathogenicity in naïve macaques because these mutants were present in the macaques after the collapse of the immune system (Kuwata et al., 2007). Therefore, we do not think

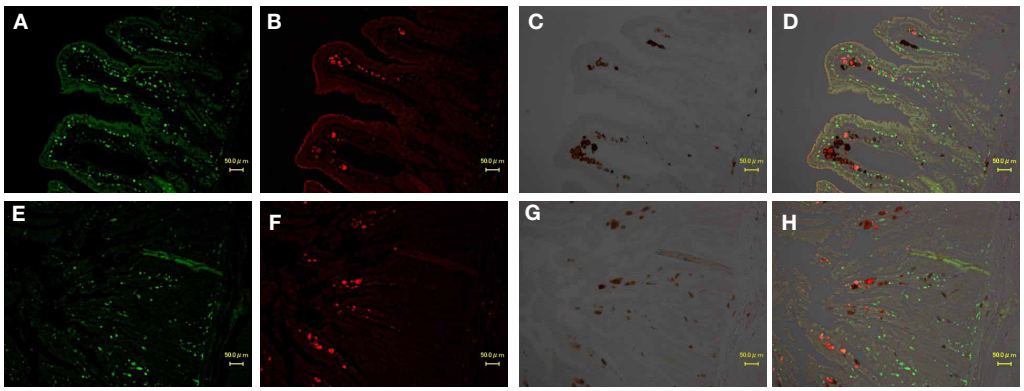


FIGURE 4 | Identification of virus-producing cells in the jejunum of two macaques (A,B,C,D: MM340, E,F,G,H: MM273). Immunohistochemical CD3 staining (**A,E**; visualized in green), CD68 (macrophage) staining (**B,F**; visualized in red) and SIV-*nef* (**C,G**; visualized in brown). More than 50% of SIV-*nef*-positive cells were co-localized with CD68+ cells in the merged images (**D,H**).

that these mutations may be characterized as association with escape from the immune system. We think they may have a substantial advantage for viral replication in CD68-positive cells; i.e., macrophages. Previous studies about HIV-1 Gag matrix suggested that *gag* targeting and assembly to multivesicular body is important step to produce the virus particles in macrophage (Ono and Freed, 2004). On the other hands, recent study about *nef* mutation demonstrated that some Nef variants were impaired virus replication in monocyte-derived macrophage (Mwimanzi et al., 2011). The significance of these mutations should be investigated further *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

This work was supported, in part, by Research on Human Immunodeficiency Virus/AIDS in Health and Labor Sciences research grants from the Ministry of Health, Labor and Welfare, Japan, a grant-in-aid for scientific research from the Ministry of Education and Science, Japan, a research grant for health sciences focusing on drug innovation for AIDS from the Japan Health Sciences Foundation, and a grant from the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan.

REFERENCES

Brenchley, J. M., Hill, B. J., Ambrozak, D. R., Price, D. A., Guenaga, F. J., Casazza, J. P., et al. (2004). T-cell subsets that harbor human immunodeficiency virus (HIV) *in vivo*: implications for HIV pathogenesis. *J. Virol.* 78, 1160–1168. doi: 10.1128/JVI.78.3.1160-1168.2004

Brown, C. R., Czapiga, M., Kabat, J., Dang, Q., Ourmanov, I., Nishimura, Y., et al. (2007). Unique pathology in simian immunodeficiency virus-infected rapid progressor macaques is consistent with a pathogenesis distinct from that of classical AIDS. *J. Virol.* 81, 5594–5606. doi: 10.1128/JVI.00202-07

Buckner, C. M., Gettie, A., Tan, R. C., Eshetu, T., Ratterree, M., Blanchard, J., et al. (2002). Infection of macaques with a molecular clone, SHIVSF33A2, provides evidence for tissue specific variants. *J. Med. Primatol.* 31, 164–170. doi: 10.1034/j.1600-0684.2002.02002.x

Clayton, F., Snow, G., Reka, S., and Kotler, D. P. (1997). Selective depletion of rectal lamina propria rather than lymphoid aggregate CD4 lymphocytes in HIV infection. *Clin. Exp. Immunol.* 107, 288–292. doi: 10.1111/j.1365-2249.1997.236-ce1111.x

Coffin, J. M. (1992). Genetic diversity and evolution of retroviruses. *Curr. Top. Microbiol. Immunol.* 176, 143–164. doi: 10.1007/978-3-642-77011-1_10

Couedel-Courteille, A., Butor, C., Juillard, V., Guillet, J. G., and Venet, A. (1999). Dissemination of SIV after rectal infection preferentially involves paracolic germinal centers. *Virology* 260, 277–294. doi: 10.1006/viro.1999.9809

Couedel-Courteille, A., Pretet, J. L., Barget, N., Jacques, S., Petitprez, K., Tulliez, M., et al. (2003). Delayed viral replication and CD4(+) T cell depletion in the rectosigmoid mucosa of macaques during primary rectal SIV infection. *Virology* 316, 290–301. doi: 10.1016/j.virol.2003.08.021

Delassus, S., Cheynier, R., and Wain-Hobson, S. (1992). Nonhomogeneous distribution of human immunodeficiency virus type 1 proviruses in the spleen. *J. Virol.* 66, 5642–5645.

Desrosiers, R. C. (1990). The simian immunodeficiency viruses. *Annu. Rev. Immunol.* 8, 557–578. doi: 10.1146/annurev.iy.08.040190.003013

Fujita, Y., Otsuki, H., Watanabe, Y., Yasui, M., Kobayashi, T., Miura, T., et al. (2013). Generation of a replication-competent chimeric simian-human immunodeficiency virus carrying env from subtype C clinical isolate through intracellular homologous recombination. *Virology* 436, 100–111. doi: 10.1016/j.virol.2012.10.036

Fukazawa, Y., Miyake, A., Ibuki, K., Inaba, K., Saito, N., Motohara, M., et al. (2008). Small intestine CD4+ T cells are profoundly depleted during acute simian-human immunodeficiency virus infection, regardless of viral pathogenicity. *J. Virol.* 82, 6039–6044. doi: 10.1128/JVI.02753-07

Harouse, J. M., Gettie, A., Tan, R. C., Blanchard, J., and Cheng-Mayer, C. (1999). Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. *Science* 284, 816–819. doi: 10.1126/science.284.5415.816

Harouse, J. M., Tan, R. C., Gettie, A., Dailey, P., Marx, P. A., Luciw, P. A., et al. (1998). Mucosal transmission of pathogenic CXCR4-utilizing SHIVSF33A variants in rhesus macaques. *Virology* 248, 95–107. doi: 10.1006/viro.1998.9236

Ho, S. H., Shek, L., Gettie, A., Blanchard, J., and Cheng-Mayer, C. (2005). V3 loop-determined coreceptor preference dictates the dynamics of CD4+-T-cell loss in simian-human immunodeficiency virus-infected macaques. *J. Virol.* 79, 12296–12303. doi: 10.1128/JVI.79.19.12296-12303.2005

Horiike, M., Iwami, S., Kodama, M., Sato, A., Watanabe, Y., Yasui, M., et al. (2012). Lymph nodes harbor viral reservoirs that cause

rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy. *Virology* 423, 107–118. doi: 10.1016/j.virol.2011.11.024

Igarashi, T., Brown, C. R., Endo, Y., Buckler-White, A., Plishka, R., Bischofberger, N., et al. (2001). Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. *Proc. Natl. Acad. Sci. U.S.A.* 98, 658–663. doi: 10.1073/pnas.98.2.658

Igarashi, T., Donau, O. K., Imamichi, H., Dumaurier, M. J., Sadjadpour, R., Plishka, R. J., et al. (2003). Macrophage-tropic simian/human immunodeficiency virus chimeras use CXCR4, not CCR5, for infections of rhesus macaque peripheral blood mononuclear cells and alveolar macrophages. *J. Virol.* 77, 13042–13052. doi: 10.1128/JVI.77.24.13042-13052.2003

Joag, S. V., Adany, I., Li, Z., Foresman, L., Pinson, D. M., Wang, C., et al. (1997). Animal model of mucosally transmitted human immunodeficiency virus type 1 disease: intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of CD4+ T cells, and AIDS. *J. Virol.* 71, 4016–4023.

Joag, S. V., Li, Z., Foresman, L., Stephens, E. B., Zhao, L. J., Adany, I., et al. (1996). Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. *J. Virol.* 70, 3189–3197.

Kodama, T., Mori, K., Kawahara, T., Ringler, D. J., and Desrosiers, R. C. (1993). Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. *J. Virol.* 67, 6522–6534.

Kozyrev, I. L., Ibuki, K., Shimada, T., Kuwata, T., Takemura, T., Hayami, M., et al. (2001). Characterization of less pathogenic infectious molecular clones derived from acute-pathogenic SHIV-89.6p stock virus. *Virology* 282, 6–13. doi: 10.1006/viro.2000.0839

Kuwata, T., Byrum, R., Whitted, S., Goeken, R., Buckler-White, A., Plishka, R., et al. (2007). A rapid progressor-specific variant clone of simian immunodeficiency virus replicates efficiently *in vivo* only in the absence of immune responses. *J. Virol.* 81, 8891–8904. doi: 10.1128/JVI.00614-07

Kuwata, T., Igarashi, T., Ido, E., Jin, M., Mizuno, A., Chen, J., et al. (1995). Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their *in vitro* and *in vivo* replication. *J. Gen. Virol.* 76, 2181–2191. doi: 10.1099/0022-1317-76-9-2181

Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., et al. (2005). Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 434, 1148–1152.

Matsuda, K., Inaba, K., Fukazawa, Y., Matsuyama, M., Ibuki, K., Horiike, M., et al. (2010). *In vivo* analysis of a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a derivative of SHIV-89.6. *Virology* 399, 134–143. doi: 10.1016/j.virol.2010.01.008

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M., and Roederer, M. (2005). Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434, 1093–1097. doi: 10.1038/nature03501

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., et al. (2004). Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J. Exp. Med.* 200, 761–770. doi: 10.1084/jem.20041196

Meng, G., Sellers, M. T., Mosteller-Barnum, M., Rogers, T. S., Shaw, G. M., and Smith, P. D. (2000). Lamina propria lymphocytes, not macrophages, express CCR5 and CXCR4 and are the likely target cell for human immunodeficiency virus type 1 in the intestinal mucosa. *J. Infect. Dis.* 182, 785–791. doi: 10.1086/315790

Miyake, A., Enose, Y., Ohkura, S., Suzuki, H., Kuwata, T., Shimada, T., et al. (2004). The quantity and diversity of infectious viruses in various tissues of SHIV-infected monkeys at the early and AIDS stages. *Arch. Virol.* 149, 943–955. doi: 10.1007/s00705-003-0252-0

Miyake, A., Ibuki, K., Enose, Y., Suzuki, H., Horiuchi, R., Motohara, M., et al. (2006). Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection. *J. Gen. Virol.* 87, 1311–1320. doi: 10.1099/vir.0.81307-0

Motohara, M., Ibuki, K., Miyake, A., Fukazawa, Y., Inaba, K., Suzuki, H., et al. (2006). Impaired T-cell differentiation in the thymus at the early stages of acute pathogenic chimeric simian-human immunodeficiency virus (SHIV) infection in contrast to less pathogenic SHIV infection. *Microbes Infect.* 8, 1539–1549. doi: 10.1016/j.micinf.2006.01.011

Mwimanzi, P., Hasan, Z., Hassan, R., Suzu, S., Takiguchi, M., and Ueno, T. (2011). Effects of naturally-arising HIV Nef mutations on cytotoxic T lymphocyte recognition and Nef's functionality in primary macrophages. *Retrovirology* 8:50. doi: 10.1186/1742-4690-8-50

Ndolo, T., Syvanen, M., Ellison, T., and Dandekar, S. (2005). Evolution of nef variants in gut associated lymphoid tissue of rhesus macaques during primary simian immunodeficiency virus infection. *Virology* 343, 1–11. doi: 10.1016/j.virol.2005.08.013

Ono, A., and Freed, E. O. (2004). Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. *J. Virol.* 78, 1552–1563. doi: 10.1128/JVI.78.3.1552-1563.2004

Oue, M., Sakabe, S., Horiike, M., Yasui, M., Miura, T., and Igarashi, T. (2013). No viral evolution in the lymph nodes of simian immunodeficiency virus-infected rhesus macaques during combined antiretroviral therapy. *J. Virol.* 87, 4789–4793. doi: 10.1128/JVI.03367-12

Overbaugh, J., Rudensey, L. M., Papenhausen, M. D., Benveniste, R. E., and Morton, W. R. (1991). Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. *J. Virol.* 65, 7025–7031.

Reimann, K. A., Li, J. T., Veazey, R., Halloran, M., Park, I. W., Karlsson, G. B., et al. (1996). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after *in vivo* passage in rhesus monkeys. *J. Virol.* 70, 6922–6928.

Reimann, K. A., Watson, A., Dailey, P. J., Lin, W., Lord, C. I., Steenbeke, T. D., et al. (1999). Viral burden and disease progression in rhesus monkeys infected with chimeric simian-human immunodeficiency viruses. *Virology* 256, 15–21. doi: 10.1006/viro.1999.9632

Ritola, K., Pilcher, C. D., Fiscus, S. A., Hoffman, N. G., Nelson, J. A., Kitrinos, K. M., et al. (2004). Multiple V1/V2 env variants are frequently present during primary infection with human immunodeficiency virus type 1. *J. Virol.* 78, 11208–11218. doi: 10.1128/JVI.78.20.11208-11218.2004

Scharko, A. M., Perlman, S. B., Hinds, P. W. 2nd., Hanson, J. M., Uno, H., and Pauza, C. D. (1996). Whole body positron emission tomography imaging of simian immunodeficiency virus-infected rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 93, 6425–6430. doi: 10.1073/pnas.93.13.6425

Scharko, A. M., Perlman, S. B., Pyzalski, R. W., Graziano, F. M., Sosman, J., and Pauza, C. D. (2003). Whole-body positron emission tomography in patients with HIV-1 infection. *Lancet* 362, 959–961. doi: 10.1016/S0140-6736(03)14366-8

Sharova, N., Swingler, C., Sharkey, M., and Stevenson, M. (2005). Macrophages archive HIV-1 virions for dissemination in trans. *EMBO J.* 24, 2481–2489. doi: 10.1038/sj.emboj.7600707

Shinohara, K., Sakai, K., Ando, S., Ami, Y., Yoshino, N., Takahashi, E., et al. (1999). A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. *J. Gen. Virol.* 80, 1231–1240.

Suryanarayana, K., Wiltrout, T. A., Vasquez, G. M., Hirsch, V. M., and Lifson, J. D. (1998). Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. *AIDS Res. Hum. Retroviruses* 14, 183–189. doi: 10.1089/aid.1998.14.183

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882. doi: 10.1093/nar/25.24.4876

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley,

D. R., Knight, H. L., et al. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 280, 427–431. doi: 10.1126/science.280.5362.427

Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E., et al. (2000). Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. *J. Virol.* 74, 11001–11007. doi: 10.1128/JVI.74.23.11001-11007.2000

Wong, J. K., Ignacio, C. C., Torriani, F., Havlir, D., Fitch, N. J., and Richman, D. D. (1997). *In vivo* compartmentalization of human immunodeficiency virus: evidence from the examination of pol sequences from autopsy tissues. *J. Virol.* 71, 2059–2071.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 May 2013; accepted: 01 July 2013; published online: 22 July 2013.
*Citation: Matsuyama-Murata M, Inaba K, Horiuchi R, Fukazawa Y, Ibuki K, Hayami M and Miura T (2013) Genetic similarity of circulating and small intestinal virus at the end stage of acute pathogenic simian-human immunodeficiency virus infection. Front. Microbiol. **4**:204. doi: 10.3389/fmicb.2013.00204*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 September 2013
doi: 10.3389/fmicb.2013.00292

A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive CD4$^+$ T cells in the hu-PBL SCID mice

Akira Kodama[1], Reiko Tanaka[1], Mineki Saito[2], Aftab A. Ansari[3] and Yuetsu Tanaka[1]*

[1] *Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan*
[2] *Department of Microbiology, Kawasaki Medical School, Kurashiki, Japan*
[3] *Department of Pathology, Emory University School of Medicine, Atlanta, GA, USA*

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Bernard A. P. Lafont, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA
Yasuko Tsunetsugu Yokota, National Institute of Infectious Diseases, Japan

Correspondence:
Yuetsu Tanaka, Department of Immunology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Okinawa 903-0215, Japan
e-mail: yuetsu@s4.dion.ne.jp

Because dendritic cells (DCs) play a critical role in the regulation of adaptive immune responses, they have been ideal candidates for cell-based immunotherapy of cancers and infections in humans. Generally, monocyte-derived DCs (MDDCs) were generated from purified monocytes by multiple steps of time-consuming physical manipulations for an extended period cultivation. In this study, we developed a novel, simple and rapid method for the generation of type-1 helper T cell (Th1)-stimulating human DCs directly from bulk peripheral blood mononuclear cells (PBMCs). PBMCs were cultivated in the presence of 20 ng/ml of granulocyte-macrophage colony-stimulating factor, 20 ng/ml of interleukin-4 (IL-4) and 1,000 U/ml of interferon-β for 24 h followed by 24 h maturation with a cytokine cocktail containing 10 ng/ml of tumor necrosis factor-α (TNF-α), 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2. The phenotype and biological activity of these new DCs for induction of allogeneic T cell proliferation and cytokine production were comparable to those of the MDDCs. Importantly, these new DCs pulsed with inactivated HIV-1 could generated HIV-1-reactive CD4$^+$ T cell responses in humanized mice reconstituted with autologous PBMCs from HIV-1-negative donors. This simple and quick method for generation of functional DCs will be useful for future studies on DC-mediated immunotherapies.

Keywords: dendritic cell, short-term culture, Th1-inducing DCs, anti-HIV-1 T cell response, hu-PBL-SCID

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) which play a critical role in the regulation of the adaptive immune response through activation and polarization of naive T cells (Banchereau et al., 2000). Since small numbers of activated DCs are highly efficient in generating immune responses against infections and cancers (Moll and Berberich, 2001; Steinman and Banchereau, 2007), the DC therapy represents a new and promising immunotherapeutic approach for treatment of advanced cancers as well as for prevention of infectious diseases. Indeed, the current clinical trials with *ex vivo*-generated DCs (so-called DC vaccine) will yield precious information regarding their potentials as vectors for immunotherapy (Gilboa, 2007; Connolly et al., 2008; Ezzelarab and Thomson, 2011). However, the general protocols to generate DCs are complicated and time consuming. Moreover, since different *ex vivo* DC generation methods affect the DC phenotype and function (Kalantari et al., 2011), it is critical to choose appropriate method for generating functional DCs. In general, the DC precursor monocytes are purified from PBMCs by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). These enriched monocytes are then induced to differentiate into DCs by 5 days-*in vitro* cultivation in medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 followed by a 2-days-maturation procedure (Sallusto and Lanzavecchia, 1994; Gilboa, 2007; Dauer et al., 2008). However, a lines of evidence are increasing that mature monocyte-derived DCs can be generated even after short-term cell culture for 2–3 days (Dauer et al., 2003a,b; Jarnjak-Jankovic et al., 2007; Zhang et al., 2008; Tawab et al., 2009).

In this study, in an attempt to simplify the methods currently being used for optimal DC generation and to develop a standardized method of preparing effective myeloid DC vaccine for immunotherapies, we explored the efficacy of using unfractionated PBMCs as a source of DC precursors and short-term *in vitro* cell culture just for 2 days.

MATERIALS AND METHODS

REAGENTS

The media used were RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (hereafter called RPMI medium) and Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island,

NY, USA) supplemented with 10% FCS with the same antibiotics (hereafter called Iscove's medium). Aldrithiol-2 (AT-2) and low-endotoxin bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). The recombinant human cytokines used included IL-4, GM-CSF, TNF-α and IL-1β (PeproTech, London, UK). Enzyme-linked immunosorbent assay (ELISA) kits for the quantitation of human IFN-γ, human IL-4, human IL-10 and human IL-12 (detecting IL-12 p75 heterodimer) were purchased from Biolegend. The human monocyte negative isolation kits and the human T cell isolation kits were purchased from Invitrogen (Carlsbad, CA, USA). The human naive $CD4^+$ T cell isolation kit was purchased from Miltenyi Biotec (Gladbach, Germany). The Vybrant CFDA SE Cell Tracer Kit was purchased from Invitrogen.

GENERATION OF DCs

Human PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by standard density gradient centrifugation. Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na_2EDTA. For select experiments, monocytes were purified from PBMCs using the $CD14^+$ monocyte negative isolation kit (Invitrogen, Carlsbad, CA, USA). An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% $CD14^+$ cells. To obtain immature MDDCs (iMDDCs), PBMCs (2.5×10^6 cells/ml) or the purified monocytes (5×10^5 cells/ml) were cultured in RPMI medium containing 20 ng/ml of human GM-CSF and 20 ng/ml of human IL-4 at 37°C in 24-well plates in a 5% CO_2 humidified incubator for 5 days. In other experiments, iDCs were generated from either purified monocytes or whole PBMCs by cultivation in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day. These iDCs were matured by incubation in the presence of either 10 ng/ml of LPS (Sigma) or a cocktail containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2 (PGE2; TIP cocktail) for 1–2 days.

FLOW CYTOMETRY

Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% sodium azide (FACS buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a predetermined optimal concentration of the appropriate fluorescent dye-labeled mAbs against human cell surface markers on ice for 30 min. The fluorescent dye-labeled monoclonal antibodies (mAbs) against human cell surface molecules used included anti-CD3, anti-CD4, anti-CD8, anti-CD14, CD20, anti-CD80, anti-HLA-DR, and isotype-matched control mAbs (Beckman Coulter, Fullerton, CA, USA), and anti-CD11c, anti-CD86, and anti-CD83 (BioLegend, San Diego, CA, USA). After washing with FACS buffer, cells were fixed in 1% paraformaldehyde (PFA) containing FACS buffer. The cells were then analyzed on FACS-Calibur flow cytometer with CellQuest software (BD Pharmingen, San Diego, CA, USA). Isotype-matched mAbs were utilized as controls to stain an aliquot of the cells to be analyzed for purposes of establishing gates and for determination of the frequency of positively stained cells.

HIV-1 PREPARATION AND INACTIVATION

$HIV\text{-}1_{IIIB}$ (virus that only use CXCR4 as chemokine co-receptor, termed X4) was harvested from Molt-4/IIIB cell cultures. Batches of each HIV-1 preparation were inactivated with Aldrithiol-2 (AT-2; Sigma) as described previously (Yoshida et al., 2003). AT-2 was removed by three successive ultrafiltration in PBS using 100-kDa-cut-off centrifugal filtration devices (Centriprep 100; Amicon, Beverly, MA, USA). Then AT-2–inactivated HIV-1 (iHIV) was purified by pelleting down the virus at 20,000 × *g* for 2 h three times in 0.1% BSA-PBS. The virus pellet was resuspended in 0.1% BSA-PBS, aliquoted, and stored at −80°C until use. The concentration of HIV-1 was estimated by measuring levels of HIV-1 p24 antigen with our in-house p24 ELISA kit (Tanaka et al., 2010). As previously described (Yoshida et al., 2003), activated human PBMCs incubated with an aliquot of 1 μg/ml of the AT-2-treated HIV-1 preparation failed to demonstrate the presence of any detectable infectious virions (data not shown).

STIMULATION OF T CELLS

Enriched populations of naive $CD4^+$ T cells and bulk T cells with >90% purity were isolated from normal human PBMCs by using appropriate negative cell isolation kits. These T cells (4×10^4 cells/well) were first labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), then co-cultured with allogeneic DCs at a T cells: DCs ratio of 50:1 in 100 μl of RPMI medium supplemented with 20 U/ml human IL-2 in 96-well, U-bottomed plates. Cell proliferation and cytokine production were determined on day 4.

hu-PBL-SCID MICE

The BALB/c-$rag2^{-/-}\gamma c^{-/-}$ mice lacking T cells, B cells and natural killer (NK) cells ($Rag2^{-/-}$ mice; Traggiai et al., 2004) were used in this study. The mice were kept in the specific-pathogen-free and P3 animal facilities of the Laboratory Animal Center, University of the Ryukyus. The protocols for the care and use of mice engrafted with human PBMCs and autologous DCs sensitized with inactivated HIV-1 or ovalbumin (OVA) were approved by the committee on animal research of the University of the Ryukyus prior to initiation of the study. Matured DCs (5×10^5 cells) pulsed with either AT-2-inactivated HIV-1 (40 ng of p24) or 100 μg of OVA in 100 μl of RPMI medium for 2 h at 37°C were mixed with autologous fresh PBMCs (3×10^6 cells) in a final volume of 100 μl in serum-free RPMI medium, and the were directly injected into the spleen of Rag2 mice as previously described (Yoshida et al., 2003). One week later, the same number of DCs pulsed with the same antigens were inoculated again into the spleen. One week later, mice were sacrificed, blood was collected by cardiocentesis, and human $CD4^+$ T cells were enriched from splenocytes using a human $CD4^+$ T cell isolation kit according to the manufacturer's instructions. For the measurement of antigen-specific human cellular immune responses, human $CD4^+$ T cell (2×10^5 cells) collected from the spleens of immunized $Rag2^{-/-}$ mice were cultured for 2 days with autologous monocytes (2×10^5 cells) in the presence or absence of inactivated HIV containing 40 ng/ml of p24 in 500 μl of RPMI medium supplemented with 20 U/ml of IL-2 in individual wells of a 48-well plate at 37°C. The concentration

of human IFN-γ or IL-4 produced in the culture supernatants was determined with ELISA kits.

STATISTICAL ANALYSIS

Data were analyzed by Student's *t* test with the with Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

GENERATION OF MYELOID MATURE DCs DIRECTLY FROM PBMCs WITHIN 2 DAYS

In order to reduce the cost, labor and any loss of potential precursors from PBMCs, we have previously established a novel culture method for generating functional human DCs from unfractionated PBMC in which whole PBMCs were cultured in the presence of IL-4 and GM-CSF for 5 days followed by a 2-day maturation in media containing poly I:C and IL-1β (Kodama et al., 2010). However, there were considerable lot variations in commercial poly:IC in the DC-maturation activity (data not shown). Therefore, we tested a previously reported maturation cytokine cocktail containing TNF-α, IL-1β, IL-6 and PGE2 (Jonuleit et al., 1997). In a preliminary study, we found that IL-6 was not necessary to mature DCs from purified monocytes in the present cell culture conditions, probably due to the use of serum-containing media. Thus, we used a cytokine cocktail

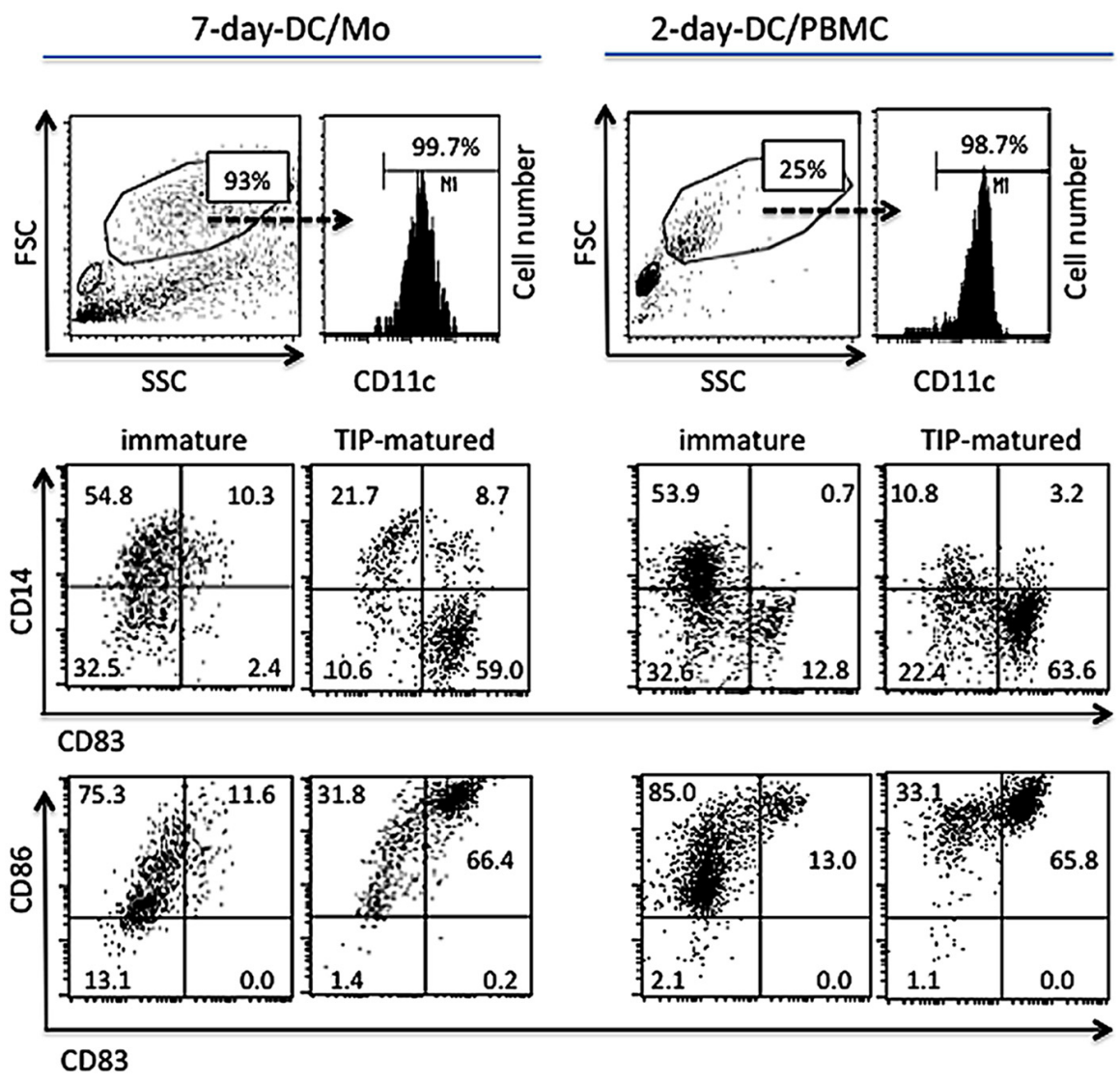


FIGURE 1 | Generation of functional human myeloid DCs directly from PBMCs *in vitro* within 2 days. MDDCs generated purified monocytes for 7 days (7-day-DC/Mo) or whole PBMCs cultured in medium containing GM-CSF, IL-4 and IFN-β (2-day-DC/PBMC), either non-treated or treated with the maturation cocktail containing TNF-α, IL-1β and PGE2 (TIP) were examined for the expression of mature DC markers by a flow cytometry. Cells were stained with antibodies to CD11c, CD14, CD83 and CD86, and analyzed on a subpopulation gated for FSC^{high}, SSC^{high} and $CD11c^{+}$. The percentages of the positive cells in the gated population were shown in the dot plots. Data shown are representative of three independent experiments using blood from three different donors.

containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of PGE2 (hereafter called TIP cocktail) throughout the present study.

Based on our previous report that monocytes can be differentiated into mature DCs within 2 days (Zhang et al., 2008), we tested whether Th1-inducing DCs could be generated from unfractionated PBMCs. PBMCs (2.5×10^6 cells/ml) were cultured in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day followed by additional 1 day cultivation in the presence or absence of the TIP cocktail. The phenotypes of $CD11c^+$ large cells in these 2-day PBMC cultures were compared with those of MDDCs derived from purified monocyte for 7 days (7-day-DC/Mo; **Figure 1**). The proportion of FSC^{high} and SSC^{high} cells in the 2-day-DC/PBMC culture was 20∼25% of total viable cells depending on donors and these cells expressed CD11c (data not shown). After maturation with the TIP cocktail, similar to the 7-day-DC/Mo, the large $CD11c^+$ cells in the 2-day PBMC cultures became $CD14^{low}$, $CD86^{high}$ and $CD83^{high}$, a typical marker of matured myeloid DCs (Ohshima et al., 1997). The other viable cell populations in the 2-day PBMC cultures were $CD3^+$ T cells (54.0∼59.2%), $CD56^+$ NK cells (8.4∼9.3%) and $CD19^+$ B cells (6.5∼8.6%; $n = 3$). These data showed that the present culture method was applicable to generate myeloid mature DCs from bulk PBMCs within 2 days (2-day-DC/PBMC).

Then we tested cytokine production by these 2-day-DC/PBMC. Interestingly, in contrast to the DCs matured in the presence of LPS, the production of IL-12 and IL-10 by the TIP matured 2-day-DC/P was minimum (**Figure 2**). To investigate whether the 2-day-DC/PBMC were immunologically functional, we examined their ability to stimulate allogeneic T cell proliferation. Like the MDDCs (7-day-DC/Mo), the 2-day-DC/PBMC could stimulate allogeneic T cell proliferation (**Figure 3A**). Then we quantitated the levels of IFN-γ and IL-4 in the culture supernatants from allogeneic $CD4^+$ T cells co-cultured with various DCs. As shown in **Figure 3B**, among the four DC preparations including the 7-day-DC/Mo, 7-day-DCs from PBMCs (7-day-DC/PBMC), 2-day-DCs from monocytes (2-day-DC/Mo) and 2-day-DC/PBMC, the 2-day-DC/PBMC were most potent in induction of IFN-γ production. The bulk 2-day-DC/PBMC alone did not produce detectable IFN-γ (<20 pg/ml) in the present culture conditions (data not shown). The levels of IL-4 and IL-10 were below detection (<5 pg/ml) in all the samples tested (data not shown). These results indicated that the 2-day-DC/PBMC had a potential to induce Th1 response.

INDUCTION OF HIV-1-REACTIVE HUMAN $CD4^+$ T CELL RESPONSES IN hu-PBL-SCID MICE

Finally, we examined whether the short-term generated 2-day-DC/PBMC could induce HIV-1-reactive immune responses *in vivo* in comparison to MDDCs (7-day-DC/Mo) using our hu-PBL-SCID mice model (Yoshida et al., 2003). SCID mice were *intra-splenically* transplanted with DCs loaded with AT-2-inactivated HIV-1 together with autologous fresh PBMCs. On day 7, these mice were received an *intra-splenic* booster injection with similarly prepared antigen-pulsed DCs. Seven days after the booster injection, mice were sacrificed and examined for antigen-specific human immune responses. **Figure 4** showed that after *in vitro* re-stimulation with autologous APCs pulsed with inactivated HIV-1, enriched human $CD4^+$ T cells from two out of three mice immunized with MDDCs (7-day-DC/Mo) pulsed with HIV-1 and those from three out of four mice immunized with 2-day-DC/PBMC pulsed with HIV-1 produced IFN-γ in antigen-dependent way, indicating that the 2-day-DC/PBMC could induce HIV-1 antigen-reactive human T responses *in vivo* as potent as MDDCs. In the re-stimulated culture supernatants, no IL-4 or IL-10 was detected (<5 pg/ml) using ELISA (data not shown). In addition, no detectable antibodies against HIV-1 were detected

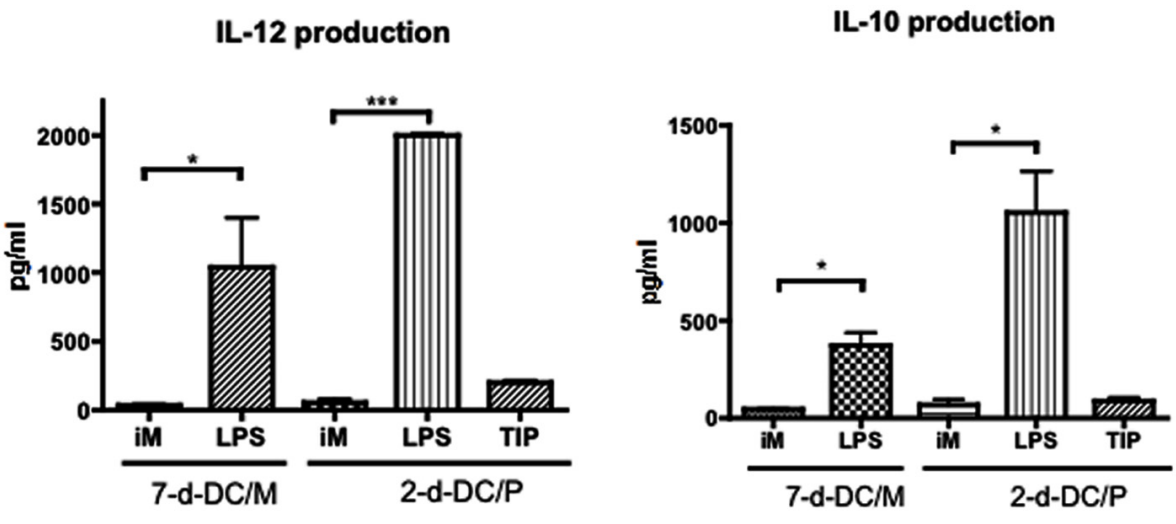


FIGURE 2 | Production of cytokines by the 2-day-DC/PBMC. The levels of IL-12 (bioactive p70 heterodimer) and IL-10 in the supernatants from MDDCs (7-d-DC/M) or 2-day-DC/PBMC (2-d-DC/P) either non-matured (iM) or matured with LPS or the TIP cocktail were determined by ELISA. Data are mean ± SD of triplicate cultures. $*P < 0.05$, $***P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

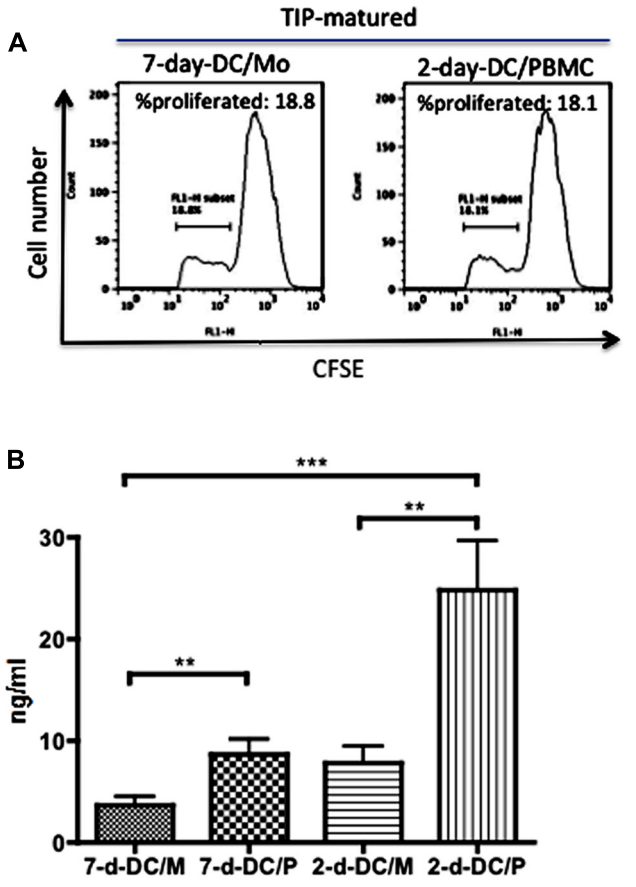


FIGURE 3 | High levels of production of IFN-γ by allogeneic T cells stimulated with 2-day-DCs generated from PBMCs. Matured MDDCs (7-day-DC/Mo) and 2-day-DCs generated from PBMCs (2-day-DC/PBMC) were co-cultured with CFSE-labeled allogeneic $CD4^+$ T cells at DC to T cell ratio of 1:50 for 4 days. The cell number of DCs in 2-day-DC/PBMC was manually counted using a Burker-Turk hemo-cytometer in which only large cells were counted. **(A)** Percentages of proliferated cells were examined by flow cytometry. **(B)** The levels of IFN-γ produced in the supernatants were quantitated by ELISA. In addition to the DCs shown in **(A)**, MDDCs generated from PBMCs for 7 days (7-d-DC/P) and 2-day-DCs generated from purified monocytes (2-d-DC/M) were also tested. Data shown are representative of three independent experiments using blood from two different donors.

as determined by using a commercial Western blot assay kit in plasma samples from all the DCs-HIV-1-immunized mice (data not shown).

Altogether, these results demonstrated that human myeloid DCs directly generated from PBMCs by the present short-term cultivation method were potent in induction of functional Th1 responses both *in vitro* and *in vivo*.

DISCUSSION

In the present study, we have developed a novel, simple and rapid protocol for generating Th1-stimulating human myeloid DCs directly from unfractionated PBMCs. These 2-day-DC/PBMC were potent in both stimulating allogeneic T cells *in vitro* and inducing HIV-1-reactive Th1 responses in hu-PBL-SCID mice. The use of whole PBMCs as DC precursors might reduce any loss of monocytes in the step of purification by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). One possible concern on using whole PBMCs was that the non-monocyte cells, such as T, B or NK cells, in the PBMCs might interfere with differentiation and function of DCs. However, in the present study there was no obvious difference in DC maturation and function between in PBMC and purified monocyte cultures.

For the final maturation, we used a cytokine cocktail containing TNF-α and IL-1β and PGE2 (TIP coctail). Simultaneous use of these three reagents in TIP was essential for maturation of DCs since use of the reagents either in single or in two combinations failed to mature DCs (data not shown). In general, IL-6 that is included in the maturation cytokine cocktail TNF-α and IL-1β and PGE2 to mature DCs was not necessary in the present culture conditions. The reason remains to be studied, but it is possible that IL-6 is required in serum-free culture conditions. The present 2-day-DC/PBMC matured by TIP produced lower IL-12 than those matured by LPS. Low levels production of IL-12 might be ascribed to the use of PGE2 that inhibits bioactive IL-12 heterodimer production (Kalinski et al., 2001; Kalim and Groettrup, 2013). Despite of the low level production of IL-12, the TIP-matured 2-day-DC/PBMC were potent in stimulating IFN-γ, but not IL-4 or IL-10, production by allogeneic T cells. The reason for higher potentials of 2-day-DC/PBMC to induce Th1 cells than MDDCs remains to be clarified. It is speculated that natural DCs contained in the 2-day-PBMC-derived DCs might enhance the activation. Indeed, 2-day-DC/PBMC culture generated from $CD14^+$ cell-depleted PBMCs were able to stimulate allogeneic $CD4^+$ T cells to a lesser extent (data not shown). However, we cannot clearly determine if the stimulation was mediated by remaining monocytes. Further study is required to solve this issue. Importantly, as the previous study (Yoshida et al., 2003), the present study showed the induction of primary HIV-1-specific human $CD4^+$ T cell immune responses in hu-PBL-SCID mice by DC-based immunization, demonstrating that the present 2-day-PBMC-derived DCs might have a potential for clinical use in DC-based immunization in humans against HIV-1. It was of interest that the levels of IFN-γ production were higher in $CD4^+$ T cells immunized with 2-day-DC/PBMC than those immunized with 7-day-DC/Mo. It is possible that 2-day-DC/PBMC could live longer than 7-day-DC/Mo *in vivo* to stimulate antigen-specific $CD4^+$ T cells. In addition, because myeloid DCs are susceptible to HIV-1 infection (Knight et al., 1990), the use of these IFN-β-treated DCs will be beneficial for HIV-1-infected individuals.

In conclusion, the present study provided a new method to generate functional human myeloid DCs directly from PBMCs in a short-term culture period. These DCs will be useful for studies exploring potentials of DC-based immunization for not only infectious diseases but also cancers *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

This work was supported by the Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

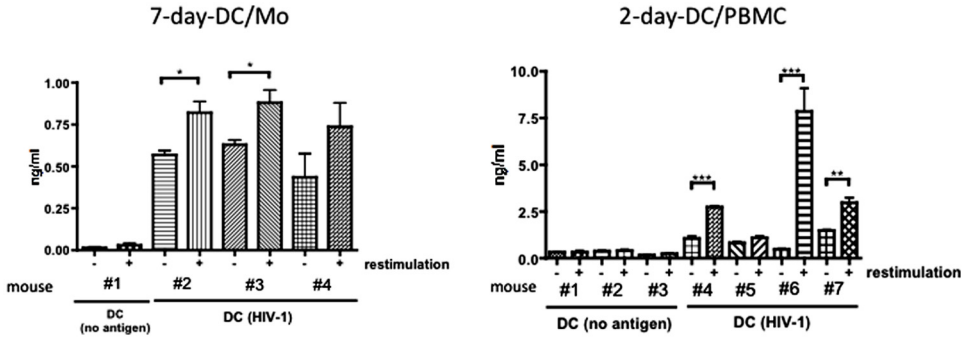


FIGURE 4 | Functional activity of the 2-days-DCs in hu-PBL-SCID mice. Fresh autologous PBMCs from normal human donors were transferred into the Rag2$^{-/-}$ mouse spleen together with autologous mature MDDCs (7-day-DC/Mo) or 2-days-PBMC-derived DCs (2-day-D/PBMC) pulsed with no antigen (no antigen) or AT-2-inactivated HIV-1 (40 ng of p24). On day 7 after the first transplantation, these mice were received an *intra splenic* booster injection with similarly prepared DCs. Seven days after the booster injection, mice were sacrificed and human CD4^{+} T cells were purified from splenocytes. These CD4^{+} T cells were co-cultured with autologous APCs (adherent PBMCs) in the presence or absence of antigens (restimulation) for 2 days at 37°C. IFN-γ levels produced in the culture supernatants were measured by ELISA. Data show mean ± SD of triplicate cultures. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

AUTHOR CONTRIBUTIONS

Akira Kodama designed and performed the experiments, analyzed the data and wrote the paper. Reiko Tanaka and Mineki Saito performed the experiments, analyzed the data and wrote the paper. Aftab A. Ansari participated in the design of the study and helped to draft the manuscript. Yuetsu Tanaka designed and supervised the research, performed experiments and wrote the paper. All authors checked the final version of this manuscript.

REFERENCES

Babatz, J., Rollig, C., Oelschlagel, U., Zhao, S., Ehninger, G., Schmitz, M., et al. (2003). Large-scale immunomagnetic selection of CD14+ monocytes to generate dendritic cells for cancer immunotherapy: a phase I study. *J. Hematother. Stem Cell Res.* 12, 515–523. doi: 10.1089/152581603322448222

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., et al. (2000). Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18, 767–811. doi: 10.1146/annurev.immunol.18.1.767

Berger, T. G., Strasser, E., Smith, R., Carste, C., Schuler-Thurner, B., Kaempgen, E., et al. (2005). Efficient elutriation of monocytes within a closed system (Elutra) for clinical-scale generation of dendritic cells. *J. Immunol. Methods* 298, 61–72. doi: 10.1016/j.jim.2005.01.005

Connolly, N. C., Whiteside, T. L., Wilson, C., Kondragunta, V., Rinaldo, C. R., and Riddler, S. A. (2008). Therapeutic immunization with human immunodeficiency virus type 1 (HIV-1) peptide-loaded dendritic cells is safe and induces immunogenicity in HIV-1-infected individuals. *Clin. Vaccine Immunol.* 15, 284–292. doi: 10.1128/CVI.00221-07

Dauer, M., Obermaier, B., Herten, J., Haerle, C., Pohl, K., Rothenfusser, S., et al. (2003a). Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J. Immunol.* 170, 4069–4076.

Dauer, M., Pohl, K., Obermaier, B., Meskendahl, T., Robe, J., Schnurr, M., et al. (2003b). Interferon-alpha disables dendritic cell precursors: dendritic cells derived from interferon-alpha-treated monocytes are defective in maturation and T-cell stimulation. *Immunology* 110, 38–47. doi: 10.1046/j.1365-2567.2003.01702.x

Dauer, M., Schnurr, M., and Eigler, A. (2008). Dendritic cell-based cancer vaccination: quo vadis? *Expert Rev. Vaccines* 7, 1041–1053. doi: 10.1586/14760584.7.7.1041

Ezzelarab, M., and Thomson, A. W. (2011). Tolerogenic dendritic cells and their role in transplantation. *Semin. Immunol.* 23, 252–263. doi: 10.1016/j.smim.2011.06.007

Gilboa, E. (2007). DC-based cancer vaccines. *J. Clin. Invest.* 117, 1195–1203. doi: 10.1172/JCI31205

Jarnjak-Jankovic, S., Hammerstad, H., Saeboe-Larssen, S., Kvalheim, G., and Gaudernack, G. (2007). A full scale comparative study of methods for generation of functional dendritic cells for use as cancer vaccines. *BMC Cancer* 7:119. doi: 10.1186/1471-2407-7-119

Jonuleit, H., Giesecke-Tuettenberg, A., Tuting, T., Thurner-Schuler, B., Stuge, T. B., Paragnik, L., et al. (2001). A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int. J. Cancer* 93, 243–251. doi: 10.1002/ijc.1323

Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., et al. (1997). Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27, 3135–3142. doi: 10.1002/eji.1830271209

Kalantari, T., Kamali-Sarvestani, E., Ciric, B., Karimi, M. H., Kalantari, M., Faridar, A., et al. (2011). Generation of immunogenic and tolerogenic clinical-grade dendritic cells. *Immunol. Res.* 51, 153–160. doi: 10.1007/s12026-011-8255-5

Kalim, K. W., and Groettrup, M. (2013). Prostaglandin E2 inhibits IL-23 and IL-12 production by human monocytes through down-regulation of their common p40 subunit. *Mol. Immunol.* 53, 274–282. doi: 10.1016/j.molimm.2012.08.014

Kalinski, P., Vieira, P. L., Schuitemaker, J. H., De Jong, E. C., and Kapsenberg, M. L. (2001). Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* 97, 3466–3469. doi: 10.1182/blood.V97.11.3466

Knight, S. C., Macatonia, S. E., and Patterson, S. (1990). HIV I infection of dendritic cells. *Int. Rev. Immunol.* 6, 163–175. doi: 10.3109/08830189009056627

Kodama, A., Tanaka, R., Zhang, L. F., Adachi, T., Saito, M., Ansari, A. A., et al. (2010). Impairment of in vitro generation of monocyte-derived human dendritic cells by inactivated human immunodeficiency virus-1: involvement of type I interferon produced from plasmacytoid dendritc cells. *Hum. Immunol.* 71, 541–550. doi: 10.1016/j.humimm.2010.02.020

Moll, H., and Berberich, C. (2001). Dendritic cells as vectors for vaccination against infectious diseases. *Int. J. Med. Microbiol.* 291, 323–329. doi: 10.1078/1438-4221-00138

Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. (1997). Expression and function of OX40 ligand on human dendritic cells. *J. Immunol.* 159, 3838–3848.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down-regulated by tumor necrosis factor alpha. *J. Exp. Med.* 179, 1109–1118. doi: 10.1084/jem.179.4.1109

Steinman, R. M., and Banchereau, J. (2007). Taking dendritic cells into

medicine. *Nature* 449, 419–426. doi: 10.1038/nature06175

Tanaka, R., Takahashi, Y., Kodama, A., Saito, M., Ansari, A. A., and Tanaka, Y. (2010). Suppression of CCR5-tropic HIV type 1 infection by OX40 stimulation via enhanced production of beta-chemokines. *AIDS Res. Hum. Retroviruses* 26, 1147–1154. doi: 10.1089/aid.2010.0043

Tawab, A., Fan, Y., Read, E. J., and Kurlander, R. J. (2009). Effect of ex vivo culture duration on phenotype and cytokine production by mature dendritic cells derived from peripheral blood monocytes. *Transfusion* 49, 536–547. doi: 10.1111/j.1537-2995.2008.02020.x

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., et al. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304, 104–107. doi: 10.1126/science.1093933

Yoshida, A., Tanaka, R., Murakami, T., Takahashi, Y., Koyanagi, Y., Nakamura, M., et al. (2003). Induction of protective immune responses against R5 human immunodeficiency virus type 1 (HIV-1) infection in hu-PBL-SCID mice by intrasplenic immunization with HIV-1-pulsed dendritic cells: possible involvement of a novel factor of human CD4(+) T-cell origin. *J. Virol.* 77, 8719–8728. doi: 10.1128/JVI.77.16.8719-8728.2003

Zhang, L. F., Okuma, K., Tanaka, R., Kodama, A., Kondo, K., Ansari, A. A., et al. (2008). Generation of mature dendritic cells with unique phenotype and function by in vitro short-term culture of human monocytes in the presence of interleukin-4 and interferon-beta. *Exp. Biol. Med. (Maywood)* 233, 721–731. doi: 10.3181/0712-RM-333

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 June 2013; accepted: 10 September 2013; published online: 27 September 2013.

Citation: Kodama A, Tanaka R, Saito M, Ansari AA and Tanaka Y (2013) A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive CD4$^+$ T cells in the hu-PBL SCID mice. Front. Microbiol. ***4****:292. doi: 10.3389/fmicb.2013.00292*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

OPINION ARTICLE
published: 29 July 2013
doi: 10.3389/fmicb.2013.00218

Growth potentials of CCR5-tropic/CXCR4-tropic HIV-1mt clones in macaque cells

Naoya Doi, Ayaka Okubo, Mizumo Yamane, Yosuke Sakai, Akio Adachi* and Masako Nomaguchi*

Department of Microbiology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan
**Correspondence: adachi@basic.med.tokushima-u.ac.jp; nomaguchi@basic.med.tokushima-u.ac.jp*

Edited by:
Tomoyuki Miura, Kyoto University, Japan

Human immunodeficiency virus type 1 (HIV-1) is strictly adapted to humans, and cause AIDS only in humans. Consequently, no experimental animals susceptible to HIV-1 and suitable for the AIDS model study are available to date (Nomaguchi et al., 2008, 2011). To overcome this issue, viruses genetically related to HIV-1 have been challenged into macaque monkeys to mimic the natural HIV-1 infection. Viruses used for these experiments are simian immunodeficiency viruses (SIVs), SIVs chimeric with parts of HIV-1 sequences (SHIVs), and macaque tropic HIV-1 derivatives carrying a small portion of SIV genome (HIV-1mt clones). Because viruses of the SHIV and HIV-1mt groups carry HIV-1 genes/sequences, their scientific/medical significance and impact are evident. Although some SHIVs indeed induce AIDS in macaques, accumulating evidences have demonstrated that the genuine CCR5-tropism of input viruses is prerequisite for superimposing the experimental outcome on the natural disease progression in humans (Feinberg and Moore, 2002; Margolis and Shattock, 2006). Therefore, a number of CCR5-tropic SHIVs currently have been generated and utilized for *in vivo* macaque experiments (Hsu et al., 2003; Humbert et al., 2008; Nishimura et al., 2010; Fujita et al., 2012).

Recently, prototype HIV-1mt clones, CXCR4-tropic NL-DT5R, and dual-tropic (CXCR4- and CCR5-tropic) stHIV-1, have been generated by us (Kamada et al., 2006) and others (Hatziioannou et al., 2006), respectively. We selected three distinct Env sequences and made three proviral constructs in the backbone of the NL-DT5R genome to obtain CCR5-tropic/dual-tropic viruses (**Figure 1A**), based on the published results (Hsu et al., 2003; Hatziioannou et al., 2006; Matsuda et al., 2010; Nishimura et al., 2010). Of the three clones constructed, while NL-DT562 grew in a cynomolgus macaeque cell line HSC-F (Akari et al., 1996; Fujita et al., 2003), the other two viruses designated NL-DT589 and NL-DT5AD did not (Doi et al., 2010; our unpublished observations). The replication efficiency in HSC-F cells of NL-DT562 was much lower than that of the parental virus NL-DT5R (Doi et al., 2010). When examined in $CD8^+$ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PBMCs), NL-DT5AD was found to be replication-competent in addition to NL-DT562 (Igarashi and Adachi, unpublished results). However, NL-DT5AD grew more poorly than NL-DT562, and NL-DT562 itself propagated much more inefficiently again than NL-DT5R in these PBMCs. Of note, NL-DT562 was confirmed to use CCR5 for cell entry (our unpublished data). To improve the replication ability of NL-DT562, we extensively modified its genome by adaptation to macaque cells and also by *in vitro* mutagenesis (Nomaguchi et al., 2008, 2011, 2013a,b; Nomaguchi et al., submitted). As a result, the same mutations were introduced into the corresponding genomic regions of NL-DT5R and NL-DT562 encoding Gag-capsid, Pol-integrase, and Vpu-transmembrane domain. Numerous growth-enhancing adaptive mutations were found to separately occur in the Env of NL-DT562, but only one in the Env of NL-DT5R (Nomaguchi et al., 2013b). Since the enhancement of virus growth by these mutations is strictly Env sequence-dependent (Nomaguchi et al., 2013b), only a single best mutation for viral replication was introduced into the *env* gene of each clone. As shown in **Figure 1B**, the final version of CCR5-tropic virus currently constructed (MN5/LSDQgtu in **Figure 1A**) surely grew extremely better than NL-DT562 in a rhesus macaque cell line M1.3S (Doi et al., 2011), but more poorly relative to MN4/LSDQgtu (Nomaguchi et al., submitted) (**Figure 1A**), a CXCR4-tropic virus derived from NL-DT5R (a virus corresponding to MN5/LSDQgtu). Taken all together, we are unable yet to have a CCR5-tropic HIV-1mt clone that grows better or equally well in macaque cells relative to CXCR4-tropic MN4/LSDQgtu. Virological and molecular basis for this negative result is presently unknown, but it is certain that the Env sequence is important for viral growth potentials. Extensive search for appropriate Env sequences to confer CCR5-tropism and high replication-ability on HIV-1mt clones is required for our final purpose, i.e., the generation of proviral clones virologically similar to viruses of the SIVmac group that are pathogenic for macaques. In this regard, it is tempting to use "intracellular homologous recombination" as a measure to readily generate recombinant HIV-1 clones (Fujita et al., 2012).

Despite the every effort of researchers, so far, no appreciable disease was induced in pig-tailed and cynomolgus macaques infected with various HIV-1mt clones (Igarashi et al., 2007; Hatziioannou et al., 2009; Saito et al., 2011, 2013; Thippeshappa et al., 2011). Although the rhesus macaque is thought to be the best macaque species for infection experiments of this kind from various virological and primatological points of view, no attempts to infect it with HIV-1mt clones have been made to date, probably due to its highly resistant nature to the viruses. Common characteristics of the non-morbifical infections as described above are low viral loads relative to those in pathogenetic infections with SIV/SHIV/HIV-1 and no apparent viral set points in the course of infection. Without initial burst of viruses in hosts to guarantee viral amount and diversity enough for persistent infection, viruses may not survive in individuals/populations. Further improvement

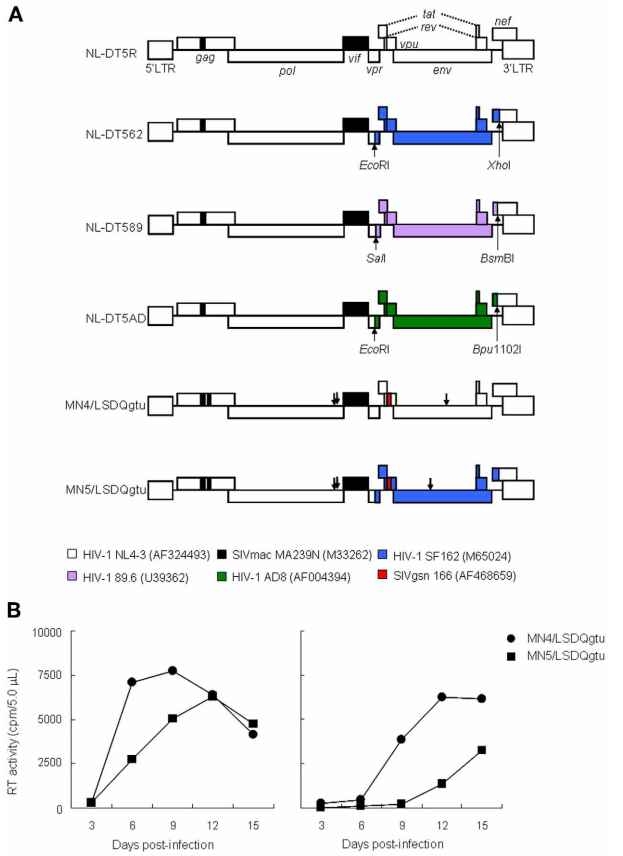


FIGURE 1 | Characteristics of HIV-1mt clones. (A) Proviral genome structure of various HIV-1mt clones. Genomes of various HIV-1mt clones generated in our laboratory are schematically illustrated. White and black (a small portion of *gag*-capsid and an entire *vif*) areas stand for the genomic regions of HIV-1 NL4-3 (Adachi et al., 1986) and SIVmac MA239N (Shibata et al., 1991; Doi et al., 2010), respectively. The other colored regions are the sequences derived from various SIV/HIV-1s as shown. Restriction enzyme sites used for the insertion of these sequences into NL-DT5R are also shown. GenBank accession numbers are indicated in parentheses. Arrows represent the adaptive mutation sites in *pol*-integrase and *env*-gp120 that enhance virus growth potentials (Nomaguchi et al., 2013b). While NL-DT5R and MN4/LSDQgtu are CXCR4-tropic viruses, NL-DT562, NL-DT5AD, and MN5/LSDQgtu are CCR5-tropic. Although examined in macaque cells, the growth ability of NL-DT589 is not yet proven (see text). mac, rhesus macaques; gsn, greater spot-nosed monkeys. **(B)** Viral replication kinetics in M1.3S cells. Cell-free viruses were prepared from 293T cells transfected with proviral clones indicated, and equal amounts [5×10^6 and 5×10^5 reverse transcriptase (RT) units for left and right panels, respectively] were inoculated into M1.3S cells (2×10^6 cells). Viral replication was monitored at intervals by RT activity in the culture supernatants. The experiments were done as described previously (Kamada et al., 2006). M1.3S is the most refractory cell line to infection with SIVmac/HIV-1mt clones to the best of our knowledge, but is CD4-, CXCR4-, and CCR5-positive. NL-DT5R and NL-DT562 do not grow at all in M1.3S cells.

of the replication ability of CCR5-tropic HIV-1mt clones would be necessary to establish HIV-1mt/macaque model systems, the rhesus system in particular, for natural infections of HIV-1, and finally for human AIDS research.

ACKNOWLEDGMENTS

We thank Ms. Kazuko Yoshida of our department for editorial assistance. We are indebted to Professor Tatsuhiko Igarashi of Kyoto University for his contribution to our study described here.

REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., et al. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* 59, 284–291.

Akari, H., Mori, K., Terao, K., Otani, I., Fukasawa, M., Mukai, R., et al. (1996). *In vitro* immortalization of Old World monkey T lymphocytes with *Herpesvirus saimiri*: its susceptibility to infection with simian immunodeficiency viruses. *Virology* 218, 382–388. doi: 10.1006/viro.1996.0207

Doi, N., Fujiwara, S., Adachi, A., and Nomaguchi, M. (2010). Growth ability in various macaque cell lines of HIV-1 with simian cell-tropism. *J. Med. Invest*. 57, 284–292. doi: 10.2152/jmi.57.284

Doi, N., Fujiwara, S., Adachi, A., and Nomaguchi, M. (2011). Rhesus M1.3S cells suitable for biological evaluation of macaque-tropic HIV/SIV clones. *Front. Microbiol.* 2:115. doi: 10.3389/fmicb.2011.00115

Feinberg, M. B., and Moore, J. P. (2002). AIDS vaccine models: challenging challenge viruses. *Nat. Med.* 3, 207–210. doi: 10.1038/nm0302-207

Fujita, M., Yoshida, A., Sakurai, A., Tatsuki, J., Ueno, F., Akari, H., et al. (2003). Susceptibility of HVS-immortalized lymphocytic HSC-F cells to various strains and mutants of HIV/SIV. *Int. J. Mol. Med.* 11, 641–644.

Fujita, Y., Otsuki, H., Watanabe, Y., Yasui, M., Kobayashi, T., Miura, T., et al. (2012). Generation of a replication-competent chimeric simian-human immunodeficiency virus carrying *env* from subtype C clinical isolate through intracellular homologous recombination. *Virology* 436, 100–111. doi: 10.1016/j.virol.2012.10.036

Hatziioannou, T., Ambrose, Z., Chung, N. P. Y., Piatak, M. Jr., Yuan, F., Trubey, C. M., et al. (2009). A macaque model of HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4425–4429. doi: 10.1073/pnas.0812587106

Hatziioannou, T., Princiotta, M., Piatak, M. Jr., Yuan, F., Zhang, F., Lifson, J. D., et al. (2006). Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* 314:95. doi: 10.1126/science.1130994

Hsu, M., Harouse, J. M., Gettie, A., Buckner, C., Blanchard, J., and Cheng-Mayer, C. (2003). Increased mucosal transmission but not enhanced pathogenicity of the CCR5-tropic, simian AIDS-inducing simian/human immunodeficiency virus SHIV (SF162P3) maps to envelope gp120. *J. Virol.* 77, 989–998. doi: 10.1128/JVI.77.2.989-998.2003

Humbert, M., Rasmussen, R. A., Song, R., Ong, H., Sharma, P., Chenine, A. L., et al. (2008). SHIV-1157i and passaged progeny viruses encoding R5 HIV-1 clade C *env* cause AIDS in rhesus monkeys. *Retrovirology* 5:94. doi: 10.1186/1742-4690-5-94

Igarashi, T., Iyengar, R., Byrum, R. A., Buckler-White, A., Dewar, R. L., Buckler, C. E., et al. (2007). Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques. *J. Virol.* 81, 11549–11552. doi: 10.1128/JVI.00960-07

Kamada, K., Igarashi, T., Martin, M. A., Khamsri, B., Hatcho, K., Yamashita, T., et al. (2006). Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16959–16964. doi: 10.1073/pnas.0608289103

Margolis, L., and Shattock, R. (2006). Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? *Nat. Rev. Microbiol.* 4, 312–317.

Matsuda, K., Inaba, K., Fukazawa, Y., Matsuyama, M., Ibuki, K., Horiike, M., et al. (2010). *In vivo* analysis of a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a

derivative of SHIV-89.6. *Virology* 399, 134–143. doi: 10.1016/j.virol.2010.01.008

Nishimura, Y., Shingai, M., Willey, R., Sadjadpour, R., Lee, W. R., Brown, C. R., et al. (2010). Generation of the pathogenic R5-tropic simian/human immunodeficiency virus $SHIV_{AD8}$ by serial passaging in rhesus macaques. *J. Virol.* 84, 4769–4781. doi: 10.1128/JVI.02279-09

Nomaguchi, M., Doi, N., Fujiwara, S., and Adachi, A. (2011). "Macaque-tropic HIV-1 derivatives: a novel experimental approach to understand viral replication and evolution *in vivo*," in *HIV-Host Interactions*, ed T. L.-Y. Chang (Rijeka: InTech), 325–348.

Nomaguchi, M., Doi, N., Kamada, K., and Adachi, A. (2008). Species barrier of HIV-1 and its jumping by virus engineering. *Rev. Med. Virol.* 18, 261–275. doi: 10.1002/rmv.576

Nomaguchi, M., Yokoyama, M., Kono, K., Nakayama, E. E., Shioda, T., Saito, A., et al. (2013a). Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells. *Microbes Infect.* 15, 56–65.

Nomaguchi, M., Doi, N., Fujiwara, S., Saito, A., Akari, H., Nakayama, E. E., et al. (2013b). Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells. *Microbes Infect.* 15, 319–328.

Saito, A., Nomaguchi, M., Iijima, S., Kuroishi, A., Yoshida, T., Lee, Y. J., et al. (2011). Improved capacity of a monkey-tropic HIV-1 derivative to replicatein cynomolgus monkeys with minimal modifications. *Microbes Infect.* 13, 58–64. doi: 10.1016/j.micinf.2010.10.001

Saito, A., Nomaguchi, M., Kono, K., Iwatani, Y., Yokoyama, M., Yasutomi, Y., et al. (2013). *TRIM5* genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceptibility to monkey-tropic human immunodeficiency virus type 1. *J. Gen. Virol.* 94, 1318–1324. doi: 10.1099/vir.0.050252-0

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., and Adachi, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* 65, 3514–3520.

Thippeshappa, R., Polacino, P., Kimata, M. T. Y., Siwak, E. B., Anderson, D., Wang, W., et al. (2011). Vif substitution enables persistent infection of pig-tailed macaques by human immunodeficiency virus type 1. *J. Virol.* 85, 3767–3779. doi: 10.1128/JVI.02438-10

Received: 09 July 2013; accepted: 12 July 2013; published online: 29 July 2013.

Citation: Doi N, Okubo A, Yamane M, Sakai Y, Adachi A and Nomaguchi M (2013) Growth potentials of CCR5-tropic/CXCR4-tropic HIV-1mt clones in macaque cells. Front. Microbiol. ***4****:218. doi: 10.3389/fmicb.2013.00218*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

METHODS ARTICLE
published: 11 October 2013
doi: 10.3389/fmicb.2013.00298

Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR

Shota Ikeno[1,2†], Moto-omi Suzuki[1,3†], Mahmod Muhsen[1], Masayuki Ishige[1], Mie Kobayashi-Ishihara[1], Shinji Ohno[4], Makoto Takeda[5], Tetsuo Nakayama[6], Yuko Morikawa[2], Kazutaka Terahara[1], Seiji Okada[7], Haruko Takeyama[3,4] and Yasuko Tsunetsugu-Yokota[1]*

[1] Department of Immunology, National Institute of Infectious Diseases, Tokyo, Japan
[2] Cooperative Major in Advanced Health Science, Tokyo University of Agriculture and Technology/Waseda University Graduate School of Collaborative Education Curriculum, Tokyo, Japan
[3] Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan
[4] Department of Virology, Faculty of Medicine, Kyushu University, Fukuoka, Japan
[5] Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan
[6] Kitasato Institute for Life Science, Kitasato University, Tokyo, Japan
[7] Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Takashi Irie, Graduate School of Biomedical Sciences, Hiroshima University, Japan
Masato Tsurudome, Mie University Graduate School of Medicine, Japan
Masae Itoh, Nagahama Institute of Bio-Science and Technology, Japan

***Correspondence:**
Yasuko Tsunetsugu-Yokota, Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan
e-mail: yyokota@nih.go.jp

† *Shota Ikeno and Moto-omi Suzuki have contributed equally to this work.*

Live attenuated measles virus (MV) has long been recognized as a safe and effective vaccine, and it has served as the basis for development of various MV-based vaccines. However, because MV is a human-tropic virus, the evaluation of MV-based vaccines has been hampered by the lack of a small-animal model. The humanized mouse, a recently developed system in which an immunodeficient mouse is transplanted with human fetal tissues or hematopoietic stem cells, may represent a suitable model. Here, we developed a sensitive one-step quantitative reverse transcription (qRT)-PCR that simultaneously measures nucleocapsid (N) and human RNase P mRNA levels. The results can be used to monitor MV infection in a humanized mouse model. Using this method, we elucidated the replication kinetics of MV expressing enhanced green fluorescent protein both *in vitro* and in humanized mice in parallel with flow-cytometric analysis. Because our qRT-PCR system was sensitive enough to detect MV expression using RNA extracted from a small number of cells, it can be used to monitor MV infection in humanized mice by sequential blood sampling.

Keywords: measles virus infection, humanized mouse, quantitative RT-PCR, EGFP expression, flow cytometry

INTRODUCTION

Measles, a highly contagious childhood disease caused by the measles virus (MV), affects more than 20 million people each year. MV infection is characterized by a high fever with typical Koplik's spots followed by the appearance of a generalized maculopapular rash, and is often associated with respiratory and neuronal complications (Griffin, 2007). Since the implementation of vaccination programs using an effective live attenuated MV vaccine, global measles deaths have decreased dramatically. Nevertheless, measles is still one of the leading causes of death among young children under the age of 5 years, especially in countries with weak health infrastructures, and approximately 158,000 measles death occurred in 2011 (http://www.who.int/mediacentre/factsheets/fs286/en/). The ongoing global vaccination strategy aims to protect small children at high risk.

The MV vaccine is safe, effective, and inexpensive. Based on its long and successful vaccination history, several groups have taken advantage of reverse-genetics technology to utilize the live attenuated MV vaccine strain as a viral vector to elicit immune responses against foreign antigens from various pathogens, such as Env or Gag of human immunodeficiency virus (HIV; Lorin et al., 2004; Stebbings et al., 2012), hepatitis B surface (S) antigen (Singh et al., 1999; Reyes-del Valle et al., 2009), fusion protein of respiratory syncytial virus (Sawada et al., 2011), and envelope glycoprotein of West Nile virus (Despres et al., 2005; Brandler et al., 2012). MV is a human-tropic virus that uses CD46, signaling of lymphocyte activation molecule (SLAM, CD150), and the recently identified epithelial-cell receptor nectin-4 (PVRL4, see review in Kato et al., 2012) as receptors. To test the immune response against MV-based recombinant vaccines, both MV receptor-transgenic mice (Singh et al., 1999; Lorin et al., 2004; Despres et al., 2005) and non-human primates have been used as animal models (Reyes-del Valle et al., 2009; Brandler et al., 2012; Stebbings et al., 2012).

Although non-human primates are susceptible to MV, and they develop pathologies similar to those that occur in humans, the expense of using monkeys in research limits the number of animals that can be used for studies. To overcome such practical problems, various types of human MV receptor-transgenic mice expressing CD46 or CD150 have been developed (review in

Sellin and Horvat, 2009). Unfortunately, MV infection of all of these human MV receptor-expressing mouse models is severely restricted by the presence of murine type I IFN; to establish MV infection, it is necessary to introduce the IFNα receptor knockout into the MV receptor-transgenic mice, even in strains expressing CD150 driven by a native human promoter (Ohno et al., 2007). The IFNα receptor knockout/CD150 knock-in mouse is highly susceptible to MV infection and reproduces some aspects of MV infection in humans, including immunosuppression (Koga et al., 2010). This makes it a useful mouse model for study purposes. However, one problem is the lack of an initial innate immune response, which may modify the outcome of MV infection. Thus, the model may not truly reflect the outcome in humans.

In the early 2000s, a series of immunodeficient mice were developed that allow efficient transplantation of human cells or tissues; these systems are collectively termed "humanized mice." A large number of studies have described the development of human hematopoietic cells and their immunological functions in humanized mice, and technical modifications have been made for the study of various human diseases (Ito et al., 2012). Currently, humanized mouse systems are widely used as alternatives to non-human primate models, especially for the study of human-tropic infectious diseases such as HIV, human T cell leukemia virus (HTLV), dengue virus, HCV, and EB virus (Akkina, 2013). Of the different humanized mice models, the BM/Liver/Thymus transplanted (BLT) mouse, which is transplanted with human fetal liver and thymus tissue in addition to hematopoietic stem cells (HSCs), is recognized as the model that most closely mimics the human immune response (Wege et al., 2008). However, the use of this model is limited, mainly because of the ethical issues surrounding human fetal organs/tissues.

We have recently established an HIV infection model in NOD/SCID/Jak3null (NOJ) mouse transplanted with human cord blood HSCs (Terahara et al., 2013). To study MV infection in humanized NOJ (hNOJ), we infected an MV vaccine strain (AIK-C) expressing enhanced green fluorescent protein (EGFP) into hNOJ and analyzed the MV-infected cells by flow cytometry. The hNOJ mouse is highly susceptible to MV infection; in that study, we observed that GFP^+ cells were present in systemic lymphoid tissues and bone marrow (BM). Because it is important to assess MV infection kinetics in an animal without sacrificing the infected mouse, we developed a highly sensitive one-step quantitative reverse transcription-PCR (qRT-PCR) system to monitor MV infection in human peripheral blood mononuclear cells (PBMCs) circulating in the blood of humanized mice. In this study, we describe how this monitoring system works and demonstrate that the results obtained reflect the actual frequency of MV-infected cells, as determined by flow cytometry.

MATERIALS AND METHODS

CELL FRACTIONATION OF PBMCs

Peripheral blood mononuclear cells were obtained from human blood samples of healthy volunteers. Samples were collected after obtaining the approval of the institutional ethical committee of the National Institute of Infectious Diseases (NIID; No. 350) and written informed consent from each subject. PBMCs were separated by Ficoll–Hypaque density-gradient centrifugation (Lymphosepal; IBL, Gunma, Japan).

To obtain monocyte-derived dendritic cells (MDDCs), monocytes were enriched from PBMCs using CD14 microbeads (Miltenyi Biotec) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; both 10 ng/ml, from PeproTech Inc., London, UK) for 1 week. T cells were isolated from CD14-negative PBMCs using the Total T Cell Enrichment Kit (STEMCELL technologies, Vancouver, BC, Canada).

PREPARATION OF RNA

Total RNA was extracted from mouse blood, BM, and spleen of humanized mice, human PBMCs, and Jurkat cells expressing human SLAM (Jurkat/hSLAM) using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) or the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA).

To prepare a standard of MV RNA, the cDNA encoding measles virus nucleocapsid (N) (MV-N: AB052821) was subcloned into the pBluescript II vector, and then MV-N RNA was produced by *in vitro* RNA transcription using the T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, followed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer. The final concentration of RNA was measured using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA).

PREPARATION OF STANDARD TEMPLATE DNA

To prepare a standard template DNA, cDNAs of human CD45 (hCD45: NG_007730) and RNase P (NM_006413) were synthesized from total RNA of CEM cells by reverse transcription (RT)-PCR using SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA, USA). The products were further amplified by PCR using TaKaRa Ex Taq Hot Start Version (TAKARA, Otsu, Shiga, Japan) for hCD45, or AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, CA, USA) for RNase P. These PCR products of hCD45 and RNase P were subcloned into plasmids using the pGeneBLAzer TOPO TA Expression kit (Invitrogen) and pGEM-T (Easy) Vector Systems (Promega), respectively.

REAL-TIME RT-PCR ASSAY

To perform real-time qRT-PCR, SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used according to the manufacturer's instructions. Briefly, each reaction contained 1× reaction mix, ROX reference dye, SuperScript III RT/Platinum TaqMix, 0.2 μM specific primers, and 0.1 μM TaqMan probe. Reactions were performed on an Mx3000P qPCR system (Agilent Technologies). Thermocycling parameters included a RT step at 50°C for 20 min, followed by a DNA polymerase activation step at 95°C for 2 min and 50 PCR cycles (95°C for 20 s, 60°C for 30 s). Threshold cycle (C_t) values were calculated for each reaction; C_t represents the cycle at which a statistically significant increase in the emission intensity of the reporter relative to the passive reference dye is first detected.

For detection of hCD45 mRNA, the following sequences were used: forward primer, 5′-GGA AGT GCT GCA ATG TGT CAT T-3′; reverse primer; 5′-CTT GAC ATG CAT ACT ATT ATC TGA TGT CA-3′; TaqMan probe; 5′-FAM-ACA ACT AAA AGT GCT CCT CCA AGC CAG GTC T-BHQ1-3′ (Hamaia et al., 2001). For detection of RNase P mRNA: forward primer, 5′-AGA TTT GGA CCT GCG AGC G-3′; reverse primer, 5′-GAG CGG CTG TCT CCA CAA GT-3′; TaqMan probe, 5′-FAM-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1-3′ (Kimberly et al., 2005). For detection of MV-N RNA: forward primer, 5′-CGA TGA CCC TGA CGT TAG CA-3′; reverse primer, 5′-GCG AAG GTA AGG CCA GAT TG-3′; TaqMan probe, 5′-FAM-AGG CTG TTA GAG GTT GTC CAG AGT GAC CAG-BHQ1-3′ (Hummel et al., 2006).

GENERATION OF HUMANIZED MICE

Humanized NOD/SCID/JAK3null mice were established as described previously (Terahara et al., 2013). In brief, NOJ mice were transplanted with human HSCs ($0.5–1 \times 10^5$ cells) enriched from human umbilical cord blood cells into the livers of irradiated (1 Gy) newborn mice within 2 days after birth. All mice were maintained under specific pathogen-free conditions in the animal facility at NIID and were treated in accordance with the guidelines issued by the Institutional Animal Care and Committee of NIID.

Human umbilical cord blood was donated by the Tokyo Cord Blood Bank (Tokyo, Japan) after obtaining informed consent. The use of human umbilical cord blood cells was approved by the Institutional Ethical Committees of NIID and the Tokyo Cord Blood Bank. Human HSCs were isolated using the CD133 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was approximately 90% as assessed by flow cytometry.

PREPARATION AND INFECTION OF MV

Recombinant wild-type MV (IC323: AB016162) expressing EGFP (IC323-EGFP; Hashimoto et al., 2002) and a recombinant vaccine strain of MV (AIK-C: S58435) expressing EGFP (AIK-C-EGFP; Fujino et al., 2007) were grown in Vero/hSLAM cells. Virus titers were determined by plaque assay using Vero/hSLAM cells.

Jurkat/hSLAM cells were infected with various doses of MV [multiplicity of infection (MOI) = 0.25, 0.05, and 0.01] by incubation at 37°C for 1 h, washed twice with phosphate buffered saline (PBS), and seeded on 24-well plates. Cells were harvested immediately after washing (time 0) or 6, 12, 18, or 24 h later. The harvested cells were either lysed for RNA extraction or analyzed by flow cytometry.

Humanized NOD/SCID/JAK3null mice were challenged intravenously (i.v.) with different doses [200, 2,000, 10,000, or 20,000 plaque-forming units (pfu)] of AIK-C-EGFP. Peripheral blood was obtained from MV-infected hNOJ mice at 3, 5, 7, 10, 14, and 21 days post-infection (p.i.). In some experiments, MV-infected hNOJ mice were sacrificed at day 7 p.i. At the time of sacrifice, peripheral blood, BM, spleen, and mesenteric lymph nodes (MLNs) were harvested, and red blood cells were lysed in ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA-2Na; pH 7.2–7.4).

FLOW-CYTOMETRIC ANALYSIS OF MV-INFECTED CELLS

PE-conjugated anti-human CD150 (A12) and Pacific Blue-conjugated anti-hCD45 (HI30) monoclonal antibodies (mAbs) were purchased from BioLegend Inc. (San Diego, CA, USA). Cells were stained with these mAbs, fixed with 2% formalin/PBS for 15 min at room temperature, washed, and kept at 4°C prior to flow-cytometric analysis. Dead cells were stained with a LIVE/DEAD Fixable Dead Cell Stain Kit (L34957; Invitrogen). Data were collected using a FACScanto (BD Biosciences, San Jose, CA, USA) and analyzed using the FACSDiva (BD Biosciences) or FlowJo (Tree Star, San Carlos, CA, USA) software.

STATISTICAL ANALYSIS

Non-parametric one-way ANOVA was performed to compare cell type-specific differences in hCD45 and RNase P mRNA expression. Spearman's rank correlation coefficient test was also performed to compare the level of MV-N expression and frequency of MV-infected cells. Prism ver.5 software (GraphPad Software, San Diego, CA, USA) was used for all analyses. $P < 0.05$ was considered statistically significant.

RESULTS

HUMAN-SPECIFIC qRT-PCR SYSTEM FOR THE DETECTION OF MV INFECTION

For the detection of MV infection in clinical specimens, Hummel et al. (2006) established a sensitive qRT-PCR system that used primer and probe sets targeting the MV-N gene. In our humanized mouse model, it is necessary to analyze endogenous mRNA expression in human PBMCs to determine the level of human cell-associated MV infection in mouse blood. We initially assumed that hCD45 expression would be suitable to discriminate human hematopoietic cells from co-existing mouse hematopoietic cells *in vivo*. On that basis, we designed human-specific primer and TaqMan probe sets for hCD45 and compared their usefulness with a primer/probe set for a widely used housekeeping gene, RNase P. RNA was extracted from humanized (hu-mouse) or non-humanized (non-hu-mouse) murine splenocytes, and the level of mRNA was measured by one-step qRT-PCR. Both hCD45 and RNase P primer/probe sets detected mRNA expression of target genes from human PBMCs present in hu-mouse spleen, at similar sensitivities, but neither set detected expression in non-hu-mouse (**Figure 1A**). Thus, both primer/probe sets are human-specific. Next, we enriched $CD14^+$ monocytes and T cells from PBMCs by positive and negative magnetic-bead selection, respectively, and then determined the copy numbers of hCD45 and RNase P in these cell fractions from each of five donors. In **Figure 1B**, the expression levels of hCD45 (left panel) and RNase P (right panel) in monocytes and T cells are depicted relative to the level in each donor's PBMCs. Because RNase P expression was less affected by cell type than CD45 expression ($^*P < 0.05$), in subsequent experiments we exclusively used RNase P primer/probe sets as an endogenous control for mRNA expression.

PARALLEL INCREASE IN THE TIME COURSE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVEL *IN VITRO*

Because wild-type MV mainly utilizes SLAM as the receptor for entry into lymphoid cells (Tatsuo et al., 2000), the kinetics of MV

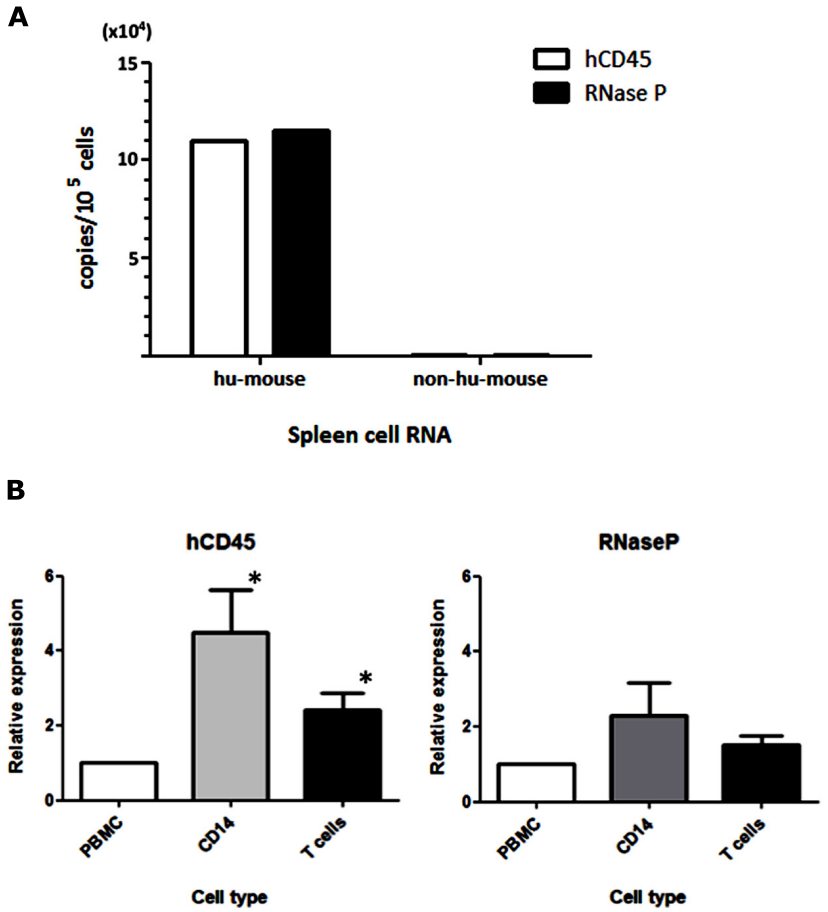


FIGURE 1 | Selection of an endogenous control for the analysis of MV-infected human PBMCs. **(A)** RNA was extracted from spleen cells of hNOJ and non-humanized NOJ, and one-step qRT-PCR was performed using primer and probe sets designed against the human-specific hCD45 and RNaseP mRNAs. To calculate copy numbers of these genes, the PCR products of human CD45 and RNase P were subcloned into plasmids and used as standard DNAs. **(B)** Human PBMCs from five donors were fractionated into $CD14^+$ monocytes and T cells. RNA from these cell populations was extracted, and the expression levels of hCD45 and RNase P were analyzed by qRT-PCR. The graph depicts the expression levels in these fractionated cells relative to the levels in PBMCs (defined as 1). Statistical differences in hCD45 and RNase P expression among these cell populations were evaluated by non-parametric one-way ANOVA test (*$P<0.05$).

infection in Jurkat/hSLAM cells can be clearly visualized by flow cytometry. We infected Jurkat/hSLAM cells with a wild-type MV encoding EGFP (IC323-EGFP) at MOI of 0.01, 0.05, and 0.25. Cells were washed and harvested at 6, 12, 18, or 24 h after MV infection. A subset of the cells in each sample was analyzed by flow cytometry, and the remainder of the sample was used for RNA extraction. The mRNA levels of MV-N and RNase P were determined by qRT-PCR, and the level of MV-N mRNA relative to RNase P RNA was calculated. Representative results of three experiments are shown in **Figure 2A** (flow cytometry) and **Figure 2B** (qRT-PCR). Because of the rapid and strong cytopathic effect by MV at the highest MOI (0.25), we omitted the flow cytometry data corresponding to that condition. At MOI 0.01, a similar frequency of GFP^+ cells was detectable at 12 and 18 h p.i., whereas at MOI 0.05, the GFP^+ cell frequency was already high at 12 h p.i. Note that the level of hSLAM was not down-modulated by MV infection. Over the time course, relative MV-N expression level at all three MOIs increased in parallel over two orders of magnitude, indicating that these two methods yield comparable results (as shown in **Figure 2C**) and are useful for monitoring the replication kinetics of MV infection *in vitro*.

PARALLEL INCREASE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVELS *IN VIVO*

We then applied these detection systems *in vivo* in MV-infected hNOJ mice. hNOJ mice were infected with an MV vaccine strain expressing EGFP (AIK-C-EGFP) at 2000 pfu, and the animals were sacrificed 7 days later. Blood PBMCs and BM cells were washed with PBS, and a subset of the cells in each sample were stained with anti-hCD45 mAb. Representative results of flow-cytometric analysis of BM cells from three mice are shown in **Figure 3A**. The percentages of GFP^+ cells in mice 127-1, 127-4, and 127-5 mice were low (0.002%), high (0.35%), and intermediate (0.028%), respectively. The number of human PBMCs obtained from mouse blood was not sufficient to determine GFP^+

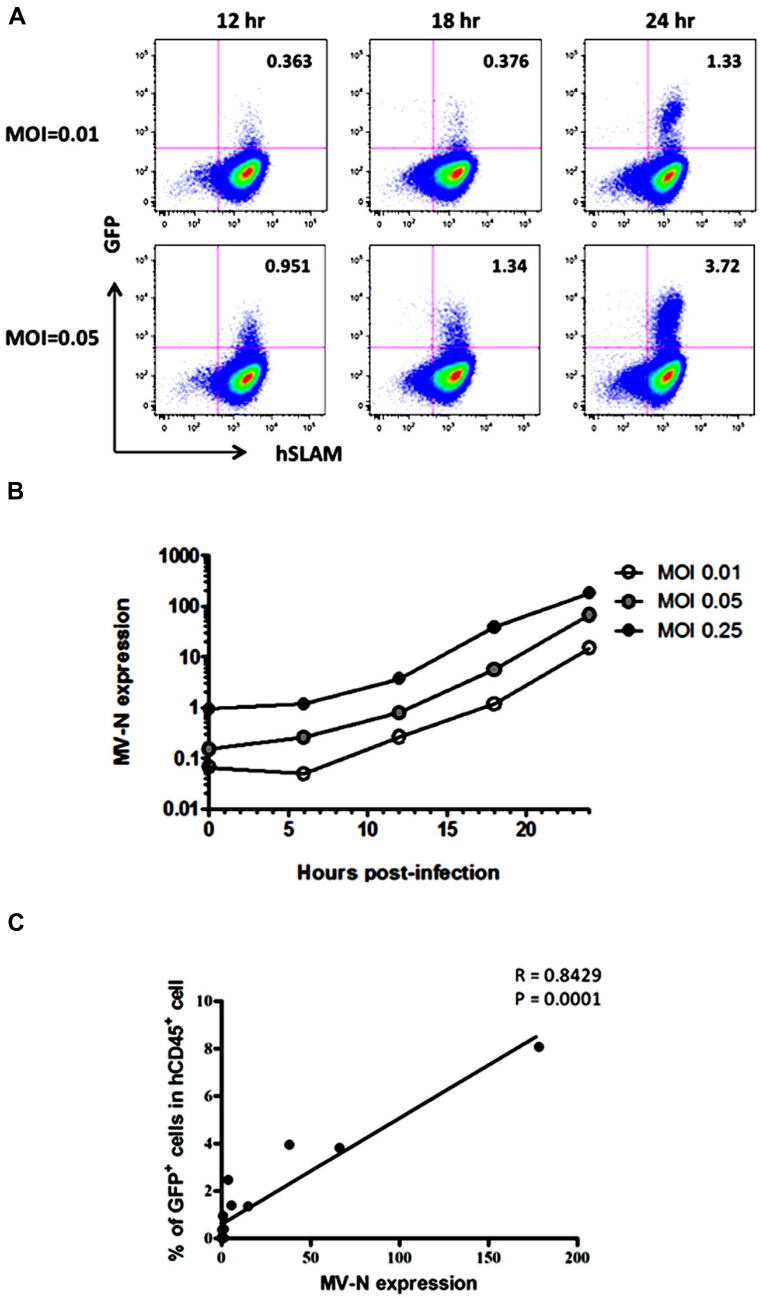


FIGURE 2 | Time course of MV infection *in vitro*. Jurkat/hSLAM cells were infected with wild-type MV IC323-EGFP at MOI of 0.01, 0.05, and 0.25, washed, and harvested at the indicated time points. **(A)** Cells were stained with PE-conjugated anti-hSLAM mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** RNA was extracted from cells, and expression levels of MV-N and RNase P were analyzed by one-step qRT-PCR. The copy numbers of MV-N and RNase P were determined, and the ratio of MV-N copies to RNase P copies is depicted on the vertical axis. **(C)** Correlation between the percentage of GFP^+ Jurkat/SLAM cells and the time course of MV-N expression. Spearman's rank correlation coefficient was used for statistical analysis.

cell frequencies by flow cytometry. Next, we extracted RNA from PBMCs and BM cells and analyzed MV-N expression by qRT-PCR, as described in the previous section. MV-N expression paralleled the GFP^+ frequencies in BM (**Figure 3B**). Notably, a high level of MV-N expression was also detected in PBMCs of mouse 127-4, suggesting that the level of MV-N expression per single hematopoietic cell is similar between blood and BM. We plotted the GFP^+ frequency and MV-N expression level in BM cells of eight mice. As shown in **Figure 3C**, these values were well correlated ($R = 0.9286$). Taken together, these data indicate that MV infection *in vivo* is detectable in BM by both flow cytometry and MV-N RNA qRT-PCR analysis, but only MV-N RNA qRT-PCR is

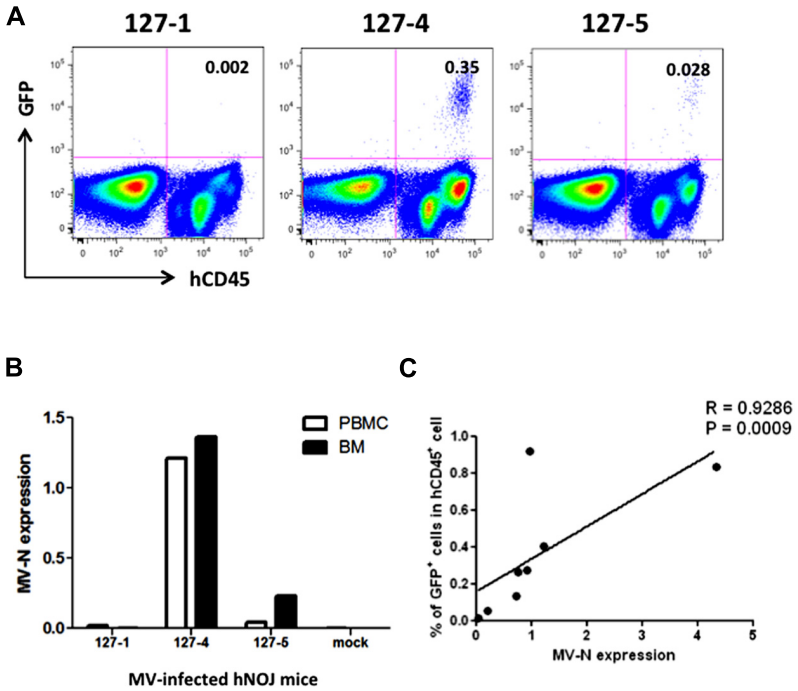


FIGURE 3 | Analysis of MV infection *in vivo*. Three hNOJ mice (127-1, -4, and -5) were infected intravenously with 2,000 pfu of the MV vaccine strain, AIK-C-EGFP. Mice were sacrificed at day 7 post-infection, and blood and bone marrow cells (BM) were obtained. **(A)** BM cells were stained with PB-anti-human CD45 mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. **(B)** PBMCs from blood and BM cells were lysed, and RNA was prepared. The expression MV-N and RNase P was analyzed as described in the legend for **Figure 2B**. **(C)** Correlation between the percentage of GFP^+ cells among $hCD45^+$ cells in BM and the level of MV-N expression in MV-infected hNOJ mice, at day 7 ($n = 4$) or day 10 ($n = 4$) p.i. Spearman's rank correlation coefficient was used for statistical analysis.

sensitive enough to detect PBMC-associated MV infection in the blood.

KINETICS OF MV GROWTH CAN BE MONITORED IN THE BLOOD OF hNOJ MOUSE

Finally, we measured MV growth kinetics *in vivo* by qRT-PCR analysis using sequential blood samples obtained from MV-infected hNOJ mice; it was not feasible to perform these measurements by flow cytometry because of the paucity of human PBMCs in the blood. Two or three hNOJ mice in each group were infected intravenously with 200, 2000, or 20,000 pfu AIK-C-EGFP and followed up to 21 days p.i. The level of PBMC-associated MV RNA in individual mice is shown in **Figure 4A**. We noticed two peaks of MV replication, the first at around day 3 p.i., and the second at day 10 p.i., irrespective of the initial inoculum. Two mice infected with 20,000 pfu MV exhibited a high level of MV replication that peaked at day 10 p.i. One mouse infected with 2,000 pfu exhibited a high level of MV replication at day 3 p.i., followed by a small peak at day 10 p.i. For some mice, we counted the number of human cells per 50 μl of blood used for RNA extraction. The data are shown in **Figure 4B**. We were able to detect high levels of MV in samples containing less than 2,000 cells, indicating that the qRT-PCR system is sensitive enough to detect low numbers of MV-infected human cells.

Although MV replication was not obvious in three mice infected with the smallest dose (200 pfu), one of these animals exhibited an increase in MV RNA expression at day 21 p.i. (gray circle). We sacrificed this particular mouse and used flow cytometry to analyze GFP expression in its blood, spleen, MLN, and BM. As shown in **Figure 4C**, GFP^+ cells were present in spleen (0.308%) and all the other tissues, albeit at a lower frequency, indicating that MV infection can occur even at a low dose (200 pfu) and spread slowly in the systemic lymphoid tissues of hNOJ.

It may be necessary to acquire at least 30,000 events to be sure of having >10,000 cells for flow cytometry analysis. This is because of the substantial amount of sample loss that occurs in this system. The flow cytometry data presented in **Figure 4C** were obtained by analyzing ~0.4 ml blood from a sacrificed mouse. However, even under these conditions, the proportion of MV-infected cells detected was only 0.056%; indeed, the cells are barely visible on the plot. Therefore, it appears that flow cytometry is not a suitable method for the sequential monitoring of infected (GFP^+) cells. Thus, the qRT-PCR system we have developed here allowed us to monitor systemic MV replication using a small volume of blood from humanized mice.

DISCUSSION

Based on a highly sensitive MV-N RNA detection method previously developed by Hummel et al. (2006), which could detect one copy of synthetic MV RNA/reaction, we developed a novel one-step real-time qRT-PCR system for the purpose of monitoring MV replication in the blood of MV-infected humanized mice.

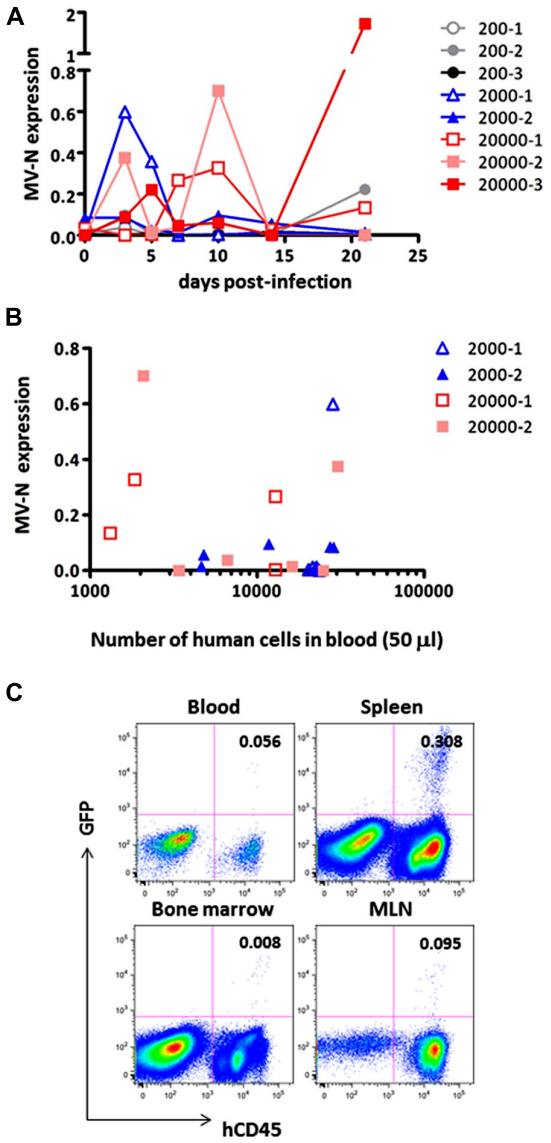


FIGURE 4 | Monitoring of MV replication *in vivo*. Two to three hNOJ mice per group were challenged with MV AIK-C-EGFP at 20,000 (squares), 2,000 (triangles), or 200 pfu (circles). The PBMCs of these mice were collected at day 3, 5, 7, 10, 14, and 21 p.i. **(A)** The level of MV-N expression in the blood of infected hNOJ. Vertical axis shows the level of MV-N relative to that of RNaseP, as described in the legend for **Figure 2B**.**(B)** For some mice (depicted using the same symbol as **(A)**), the number of human cells per 50 µl of blood used for RNA extraction and analyzed for MV-N expression was plotted on the X-axis. **(C)** An hNOJ mouse infected with MV at a low dose (gray closed circle, 200-2) exhibited an increased level of MV-N. At day 21 p.i., the mouse was sacrificed; cells from blood, spleen, BM, and mesenteric lymph node (MLN) were prepared. Cells were stained and analyzed as described in the legend for **Figure 2A**.

Because MV replication usually occurs in association with cells (Griffin, 2007), it is necessary to evaluate the endogenous RNA expression level of human PBMCs that co-exist with mouse blood cells. To this end, we designed human-specific primer/probe sets for the CD45 and RNase P mRNAs. When we analyzed the detection efficiencies of these two primer/probe sets using distinct cell types present in human PBMCs, we found that RNase P expression was less dependent than CD45 expression on cell type. Using this qRT-PCR system with RNase P as an internal control, we can reliably detect MV replication with high sensitivity in humanized mice *in vivo*. When MV expressing GFP was used for infections *in vitro* or *in vivo*, the level of MV-N RNA was closely correlated with the frequencies of GFP^+ MV-infected cells determined by flow cytometry.

Our qRT-PCR system allowed us to follow MV replication *in vivo* using a small amount of blood, with no need to sacrifice mice at each time point. Although flow-cytometric analysis provides valuable information, such as the proportions of various cell types and the surface phenotypes of MV-infected cells, the small number of human cells circulating in the mouse blood may not be sufficient for precise estimation of MV-infected cells by flow cytometry. By contrast, our qRT-PCR system was able to detect MV-N RNA in fewer than 2,000 human PBMCs (**Figure 4B**). This is an important technological advantage considering that individual humanized mice exhibit variable levels of human cell engraftment, i.e., chimerism (Terahara et al., 2013); moreover, there may exist donor-to-donor variation in susceptibility to MV infection. Thus, it should be possible to select humanized mice with a degree of MV infection appropriate for the purpose of a given experiment.

In this study, MV was inoculated through the tail vein, and infected cells were distributed to systemic lymphoid tissues as well as BMs, where human hematopoietic cells localize in humanized mice (Traggiai et al., 2004). MV may also be distributed to other organs, such as lung and intestinal tissue, as demonstrated in the case of HIV infection using the BLT mouse (Sun et al., 2007). To our surprise, by monitoring MV replication in PBMCs of humanized mice, we noticed two peaks of MV replication, at around 3 and 10 days p.i., in some mice. This pattern of MV replication did not depend on the initial dose of MV inoculum. We do not know why MV replication showed two peaks in many animals. However, it was recently reported in a monkey model that MV RNA persists in PBMCs for more than 1 month after primary infection, and declined in three phases (Lin et al., 2012). The authors of that study hypothesized that both T cells, including regulatory T cells (Treg), and antibody responses contributed to the dynamics of MV replication *in vivo*. Although hNOJ mice are reported to show poor immune responses, the role of regulatory T cells should be considered. This is because these cells regulate HIV-1 infection in humanized mice (Jiang et al., 2008). Alternatively, it may be that the intravenous injection of MV rapidly kills the target cells (probably those showing an activated phenotype) within 3 days. The low number of MV-infected cells then gradually transmits the virus to the human cells that are replenished from the BM stem cell pool. Further investigations are required to clarify this issue.

The humanized mouse model is expected to be a useful tool for studying virus infection (Akkina, 2013). Although the human immune system is not fully reconstructed by the transplantation of human HSCs alone, we believe that further improvements are possible, which will allow us to utilize this mouse model to not only evaluate vaccine and drug efficacy but also to increase our understanding of the pathogenesis of MV infection. The described novel method of monitoring MV-infected human cells in the blood will

be useful for studying MV-based vaccines in humanized mouse models without the need to sacrifice the mice.

ACKNOWLEDGMENTS

We thank Prof. Yusuke Yanagi for providing IC323-EGFP, and Ms. Kahori Okano for her excellent technical assistance. We also thank the Tokyo Cord Blood Bank for donating human umbilical cord blood. This work was supported in part by a grant from the Ministry of Health, Labor, and Welfare of Japan, and in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan. Shota Ikeno is supported by the Global COE Program (Practical Chemical Wisdom) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

Akkina, R. (2013). New generation humanized mice for virus research: comparative aspects and future prospects. *Virology* 435, 14–28. doi: 10.1016/j.virol.2012.10.007

Brandler, S., Marianneau, P., Loth, P., Lacote, S., Combredet, C., Frenkiel, M. P., et al. (2012). Measles vaccine expressing the secreted form of West Nile virus envelope glycoprotein induces protective immunity in squirrel monkeys, a new model of West Nile virus infection. *J. Infect. Dis.* 206, 212–219. doi: 10.1093/infdis/jis328

Despres, P., Combredet, C., Frenkiel, M. P., Lorin, C., Brahic, M., and Tangy, F. (2005). Live measles vaccine expressing the secreted form of the West Nile virus envelope glycoprotein protects against West Nile virus encephalitis. *J. Infect. Dis.* 191, 207–214. doi: 10.1086/426824

Fujino, M., Yoshida, N., Kimura, K., Zhou, J., Motegi, Y., Komase, K., et al. (2007). Development of a new neutralization test for measles virus. *J. Virol. Methods* 142, 15–20. doi: 10.1016/j.jviromet.2007.01.001

Griffin, D. E. (2007). "Measles virus," in *Fields Virology*, eds D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus, 5 Edn (Philadelphia: Lippincott Williams & Wilkins, a Wolters Kluwer Business), 1551–1585.

Hamaia, S., Li, C., and Allain, J. P. (2001). The dynamics of hepatitis C virus binding to platelets and 2 mononuclear cell lines. *Blood* 98, 2293–2300. doi: 10.1182/blood.V98.8.2293

Hashimoto, K., Ono, N., Tatsuo, H., Minagawa, H., Takeda, M., Takeuchi, K., et al. (2002). SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. *J. Virol.* 76, 6743–6749. doi: 10.1128/JVI.76.13.6743-6749.2002

Hummel, K. B., Lowe, L., Bellini, W. J., and Rota, P. A. (2006). Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. *J. Virol. Methods* 132, 166–173. doi: 10.1016/j.jviromet.2005.10.006

Ito, R., Takahashi, T., Katano, I., and Ito, M. (2012). Current advances in humanized mouse models. *Cell. Mol. Immunol.* 9, 208–214. doi: 10.1038/cmi.2012.2

Jiang, Q., Zhang, L., Wang, R., Jeffrey, J., Washburn, M. L., Brouwer, D., et al. (2008). FoxP3+CD4+ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2−/−gammaC−/− mice in vivo. *Blood* 112, 2858–2868. doi: 10.1182/blood-2008-03-145946

Kato, S. I., Nagata, K., and Takeuchi, K. (2012). Cell tropism and pathogenesis of measles virus in monkeys. *Front. Microbiol.* 3:14. doi: 10.3389/fmicb.2012.00014

Kimberly, W. T., Zheng, J. B., Town, T., Flavell, R. A., and Selkoe, D. J. (2005). Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J. Neurosci.* 25, 5533–5543. doi: 10.1523/JNEUROSCI.4883-04.2005

Koga, R., Ohno, S., Ikegame, S., and Yanagi, Y. (2010). Measles virus-induced immunosuppression in SLAM knock-in mice. *J. Virol.* 84, 5360–5367. doi: 10.1128/JVI.02525-09

Lin, W. H., Kouyos, R. D., Adams, R. J., Grenfell, B. T., and Griffin, D. E. (2012). Prolonged persistence of measles virus RNA is characteristic of primary infection dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14989–14994. doi: 10.1073/pnas.1211138109

Lorin, C., Mollet, L., Delebecque, F., Combredet, C., Hurtrel, B., Charneau, P., et al. (2004). A single injection of recombinant measles virus vaccines expressing human immunodeficiency virus (HIV) type 1 clade B envelope glycoproteins induces neutralizing antibodies and cellular immune responses to HIV. *J. Virol.* 78, 146–157. doi: 10.1128/JVI.78.1.146-157.2004

Ohno, S., Ono, N., Seki, F., Takeda, M., Kura, S., Tsuzuki, T., et al. (2007). Measles virus infection of SLAM (CD150) knockin mice reproduces tropism and immunosuppression in human infection. *J. Virol.* 81, 1650–1659. doi: 10.1128/JVI.02134-06

Reyes-del Valle, J., Hodge, G., Mcchesney, M. B., and Cattaneo, R. (2009). Protective anti-hepatitis B virus responses in rhesus monkeys primed with a vectored measles virus and boosted with a single dose of hepatitis B surface antigen. *J. Virol.* 83, 9013–9017. doi: 10.1128/JVI.00906-09

Sawada, A., Komase, K., and Nakayama, T. (2011). AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats. *Vaccine* 29, 1481–1490. doi: 10.1016/j.vaccine.2010.12.028

Sellin, C. I., and Horvat, B. (2009). Current animal models: transgenic animal models for the study of measles pathogenesis. *Curr. Top. Microbiol. Immunol.* 330, 111–127. doi: 10.1007/978-3-540-70617-5_6

Singh, M., Cattaneo, R., and Billeter, M. A. (1999). A recombinant measles virus expressing hepatitis B virus surface antigen induces humoral immune responses in genetically modified mice. *J. Virol.* 73, 4823–4828.

Stebbings, R., Fevrier, M., Li, B., Lorin, C., Koutsoukos, M., Mee, E., et al. (2012). Immunogenicity of a recombinant measles-HIV-1 clade B candidate vaccine. *PLoS ONE* 7:e50397. doi: 10.1371/journal.pone.0050397

Sun, Z., Denton, P. W., Estes, J. D., Othieno, F. A., Wei, B. L., Wege, A. K., et al. (2007). Intrarectal transmission, systemic infection, and CD4+ T cell depletion in humanized mice infected with HIV-1. *J. Exp. Med.* 204, 705–714. doi: 10.1084/jem.20062411

Tatsuo, H., Ono, N., Tanaka, K., and Yanagi, Y. (2000). SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 406, 893–897. doi: 10.1038/35022579

Terahara, K., Ishige, M., Ikeno, S., Mitsuki, Y. Y., Okada, S., Kobayashi, K., et al. (2013). Expansion of activated memory $CD4^+$ T cells affects infectivity of CCR5-tropic HIV-1 in humanized NOD/SCID/JAK3^{null} mice. *PLoS ONE* 8:e53495. doi: 10.1371/journal.pone.0053495

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., et al. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304, 104–107. doi: 10.1126/science.1093933

Wege, A. K., Melkus, M. W., Denton, P. W., Estes, J. D., and Garcia, J. V. (2008). Functional and phenotypic characterization of the humanized BLT mouse model. *Curr. Top. Microbiol. Immunol.* 324, 149–165. doi: 10.1007/978-3-540-75647-7_10

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 August 2013; accepted: 17 September 2013; published online: 11 October 2013.

*Citation: Ikeno S, Suzuki M, Muhsen M, Ishige M, Kobayashi-Ishihara M, Ohno S, Takeda M, Nakayama T, Morikawa Y, Terahara K, Okada S, Takeyama H and Tsunetsugu-Yokota Y (2013) Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR. Front. Microbiol. **4**:298. doi: 10.3389/fmicb.2013.00298*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 10 September 2013
doi: 10.3389/fmicb.2013.00264

Quantification of viral infection dynamics in animal experiments

Shingo Iwami[1]*, Yoshiki Koizumi[1,2], Hiroki Ikeda[1] and Yusuke Kakizoe[1]

[1] *Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka, Japan*
[2] *School of Medicine, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan*

Edited by:
Tomoyuki Miura, Kyoto University, Japan

Reviewed by:
Dorian McILroy, University of Nantes, France
Shinji Nakaoka, RIKEN Center for Integrative Medical Science Center, Japan

****Correspondence:***
Shingo Iwami, Department of Biology, Faculty of Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, Fukuoka 812-8581, Japan
e-mail: siwami@kyushu-u.org

Analyzing the time-course of several viral infections using mathematical models based on experimental data can provide important quantitative insights regarding infection dynamics. Over the past decade, the importance and significance of mathematical modeling has been gaining recognition among virologists. In the near future, many animal models of human-specific infections and experimental data from high-throughput techniques will become available. This will provide us with the opportunity to develop new quantitative approaches, combining experimental and mathematical analyses. In this paper, we review the various quantitative analyses of viral infections and discuss their possible applications.

Keywords: virus infection, mathematical modeling, computer simulation, animal experiment, quantification

INTRODUCTION

Based on a decline in the viral load of human immunodeficiency virus type-1 (HIV-1) patients following the initiation of antiviral therapy, the turnover of HIV infected cells *in vivo* was estimated through mathematical modeling (Ho et al., 1995; Wei et al., 1995). Starting with these landmark papers, mathematical modeling has evolved into an important tool in modern virology. Developing a quantitative understanding of virus infection dynamics is useful for determining the pathogenesis and transmissibility of viruses, predicting the course of disease, and evaluating the effects of antiviral therapy in HIV (Perelson, 2002; Simon and Ho, 2003; Rong and Perelson, 2009), hepatitis B/C virus (HBV/HCV; Dahari et al., 2008, 2011; Rong and Perelson, 2010; Chatterjee et al., 2012) and influenza virus infection (Beauchemin and Handel, 2011; Murillo et al., 2013). The importance and significance of mathematical modeling work is slowly being recognized by virologists. In addition, in recent years, data from animal experiments have been analyzed using mathematical models (Igarashi et al., 1999; Chen et al., 2007; Dahari and Perelson, 2007; Dinoso et al., 2009; Klatt et al., 2010; Miao et al., 2010; Wong et al., 2010; Graw et al., 2011; Horiike et al., 2012; Pinilla et al., 2012; Oue et al., 2013). A synergistic approach, combining animal experiments and mathematical models, has strong potential applications for researching various viral infections. For example, to determine certain aspects of virus infection, such as sites of infection, target cells (Dinoso et al., 2009; Horiike et al., 2012), and viral gene functions (Sato et al., 2010, 2012; Pinilla et al., 2012), designing an animal experiment and estimating numerous parameters with a mathematical model are useful and important. In the future, to understand the pathophysiology of untreatable or (re-)emerging virus infections, and to effectively develop therapeutic strategies against these viruses, we need to establish a platform involving quantitative analyses that are based on data from animal experiments (Perelson, 2002; Simon and Ho, 2003; Dahari et al., 2008, 2011; Rong and Perelson, 2009, 2010; Beauchemin and Handel, 2011; Chatterjee et al., 2012; Murillo et al., 2013). In this paper, we briefly review a history of quantitative approaches to virology and discuss the possible applications of these in combination with animal experiments.

QUANTIFICATION OF VIRUS INFECTION DYNAMICS

Virological research has typically been conducted with a small number of experiments. For example, in order to investigate the fitness of virus strains, one typically measured viral loads (e.g., the amount of viral protein and viral infectivity) at a few times during infection and determined whether one strain produces significantly more virus than the other. However, the entire time-course of an infection reflects complex processes involving interactions between viruses, target cells, and infected cells. Therefore, viral load detection at one time point ignores the complexity of the aforementioned processes during an entire infection (Iwami et al., 2012b). It would be useful to translate virus infection quantitatively into the parameters identifying the multi-composed kinetics of viral infection from time-course data (Perelson, 2002; Simon and Ho, 2003; Dahari et al., 2008, 2011; Rong and Perelson, 2009, 2010; Beauchemin and Handel, 2011; Chatterjee et al., 2012; Murillo et al., 2013). Mathematical modeling of the entire time-course of infection would allow us to estimate several parameters underlying the kinetics of virus infection, including burst size and basic reproductive number (Nowak and May, 2000). These parameters cannot be directly obtained through experimental and clinical studies.

HUMAN IMMUNODEFICIENCY VIRUS AND SIMIAN IMMUNODEFICIENCY VIRUS

On average it takes about, 10 years for an HIV infection to possibly progress to acquired immunodeficiency syndrome (AIDS;

Richman, 2001). Because of this slow disease progression, HIV is classified as a slowly replicating virus (Coffin, 1995; Richman, 2001). Several studies have indicated that slow disease progression is not due to inactive viral replication, but is a result of aggressive viral replication and its clearance (Coffin, 1995; Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996). Interestingly, these results were based on mathematical analyses of clinical data. Estimating the decline in viral load of patients following the initiation of antiviral therapy (or plasma removal by apheresis technique; Ramratnam et al., 1999) shows us that HIV is cleared from patients at a rapid rate, with a half-life of around 6 h (Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996). This estimation of rapid virus turnover implies that HIV resistance to any single drug could quickly emerge, highlighting the importance of combination therapies as they reduce the chances of drug resistance developing (Coffin, 1995; Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996; Perelson and Nelson, 1999).

The success of mathematical modeling, especially with respect to HIV infection dynamics has led to the development of a field called "viral dynamics" (Nowak and May, 2000; Perelson, 2002; Simon and Ho, 2003; Rong and Perelson, 2009) and has provided us with further quantitatively novel insights. In 1997, combinations of three antiretroviral drugs successfully reduced plasma HIV levels to below the limit of detection in clinical assays (50 copies of HIV RNA/ml; Perelson et al., 1997; Eisele and Siliciano, 2012). This approach, known as highly active antiretroviral therapy (HAART), is currently the primary choice of therapeutic intervention for HIV-1 infected patients, and dramatically decreases mortality associated with HIV-1 infection (Richman, 2001; Trono et al., 2010; Eisele and Siliciano, 2012). After the initiation of HAART, the viral load decays with an initially rapid and exponential decline, followed by a slower exponential decline (Perelson et al., 1997; Perelson, 2002; Murray et al., 2007; Keele et al., 2008; Palmer et al., 2008; Rong and Perelson, 2010). Modeling the effects of drug therapy allowed for the quantitation of virus clearance rates (Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996), and the death rate of several cell types (e.g., the productively infected $CD4^+$ T cells; Perelson et al., 1996, 1997; Markowitz et al., 2003), the productively long-lived infected cells (Perelson et al., 1997; Palmer et al., 2008), latently infected cells (Perelson et al., 1997; Zhang et al., 1999; Havlir et al., 2003; Palmer et al., 2008 and so on). This modeling also estimates the period of infectiousness for follicular dendritic cell-trapping viruses (Hlavacek et al., 2000), and assists with designing an optimal therapy (Murray et al., 2007; Rosenbloom et al., 2012). A simple assessment of HIV RNA data yields certain information, but mathematical approaches allow for the extraction of much more information from raw data. Some excellent reviews regarding the quantitation of HIV dynamics and its importance have been reported (Perelson and Nelson, 1999; Nowak and May, 2000; Perelson, 2002; Simon and Ho, 2003; Rong and Perelson, 2009).

Potent HAART effectively suppresses *de novo* replication of HIV but fails to eradicate an HIV-1 infection. Recent studies have revealed that HIV RNA persists over several years in most infected patients on suppressive HAART (Havlir et al., 2003; Palmer et al., 2008). Furthermore, virus loads rapidly rebound to pretreatment levels after discontinuation of HAART (Chun et al., 1999; Imamichi et al., 2001). These observations suggest the persistence of viral reservoirs during combination antiviral therapy. To completely cure HIV-1 infection, it is essential to identify these viral reservoirs and to eradicate them (Richman, 2001; Trono et al., 2010; Eisele and Siliciano, 2012). In addition to existing studies looking at peripheral blood (Chun et al., 1999; Imamichi et al., 2001; Sharkey et al., 2011), systemic analysis is required to elucidate the mechanisms underlying rebound of plasma viremia upon discontinuation of HAART. Because it is unethical to collect various tissues from patients, or to deplete certain cell populations in patients for analysis, the simian immunodeficiency virus (SIV)/macaque model (which has been useful in understanding HIV-1 infection) with HAART is suitable for investigating poorly understood aspects of HIV-1 infection (Dinoso et al., 2009; North et al., 2010; Horiike et al., 2012; Oue et al., 2013). Using the SIV/macaque model, for example, it has been recently reported that the cytotoxic effects of $CD8^+$ T cells on virus-infected cells during HAART is limited despite suppression of viral load *in vivo* (Klatt et al., 2010; Wong et al., 2010). Similarly, analyzing the levels of viral RNA in plasma and infected cells (e.g., macrophage and resting memory $CD4^+$ T cells) of certain tissues such as lung and lymph nodes in SIV-infected macaques using mathematical models might reveal the precise dynamics of the viral reservoir, and provide several valuable clues for HIV eradication in patients on HAART.

HEPATITIS B VIRUS AND HEPATITIS C VIRUS

The theoretical framework for quantifying HIV infection has also been applied to understand the dynamics of HBV (Nowak et al., 1996; Lewin et al., 2001; Murray et al., 2006; Dahari et al., 2009b) and HCV (Neumann et al., 1998; Dixit et al., 2004; Guedj and Perelson, 2011; Guedj et al., 2012) infections during antiviral therapy. These approaches have estimated the key parameters of the viral life cycle such as the rate of virus production and clearance and the death rate of infected cells, that explained the mechanism of action of antiviral drugs such as interferon, ribavirin, and protease inhibitor against HCV (Neumann et al., 1998; Dixit et al., 2004; Guedj and Perelson, 2011; Guedj et al., 2012) and reverse transcriptase inhibitor against HBV (Nowak et al., 1996; Lewin et al., 2001; Murray et al., 2006; Dahari et al., 2009b). These analyses have mainly focused on extracellular viral dynamics based on clinical studies, while several researchers have investigated the intracellular replication of HCV (Dahari et al., 2007, 2009a; McLean et al., 2010; Nakabayashi, 2012) and HBV (Nakabayashi and Sasaki, 2011) based on experimentally established HBV/HCV cell culture system. These studies have provided novel insights into the detailed dynamics of intracellular HBV/HCV replication, and revealed some important processes of the HCV life cycle such as the subcellular localization of HCV RNA to the replication complex for RNA replication and viral assembly. The above findings are helpful in understanding HCV turnover and determining new drug targets with fewer side effects. A number of reviews have been published detailing the mathematical modeling of HCV infection (Dahari et al., 2008; Guedj et al., 2010; Rong and Perelson, 2010; Guedj and Perelson, 2011; Chatterjee et al., 2012).

Although mathematical models were successfully used to understand the viral dynamics of HBV/HCV during antiviral

therapy, these models considered only one level of extracellular or intracellular viral replication. Recently, for HCV infection, several researchers have developed a new mathematical model, known as the multi-scale model, that combines extracellular virus infection dynamics with the key features of intracellular viral replication (Guedj and Neumann, 2010; Guedj et al., 2013; Rong et al., 2013). This model incorporates two different time scales: one is for viral replication within a cell, and the other is for free viral infection among cells. Using this model in conjunction with clinical trials, it is possible to verify the mechanism of action of direct-acting antiviral agents (DAAs) that target specific viral proteins in a cell. Estimating the effectiveness of DAAs using multi-scale model takes into account intracellular viral dynamics (Guedj et al., 2013; Rong et al., 2013). Additionally, the multi-scale model has the potential to describe the emergence of viral drug resistance against DAAs at an intracellular and extracellular level (Guedj and Neumann, 2010). In the era of developing DAAs, multi-scale models could provide a new theoretical framework that combines findings from several studies of intra- and extracellular viral dynamics; this can be applied to HBV, HIV (Althaus and De Boer, 2010), and influenza virus (Murillo et al., 2013).

Using animal and cell culture systems with mathematical models paves the way to investigate new vaccines against HCV. It would also assist with understanding the mechanisms of antiviral drug therapy. HBV vaccines are available, but there is no effective vaccine against HCV infection. The development of an effective HCV vaccine has been hampered by the high mutation rate of viral proteins, the genetic diversity of HCV, and the lack of usable small animal models for HCV infection (Houghton and Abrignani, 2005; Klenerman and Gupta, 2012). Recently, a uPA-TG/severe combined immunodeficiency (SCID) mouse model for HCV infection has been developed (Mercer et al., 2001). Although an authentic immune response against HCV does not occur in these models (and therefore cannot be directly suitable for vaccine studies), mathematical modeling could compensate for the lack of information regarding key processes of HCV immune interactions and promote further development of small animal models of HCV. These and other animal models could be alternatives to chimpanzees for investigating the effects of candidates drugs and vaccines against HCV (Bukh, 2012; chimpanzees are endangered species and now cannot be used for animal experiments). On the other hand, mathematical modeling of the immune response against HBV in patients has estimated the contribution of the host response for viral clearance (Ciupe et al., 2007a,b), and the optimal vaccination schedule (Gesemann and Scheiermann, 1995; Wilson et al., 2007). Taken together, the combination of a small animal model and mathematical modeling can overcome the ethical and financial limitations of clinical trials and help develop new effective therapies against HBV and HCV.

INFLUENZA VIRUS

In epidemiology, many mathematical models have been developed and been used to determine the dynamics of influenza virus infections on the population level (Anderson, 1991; Ferguson et al., 2006; Hatchett et al., 2007; Beauchemin and Handel, 2011; Murillo et al., 2013). A small number of models have also been generated to describe influenza virus infections at the host level (Baccam et al., 2006; Miao et al., 2010; Dobrovolny et al., 2011; Pinilla et al., 2012; and at the individual cell level; Hatada et al., 1989; Heldt et al., 2012). The purpose of these models is to describe the time-course of influenza virus infections as accurately as possible. This allows for the calculation of the half-life of infected cells, the number of virus particles released per infected cell (i.e., the burst size), and the number of infected cells produced per infected cell (i.e., the basic reproductive number; Baccam et al., 2006; Beauchemin et al., 2008; Mitchell et al., 2011; Pinilla et al., 2012). This information has been used to understand the severity and duration of infections (Bocharov and Romanyukha, 1994; Hancioglu et al., 2007; Canini and Carrat, 2011), and has provided us with an optimal antiviral therapy (Baccam et al., 2006; Handel et al., 2007; Dobrovolny et al., 2011; Perelson et al., 2012).

Although influenza viruses have been studied extensively *in vivo*, it is difficult to determine the exact date of infection, influenza virus loads prior to a peak, and pre-hemagglutination inhibition antibody titers. All these factors are crucial in quantifying virus infection dynamics (Murphy et al., 1980; Carrat et al., 2008). Experimental infection of healthy volunteers with influenza viruses provides a unique opportunity to elucidate the dynamics of natural influenza infections. The first mathematical model proposed to describe the dynamics of influenza infections, using influenza A/Hong Kong/123/77 (H1N1), was conducted in 2006 (Baccam et al., 2006). This simple mathematical model revealed several important and novel quantities corresponding to biological processes of influenza virus infection (Mohler et al., 2005; Baccam et al., 2006; Schulze-Horsel et al., 2009; Smith et al., 2010; Beauchemin and Handel, 2011; Holder and Beauchemin, 2011; Murillo et al., 2013). For example, if a basic reproductive number (R_0) was obtained, the critical inhibition rate ($1 - 1/R_0$) could be estimated for protection against virus infection (Anderson, 1991; Iwami et al., 2012a,b). This implies that reducing viral growth with antiviral interventions, such as vaccines or drugs, could prevent viral spread *in vivo*. More biologically realistic mathematical models incorporating the eclipse phase of infected cells (Holder and Beauchemin, 2011; Pinilla et al., 2012), innate or adaptive immune responses (Bocharov and Romanyukha, 1994; Hancioglu et al., 2007; Handel et al., 2007; Miao et al., 2010; Canini and Carrat, 2011), and several distributed delays for each biological process (Holder and Beauchemin, 2011; e.g., a time from virus entry to progeny virus producing) have been developed. Furthermore, using a mathematical model, a relationship between virus and symptom dynamics during influenza infections has been described recently (Canini and Carrat, 2011). Reviews regarding quantitation of influenza virus dynamics and its importance have been published previously (Beauchemin and Handel, 2011; Murillo et al., 2013).

These quantitative analyses of influenza viruses have yielded useful insights (Bocharov and Romanyukha, 1994; Mohler et al., 2005; Baccam et al., 2006; Hancioglu et al., 2007; Handel et al., 2007; Schulze-Horsel et al., 2009; Miao et al., 2010; Smith et al., 2010; Canini and Carrat, 2011; Dobrovolny et al., 2011; Holder and Beauchemin, 2011; Perelson et al., 2012; Pinilla et al., 2012; Murillo et al., 2013). The development of reliable within-host models is critical in improving epidemiological models because the dynamics of viral shedding and symptoms following influenza

virus infection are key factors (Ferguson et al., 2006; Hatchett et al., 2007; Murillo et al., 2013). However, a major difficulty, over-parameterization (Beauchemin and Handel, 2011), arises when only viral load data are available, especially in human volunteer studies. One possible approach to overcome this limitation is to conduct animal experiments using rhesus macaques (Watanabe et al., 2011), ferrets (Kiso et al., 2010), and mice (Imai et al., 2008; Martin and Wurfel, 2008; Morita et al., 2013), and to measure the time-course data for these analyses (Miao et al., 2010; Pinilla et al., 2012). It is possible that the number of uninfected and infected cells from the lung or respiratory tract can be measured. Although it is still currently not feasible to obtain sufficient time-course data during the acute phase of infections, we have recently developed a novel but simple mathematical model to robustly estimate virus replication rates (Ikeda et al., unpublished). In this model, a relatively few time-course data of both the number of uninfected cells and viral load is required. A new model and diverse data will promote knowledge of influenza virus infection dynamics, which is important for future research.

OTHER VIRUSES

Mathematical models have also been applied for understanding the dynamics of other virus infections. For example, during acute lymphocytic choriomeningitis virus (LCMV, which is a common infection of rodents and is best known for its application in immunological studies) infection, the dynamics of the specific $CD8^+$ T-cell response such as proliferation and apoptosis rate was estimated in infected mice (De Boer et al., 2001). Quantitative analyses suggested that the specific $CD8^+$ T-cell response is controlled via the number of $CD8^+$ T-cells, rather than their individual function during persistent LCMV infection compared with those in acute LCMV infections (in fact, the immune response with a high killing effect is necessary to clear the LCMV infection; Graw et al., 2011). On the other hand, modeling and fitting data from patients revealed that the doubling time of cytomegaloviruses (CMV, which is assumed to cause asymptomatic infection in normal hosts) in human hosts is around 1 day, similar to that for HIV (Emery et al., 1999; Perelson, 2002). More recently, it has been revealed that viral productivity and transmissibility, but not cytotoxicity, differ among Enterovirus 71 (EV71, which is the causative agent of hand-foot-and-mouth disease and can trigger neurological disorders) strains in cell culture and could be associated with their epidemiological backgrounds (Fukuhara et al., 2013). Animal experiments using monkeys and mice are available to investigate the pathogenesis and symptoms of these and numerous other viruses (Farrell et al., 1997; De Boer et al., 2001; Arita et al., 2007, 2008; Graw et al., 2011; Sato et al., 2011). We have a chance to establish a platform that will allow for quantitative understanding of various virus infections based on animal experiments. Accumulation of knowledge regarding viral dynamics should be useful in understanding untreatable or (re-)emerging virus infections.

CONCLUSION

Studies of virus infection dynamics have significantly contributed to our understanding of many diseases. Merging animal experiment results with mathematical models is a desirable direction for virology research (**Figure 1**). In particular, quantifying viral dynamics in "humanized mice" (Shultz et al., 2007; Sato and Koyanagi, 2011), which are the most practical and relevant model available, will provide us with novel insights. Using humanized mice as models of specific human viral infections (Sato et al., 2010, 2011, 2012) or human diseases (Ishikawa et al., 2007), we were able to investigate mechanisms of disease symptoms (e.g., a relation between function of regulatory T cells and depletion of $CD4^+$ T cells in HIV-1 infection), and the potency/mechanism of action for drug/host factors (e.g., an effect of anticancer drug in human T cell leukemia virus type-1 infection) based on virus infection dynamics. Mathematical models can be used to explore a complicated dynamical system of virus infection. Estimation of key parameters during a virus infection provides us with many details regarding the infection. If we can obtain these estimated parameters and calculate burst size and basic reproductive numbers, we could easily compare the dynamics of various viral infections. Qualitative data (in fact, most experiments are not designed from a quantitative point of view) are difficult to understand and compare with results from other studies. Based on the theoretical analysis of experimental data, we are able to determine optimum frequencies of sampling for the highest quality data possible. Once we establish a mathematical model and reasonably fit that model to experimental data, we can predict outcomes of animal experiments under different conditions and determine factors that control several phenomena during virus infection (e.g., peak of viral load and mode of virus spread) through simulations. Further associations between animal experiments and mathematical models are required to overcome untreatable diseases.

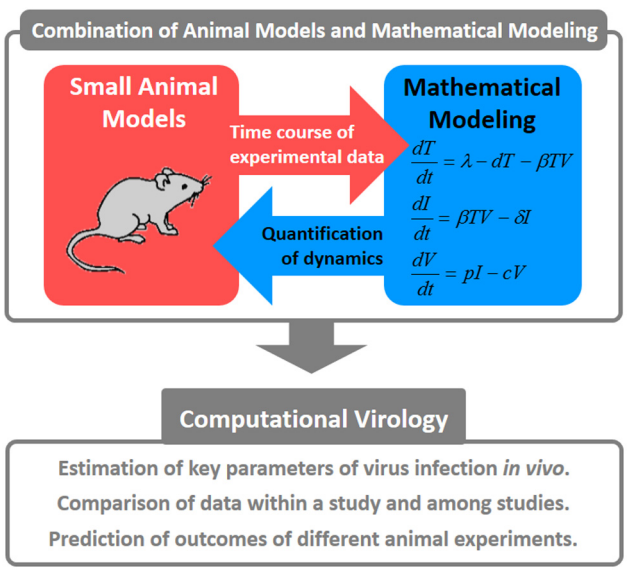


FIGURE 1 | Concepts for combining animal models with mathematical modeling. These quantitative approaches are referred to as "computational virology." This has the potential to provide novel insights into viral pathogenesis, the development of antiviral drugs, and the establishment of effective therapies against viral infections.

ACKNOWLEDGMENTS

This work was supported, in part, by Kyushu University Interdisciplinary Programs in Education and Projects in Research Development (to Shingo Iwami); a grant-in-aid for Young Scientists B25800092 (to Shingo Iwami) from the Japan Society for the Promotion of Science (JSPS); and by the Aihara Innovative Mathematical Modeling Project, JSPS, through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)," initiated by the Council for Science and Technology Policy (to Shingo Iwami) and a Kyushu University Fund (to Hiroki Ikeda).

REFERENCES

Althaus, C. L., and De Boer, R. J. (2010). Intracellular transactivation of HIV can account for the decelerating decay of virus load during drug therapy. *Mol. Syst. Biol.* 6, 348. doi: 10.1038/msb.2010.4

Anderson, R. M. (1991). The Kermack–McKendrick epidemic threshold theorem. *Bull. Math. Biol.* 53, 3–32. doi: 10.1016/S0092-8240(05)80039-4

Arita, M., Ami, Y., Wakita, T., and Shimizu, H. (2008). Cooperative effect of the attenuation determinants derived from poliovirus sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. *J. Virol.* 82, 1787–1797. doi: 10.1128/JVI.01798-07

Arita, M., Nagata, N., Iwata, N., Ami, Y., Suzaki, Y., Mizuta, K., et al. (2007). An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. *J. Virol.* 81, 9386–9395. doi: 10.1128/JVI.02856-06

Baccam, P., Beauchemin, C. A., Macken, C. A., Hayden, F. G., and Perelson, A. S. (2006). Kinetics of influenza A virus infection in humans. *J. Virol.* 80, 7590–7599. doi: 10.1128/JVI.01623-05

Beauchemin, C. A., and Handel, A. (2011). A review of mathematical models of influenza A infections within a host or cell culture: lessons learned and challenges ahead. *BMC Public Health* 11(Suppl. 1):S7. doi: 10.1186/1471-2458-11-S1-S7

Beauchemin, C. A., McSharry, J. J., Drusano, G. L., Nguyen, J. T., Went, G. T., Ribeiro, R. M., et al. (2008). Modeling amantadine treatment of influenza A virus *in vitro*. *J. Theor. Biol.* 254, 439–451. doi: 10.1016/j.jtbi.2008.05.031

Bocharov, G. A., and Romanyukha, A. A. (1994). Mathematical model of antiviral immune response III. Influenza A virus infection. *J. Theor. Biol.* 167, 323–360. doi: 10.1006/jtbi.1994.1074

Bukh, J. (2012). Animal models for the study of hepatitis C virus infection and related liver disease. *Gastroenterology* 142, 1279–1287. doi: 10.1053/j.gastro.2012.02.016

Canini, L., and Carrat, F. (2011). Population modeling of influenza A/H1N1 virus kinetics and symptom dynamics. *J. Virol.* 85, 2764–2770. doi: 10.1128/JVI.01318-10

Carrat, F., Vergu, E., Ferguson, N. M., Lemaitre, M., Cauchemez, S., Leach, S., et al. (2008). Time lines of infection and disease in human influenza: a review of volunteer challenge studies. *Am. J. Epidemiol.* 167, 775–785. doi: 10.1093/aje/kwm375

Chatterjee, A., Guedj, J., and Perelson, A. S. (2012). Mathematical modelling of HCV infection: what can it teach us in the era of direct-acting antiviral agents? *Antivir. Ther.* 17, 1171–1182. doi: 10.3851/IMP2428

Chen, H. Y., Di Mascio, M., Perelson, A. S., Ho, D. D., and Zhang, L. (2007). Determination of virus burst size *in vivo* using a single-cycle SIV in rhesus macaques. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19079–19084. doi: 10.1073/pnas.0707449104

Chun, T. W., Davey, R. T. Jr., Engel, D., Lane, H. C., and Fauci, A. S. (1999). Re-emergence of HIV after stopping therapy. *Nature* 401, 874–875. doi: 10.1038/44755

Ciupe, S. M., Ribeiro, R. M., Nelson, P. W., Dusheiko, G., and Perelson, A. S. (2007a). The role of cells refractory to productive infection in acute hepatitis B viral dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5050–5055. doi: 10.1073/pnas.0603626104

Ciupe, S. M., Ribeiro, R. M., Nelson, P. W., and Perelson, A. S. (2007b). Modeling the mechanisms of acute hepatitis B virus infection. *J. Theor. Biol.* 247, 23–35. doi: 10.1016/j.jtbi.2007.02.017

Coffin, J. M. (1995). HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy. *Science* 267, 483–489. doi: 10.1126/science.7824947

Dahari, H., Guedj, J., Perelson, A. S., and Layden, T. J. (2011). Hepatitis C viral kinetics in the era of direct acting antiviral agents and IL28B. *Curr. Hepat. Rep.* 10, 214–227. doi: 10.1007/s11901-011-0101-7

Dahari, H., Layden-Almer, J. E., Perelson, A. S., and Layden, T. J. (2008). Hepatitis C viral kinetics in special populations. *Curr. Hepat. Rep.* 7, 97–105. doi: 10.1007/s11901-008-0022-2

Dahari, H., and Perelson, A. S. (2007). Hepatitis C virus kinetics in chimeric mice during antiviral therapy. *Hepatology* 46, 2048–2049. doi: 10.1002/hep.21798

Dahari, H., Ribeiro, R. M., Rice, C. M., and Perelson, A. S. (2007). Mathematical modeling of subgenomic hepatitis C virus replication in Huh-7 cells. *J. Virol.* 81, 750–760. doi: 10.1128/JVI.01304-06

Dahari, H., Sainz, B. Jr., Perelson, A. S., and Uprichard, S. L. (2009a). Modeling subgenomic hepatitis C virus RNA kinetics during treatment with alpha interferon. *J. Virol.* 83, 6383–6390. doi: 10.1128/JVI.02612-08

Dahari, H., Shudo, E., Ribeiro, R. M., and Perelson, A. S. (2009b). Modeling complex decay profiles of hepatitis B virus during antiviral therapy. *Hepatology* 49, 32–38. doi: 10.1002/hep.22586

De Boer, R. J., Oprea, M., Antia, R., Murali-Krishna, K., Ahmed, R., and Perelson, A. S. (2001). Recruitment times, proliferation, and apoptosis rates during the CD8(+) T-cell response to lymphocytic choriomeningitis virus. *J. Virol.* 75, 10663–10669. doi: 10.1128/JVI.75.22.10663-10669.2001

Dinoso, J. B., Rabi, S. A., Blankson, J. N., Gama, L., Mankowski, J. L., Siliciano, R. F., et al. (2009). A simian immunodeficiency virus-infected macaque model to study viral reservoirs that persist during highly active antiretroviral therapy. *J. Virol.* 83, 9247–9257. doi: 10.1128/JVI.00840-09

Dixit, N. M., Layden-Almer, J. E., Layden, T. J., and Perelson, A. S. (2004). Modelling how ribavirin improves interferon response rates in hepatitis C virus infection. *Nature* 432, 922–924. doi: 10.1038/nature03153

Dobrovolny, H. M., Gieschke, R., Davies, B. E., Jumbe, N. L., and Beauchemin, C. A. (2011). Neuraminidase inhibitors for treatment of human and avian strain influenza: a comparative study. *J. Theor. Biol.* 269, 234–244. doi: 10.1016/j.jtbi.2010.10.017

Eisele, E., and Siliciano, R. F. (2012). Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* 37, 377–388. doi: 10.1016/j.immuni.2012.08.010

Emery, V. C., Cope, A. V., Bowen, E. F., Gor, D., and Griffiths, P. D. (1999). The dynamics of human cytomegalovirus replication *in vivo*. *J. Exp. Med.* 190, 177–182. doi: 10.1084/jem.190.2.177

Farrell, H. E., Vally, H., Lynch, D. M., Fleming, P., Shellam, G. R., Scalzo, A. A., et al. (1997). Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue *in vivo*. *Nature* 386, 510–514. doi: 10.1038/386510a0

Ferguson, N. M., Cummings, D. A. T., Fraser, C., Cajka, J. C., Cooley, P. C., and Burke, D. S. (2006). Strategies for mitigating an influenza pandemic. *Nature* 442, 448–452. doi: 10.1038/nature04795

Fukuhara, M., Iwami, S., Sato, K., Nishimura, Y., Shimizu, H., Aihara, K., et al. (2013). Quantification of the dynamics of enterovirus 71 infection by experimental-mathematical investigation. *J. Virol.* 87, 701–705. doi: 10.1128/JVI.01453-12

Gesemann, M., and Scheiermann, N. (1995). Quantification of hepatitis B vaccine-induced antibodies as a predictor of anti-HBs persistence. *Vaccine* 13, 443–447. doi: 10.1016/0264-410X(94)00010-K

Graw, F., Richter, K., Oxenius, A., and Regoes, R. R. (2011). Comparison of cytotoxic T lymphocyte efficacy in acute and persistent lymphocytic choriomeningitis virus infection. *Proc. Biol. Sci.* 278, 3395–3402. doi: 10.1098/rspb.2011.0453

Guedj, J., Dahari, H., Rong, L., Sansone, N. D., Nettles, R. E., Cotler, S. J., et al. (2013). Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3991–3996. doi: 10.1073/pnas.1203110110

Guedj, J., Dahari, H., Shudo, E., Smith, P., and Perelson, A. S. (2012). Hepatitis C viral kinetics with the nucleoside polymerase inhibitor mericitabine (RG7128). *Hepatology* 55, 1030–1037. doi: 10.1002/hep.24788

Guedj, J., and Neumann, A. U. (2010). Understanding hepatitis C viral dynamics with direct-acting antiviral agents due to the interplay between intracellular replication

and cellular infection dynamics. *J. Theor. Biol.* 267, 330–340. doi: 10.1016/j.jtbi.2010.08.036

Guedj, J., and Perelson, A. S. (2011). Second-phase hepatitis C virus RNA decline during telaprevir-based therapy increases with drug effectiveness: implications for treatment duration. *Hepatology* 53, 1801–1808. doi: 10.1002/hep.24272

Guedj, J., Rong, L., Dahari, H., and Perelson, A. S. (2010). A perspective on modelling hepatitis C virus infection. *J. Viral Hepat.* 17, 825–833. doi: 10.1111/j.1365-2893.2010.01348.x

Hancioglu, B., Swigon, D., and Clermont, G. (2007). A dynamical model of human immune response to influenza A virus infection. *J. Theor. Biol.* 246, 70–86. doi: 10.1016/j.jtbi.2006.12.015

Handel, A., Longini, I. M., and Antia, R. (2007). Neuraminidase inhibitor resistance in influenza: assessing the danger of its generation and spread. *PLoS Comput. Biol.* 3:e240. doi: 10.1371/journal.pcbi.0030240

Hatada, E., Hasegawa, M., Mukaigawa, J., Shimizu, K., and Fukuda, R. (1989). Control of influenza virus gene expression: quantitative analysis of each viral RNA species in infected cells. *J. Biochem.* 105, 537–546.

Hatchett, R. J., Mecher, C. E., and Lipsitch, M. (2007). Public health interventions and epidemic intensity during the 1918 influenza pandemic. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7582–7587. doi: 10.1073/pnas.0610941104

Havlir, D. V., Strain, M. C., Clerici, M., Ignacio, C., Trabattoni, D., Ferrante, P., et al. (2003). Productive infection maintains a dynamic steady state of residual viremia in human immunodeficiency virus type 1-infected persons treated with suppressive antiretroviral therapy for five years. *J. Virol.* 77, 11212–11219. doi: 10.1128/JVI.77.20.11212-11219.2003

Heldt, F. S., Frensing, T., and Reichl, U. (2012). Modeling the intracellular dynamics of influenza virus replication to understand the control of viral RNA synthesis. *J. Virol.* 86, 7806–7817. doi: 10.1128/JVI.00080-12

Hlavacek, W. S., Stilianakis, N. I., Notermans, D. W., Danner, S. A., and Perelson, A. S. (2000). Influence of follicular dendritic cells on decay of HIV during antiretroviral therapy. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10966–10971. doi: 10.1073/pnas.190065897

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 373, 123–126. doi: 10.1038/373123a0

Holder, B. P., and Beauchemin, C. A. A. (2011). Exploring the effect of biological delays in kinetic models of influenza within a host or cell culture. *BMC Public Health* 11:S10. doi: 10.1186/1471-2458-11-S1-S10

Horiike, M., Iwami, S., Kodama, M., Sato, A., Watanabe, Y., Yasui, M., et al. (2012). Lymph nodes harbor viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy. *Virology* 423, 107–118. doi: 10.1016/j.virol.2011.11.024

Houghton, M., and Abrignani, S. (2005). Prospects for a vaccine against the hepatitis C virus. *Nature* 436, 961–966. doi: 10.1038/nature04081

Igarashi, T., Brown, C., Azadegan, A., Haigwood, N., Dimitrov, D., Martin, M. A., et al. (1999). Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. *Nat. Med.* 5, 211–216. doi: 10.1038/5576

Imai, Y., Kuba, K., Neely, G. G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., et al. (2008). Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 133, 235–249. doi: 10.1016/j.cell.2008.02.043

Imamichi, H., Crandall, K. A., Natarajan, V., Jiang, M. K., Dewar, R. L., Berg, S., et al. (2001). Human immunodeficiency virus type 1 quasi species that rebound after discontinuation of highly active antiretroviral therapy are similar to the viral quasi species present before initiation of therapy. *J. Infect. Dis.* 183, 36–50. doi: 10.1086/317641

Ishikawa, F., Yoshida, S., Saito, Y., Hijikata, A., Kitamura, H., Tanaka, S., et al. (2007). Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* 25, 1315–1321. doi: 10.1038/nbt1350

Iwami, S., Holder, B. P., Beauchemin, C. A., Morita, S., Tada, T., Sato, K., et al. (2012a). Quantification system for the viral dynamics of a highly pathogenic simian/human immunodeficiency virus based on an *in vitro* experiment and a mathematical model. *Retrovirology* 9, 18. doi: 10.1186/1742-4690-9-18

Iwami, S., Sato, K., De Boer, R. J., Aihara, K., Miura, T., and Koyanagi, Y. (2012b). Identifying viral parameters from *in vitro* cell cultures. *Front. Microbiol.* 3:319. doi: 10.3389/fmicb.2012.00319

Keele, B. F., Tazi, L., Gartner, S., Liu, Y., Burgon, T. B., Estes, J. D., et al. (2008). Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1. *J. Virol.* 82, 5548–5561. doi: 10.1128/JVI.00124-08

Kiso, M., Shinya, K., Shimojima, M., Takano, R., Takahashi, K., Katsura, H., et al. (2010). Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. *PLoS Pathog.* 6:e1001079. doi: 10.1371/journal.ppat.1001079

Klatt, N. R., Shudo, E., Ortiz, A. M., Engram, J. C., Paiardini, M., Lawson, B., et al. (2010). $CD8^+$ lymphocytes control viral replication in SIVmac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. *PLoS Pathog.* 6:e1000747. doi: 10.1371/journal.ppat.1000747

Klenerman, P., and Gupta, P. K. (2012). Hepatitis C virus: current concepts and future challenges. *Q. J. Med.* 105, 29–32. doi: 10.1093/qjmed/hcr231

Lewin, S. R., Ribeiro, R. M., Walters, T., Lau, G. K., Bowden, S., Locarnini, S., et al. (2001). Analysis of hepatitis B viral load decline under potent therapy: complex decay profiles observed. *Hepatology* 34, 1012–1020. doi: 10.1053/jhep.2001.28509

Markowitz, M., Louie, M., Hurley, A., Sun, E., Di Mascio, M., Perelson, A. S., et al. (2003). A novel antiviral intervention results in more accurate assessment of human immunodeficiency virus type 1 replication dynamics and T-cell decay *in vivo*. *J. Virol.* 77, 5037–5038. doi: 10.1128/JVI.77.8.5037-5038.2003

Martin, T. R., and Wurfel, M. M. (2008). A TRIFfic perspective on acute lung injury. *Cell* 133, 208–210. doi: 10.1016/j.cell.2008.04.006

McLean, A. K., Luciani, F., and Tanaka, M. M. (2010). Trade-offs in resource allocation in the intracellular life-cycle of hepatitis C virus. *J. Theor. Biol.* 267, 565–572. doi: 10.1016/j.jtbi.2010.09.031

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., et al. (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* 7, 927–933. doi: 10.1038/90968

Miao, H., Hollenbaugh, J. A., Zand, M. S., Holden-Wiltse, J., Mosmann, T. R., Perelson, A. S., et al. (2010). Quantifying the early immune response and adaptive immune response kinetics in mice infected with influenza A virus. *J. Virol.* 84, 6687–6698. doi: 10.1128/JVI.00266-10

Mitchell, H., Levin, D., Forrest, S., Beauchemin, C. A., Tipper, J., Knight, J., et al. (2011). Higher level of replication efficiency of 2009 (H1N1) pandemic influenza virus than those of seasonal and avian strains: kinetics from epithelial cell culture and computational modeling. *J. Virol.* 85, 1125–1135. doi: 10.1128/JVI.01722-10

Mohler, L., Flockerzi, D., Sann, H., and Reichl, U. (2005). Mathematical model of influenza A virus production in large-scale microcarrier culture. *Biotechnol. Bioeng.* 90, 46–58. doi: 10.1002/bit.20363

Morita, M., Kuba, K., Ichikawa, A., Nakayama, M., Katahira, J., Iwamoto R., et al. (2013). The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza. *Cell* 153, 112–125. doi: 10.1016/j.cell.2013.02.027

Murillo, L. N., Murillo, M. S., and Perelson, A. S. (2013). Towards multiscale modeling of influenza infection. *J. Theor. Biol.* 332, 267–290. doi: 10.1016/j.jtbi.2013.03.024

Murphy, B. R., Rennels, M. B., Douglas, R. G. Jr., Betts, R. F., Couch, R. B., Cate, T. R. Jr., et al. (1980). Evaluation of influenza A/Hong Kong/123/77 (H1N1) ts-1A2 and cold-adapted recombinant viruses in seronegative adult volunteers. *Infect. Immun.* 29, 348–355.

Murray, J. M., Emery, S., Kelleher, A. D., Law, M., Chen, J., Hazuda, D. J., et al. (2007). Antiretroviral therapy with the integrase inhibitor raltegravir alters decay kinetics of HIV, significantly reducing the second phase. *AIDS* 21, 2315–2321. doi: 10.1097/QAD.0b013e3282f12377

Murray, J. M., Purcell, R. H., and Wieland, S. F. (2006). The half-life of hepatitis B virions. *Hepatology* 44, 1117–1121. doi: 10.1002/hep.21364

Nakabayashi, J. (2012). A compartmentalization model of hepatitis C virus replication: an appropriate distribution of HCV RNA for the effective replication. *J. Theor. Biol.* 300, 110–117. doi: 10.1016/j.jtbi.2012.01.023

Nakabayashi, J., and Sasaki, A. (2011). A mathematical model of the intracellular replication and within host evolution of hepatitis type B virus: understanding the long time course of chronic hepatitis. *J. Theor. Biol.* 269, 318–329. doi: 10.1016/j.jtbi.2010.10.024

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J., et al. (1998). Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-alpha therapy. *Science* 282, 103–107. doi: 10.1126/science.282.5386.103

North, T. W., Higgins, J., Deere, J. D., Hayes, T. L., Villalobos, A., Adamson, L., et al. (2010). Viral sanctuaries during highly active antiretroviral therapy in a nonhuman primate model for AIDS. *J. Virol.* 84, 2913–2922. doi: 10.1128/JVI.02356-09

Nowak, M. A., Bonhoeffer, S., Hill, A. M., Boehme, R., Thomas, H. C., and McDade H. (1996). Viral dynamics in hepatitis B virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4398–4402. doi: 10.1073/pnas.93.9.4398

Nowak, M. A., and May, R. M. (2000). *Virus Dynamics.* New York, NY: Oxford University Press.

Oue, M., Sakabe, S., Horiike, M., Yasui, M., Miura, T., and Igarashi, T. (2013). No viral evolution in the lymph nodes of simian immunodeficiency virus-infected rhesus macaques during combined antiretroviral therapy. *J. Virol.* 87, 4789–4793. doi: 10.1128/JVI.03367-12

Palmer, S., Maldarelli, F., Wiegand, A., Bernstein, B., Hanna, G. J., Brun, S. C., et al. (2008). Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3879–3884. doi: 10.1073/pnas.0800050105

Perelson, A. S. (2002). Modelling viral and immune system dynamics. *Nat. Rev. Immunol.* 2, 28–36. doi: 10.1038/nri700

Perelson, A. S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., et al. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* 387, 188–191. doi: 10.1038/387188a0

Perelson, A. S., and Nelson, P. W. (1999). Mathematical analysis of HIV-1 dynamics *in vivo*. *SIAM Rev.* 41, 3–44. doi: 10.1137/S0036144598335107

Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M., and Ho, D. D. (1996). HIV-1 dynamics *in vivo*: virion clearance rate, infected cell life-span, and viral generation time. *Science* 271, 1582–1586. doi: 10.1126/science.271.5255.1582

Perelson, A. S., Rong, L., and Hayden, F. G. (2012). Combination antiviral therapy for influenza: predictions from modeling of human infections. *J. Infect. Dis.* 205, 1642–1645. doi: 10.1093/infdis/jis265

Pinilla, L. T., Holder, B. P., Abed, Y., Boivin, G., and Beauchemin, C. A. (2012). The H275Y neuraminidase mutation of the pandemic A/H1N1 influenza virus lengthens the eclipse phase and reduces viral output of infected cells, potentially compromising fitness in ferrets. *J. Virol.* 86, 10651–10660. doi: 10.1128/JVI.07244-11

Ramratnam, B., Bonhoeffer, S., Binley, J., Hurley, A., Zhang, L., Mittler, J. E., et al. (1999). Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. *Lancet* 354, 1782–1785. doi: 10.1016/S0140-6736(99)02035-8

Richman, D. D. (2001). HIV chemotherapy. *Nature* 410, 995–1001. doi: 10.1038/35073673

Rong, L., Guedj, J., Dahari, H., Coffield, D. J. Jr., Levi, M., Smith, P., et al. (2013). Analysis of hepatitis C virus decline during treatment with the protease inhibitor danoprevir using a multiscale model. *PLoS Comput. Biol.* 9:e1002959. doi: 10.1371/journal.pcbi.1002959

Rong, L., and Perelson, A. S. (2009). Modeling HIV persistence, the latent reservoir, and viral blips. *J. Theor. Biol.* 260, 308–331. doi: 10.1016/j.jtbi.2009.06.011

Rong, L., and Perelson, A. S. (2010). Treatment of hepatitis C virus infection with interferon and small molecule direct antivirals: viral kinetics and modeling. *Crit. Rev. Immunol.* 30, 131–148. doi: 10.1615/CritRevImmunol.v30.i2.30

Rosenbloom, D. I., Hill, A. L., Rabi, S. A., Siliciano, R. F., and Nowak, M. A. (2012). Antiretroviral dynamics determines HIV evolution and predicts therapy outcome. *Nat. Med.* 18, 1378–1385. doi: 10.1038/nm.2892

Sato, K., Izumi, T., Misawa, N., Kobayashi, T., Yamashita, Y., Ohmichi, M., et al. (2010). Remarkable lethal G-to-A mutations in vif-proficient HIV-1 provirus by individual APOBEC3 proteins in humanized mice. *J. Virol.* 84, 9546–9556. doi: 10.1128/JVI.00823-10

Sato, K., and Koyanagi, Y. (2011). The mouse is out of the bag: insights and perspectives on HIV-1-infected humanized mouse models. *Exp. Biol. Med.* 236, 977–985. doi: 10.1258/ebm.2011.010294

Sato, K., Misawa, N., Fukuhara, M., Iwami, S., An, D. S., Ito, M., et al. (2012). Vpu augments the initial burst phase of HIV-1 propagation and downregulates BST2 and CD4 in humanized mice. *J. Virol.* 86, 5000–5013. doi: 10.1128/JVI.07062-11

Sato, K., Misawa, N., Nie, C., Satou, Y., Iwakiri, D., Matsuoka, M., et al. (2011). A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. *Blood* 117, 5663–5673. doi: 10.1182/blood-2010-09-305979

Schulze-Horsel, J., Schulze, M., Agalaridis, G., Genzel, Y., and Reichl, U. (2009). Infection dynamics and virus-induced apoptosis in cell culture-based influenza vaccine production: flow cytometry and mathematical modeling. *Vaccine* 27, 2712–2722. doi: 10.1016/j.vaccine.2009.02.027

Sharkey, M., Babic, D. Z., Greenough, T., Gulick, R., Kuritzkes, D. R., and Stevenson, M. (2011). Episomal viral cDNAs identify a reservoir that fuels viral rebound after treatment interruption and that contributes to treatment failure. *PLoS Pathog.* 7:e1001303. doi: 10.1371/journal.ppat.1001303

Shultz, L. D., Ishikawa, F., and Greiner, D. L. (2007). Humanized mice in translational biomedical research. *Nat. Rev. Immunol.* 7, 118–130. doi: 10.1038/nri2017

Simon, V., and Ho, D. D. (2003). HIV-1 dynamics *in vivo*: implications for therapy. *Nat. Rev. Microbiol.* 1, 181–190. doi: 10.1038/nrmicro772

Smith, A. M., Adler, F. R., and Perelson, A. S. (2010). An accurate two-phase approximate solution to an acute viral infection model. *J. Math. Biol.* 60, 711–726. doi: 10.1007/s00285-009-0281-8

Trono, D., Van Lint, C., Rouzioux, C., Verdin, E., Barré-Sinoussi, F., Chun, T. W., et al. (2010). HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals. *Science* 329, 174–180. doi: 10.1126/science.1191047

Watanabe, T., Shinya, K., Watanabe, S., Imai, M., Hatta, M., Li, C., et al. (2011). Avian-type receptor-binding ability can increase influenza virus pathogenicity in macaques. *J. Virol.* 85, 13195–13203. doi: 10.1128/JVI.00859-11

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., et al. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 373, 117–122. doi: 10.1038/373117a0

Wilson, J. N., Nokes, D. J., Medley, G. F., and Shouval, D. (2007). Mathematical model of the antibody response to hepatitis B vaccines: implications for reduced schedules. *Vaccine* 25, 3705–3712. doi: 10.1016/j.vaccine.2007.01.012

Wong, J. K., Strain, M. C., Porrata, R., Reay, E., Sankaran-Walters, S., Ignacio, C. C., et al. (2010). *In vivo* $CD8^+$ T-cell suppression of SIV viremia is not mediated by CTL clearance of productively infected cells. *PLoS Pathog.* 6:e1000748. doi: 10.1371/journal.ppat.1000748

Zhang, L., Ramratnam, B., Tenner-Racz, K., He, Y., Vesanen, M., Lewin, S., et al. (1999). Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N. Engl. J. Med.* 340, 1605–1613. doi: 10.1056/NEJM199905273402101

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 June 2013; accepted: 16 August 2013; published online: 10 September 2013.

Citation: Iwami S, Koizumi Y, Ikeda H and Kakizoe Y (2013) Quantification of viral infection dynamics in animal experiments. Front. Microbiol. ***4****:264. doi: 10.3389/fmicb.2013.00264*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 17 December 2013
doi: 10.3389/fmicb.2013.00377

Transgenic expression of the human LEDGF/p75 gene relieves the species barrier against HIV-1 infection in mouse cells

Takuya Tada [1,2,3,4], ***Motohiko Kadoki*** [1,5], ***Yang Liu*** [1,6], ***Kenzo Tokunaga*** [4] **** and Yoichiro Iwakura*** [1,2,3,5] ******

[1] *Center for Experimental Medicine and System Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[2] *Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo, Japan*
[3] *Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan*
[4] *Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan*
[5] *Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba, Japan*
[6] *Stem Cell Research Center, Shanghai Jiao Tong University School of Medicine Renji Hospital, Shanghai, China*

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Akio Adachi, The University of Tokushima Graduate School, Japan
Masako Nomaguchi, The University of Tokushima Graduate School, Japan

****Correspondence:***
Kenzo Tokunaga, Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
e-mail: tokunaga@nih.go.jp;
Yoichiro Iwakura, Center for Animal Disease Models, Research Institute for Biomedical Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan
e-mail: iwakura@rs.tus.ac.jp

Attempts to create mouse models for AIDS have been hampered by species barriers in HIV-1 infection. We previously showed that the nuclear accumulation of HIV-1 preintegration complex (PIC) was suppressed in mouse cells. Lens epithelium-derived growth factor (LEDGF/p75) is a host factor identified as a binding partner of integrase (IN), and has been suggested to be involved in promoting viral integration by tethering PIC to the chromatin, which are observed as nuclear accumulation of IN by LEDGF/p75. Therefore, we here hypothesized that this host factor might act as one of the species-specific barriers in mouse cells. We generated transgenic (Tg) mice that constitutively express human (h) LEDGF/p75. The GFP-fused IN was efficiently accumulated into the nucleus of hLEDGF/p75 expressing Tg mouse embryonic fibroblast (MEF) cells in contrast to the control MEF cells. Importantly, hLEDGF/p75 Tg MEF cells were significantly more susceptible to HIV-1 infection. These results suggest that LEDGF/p75 is one of the host factors that constitute species barrier against HIV-1 in mouse cells.

Keywords: HIV-1, LEDGF/p75, IN, transgenic mouse, species barrier

INTRODUCTION

The number of patients with HIV/AIDS has been increasing throughout the world. In order to study AIDS pathogenesis and to evaluate antiviral drugs and vaccines, animal models for HIV-1 infection need to be established. A mouse model has been considered as one of such candidates because of the availability of inbred and gene-manipulated strains. As a matter of fact, mice are non-permissive for HIV-1 infection because of the species barriers against both the early, and late phases of HIV-1 infection (van Maanen and Sutton, 2003), although the precise mechanisms for the non-permissiveness remain unclear. The finding that the heterokaryons created between human and mouse cells were susceptible to HIV-1 infection (Dragic et al., 1992; Mariani et al., 2001) suggested that mouse cells lack some human-specific cofactors that can support HIV-1 replication.

A number of factors in host cells have been implicated to be involved in the early and late phases of HIV-1 infection; e.g., CD4 as the major receptor for HIV-1 entry (Maddon et al., 1985; Lores et al., 1992), chemokine receptors as coreceptors (Berson et al., 1996; Feng et al., 1996) and cyclin T1 (CycT1) for the efficient viral transcription through binding to HIV-1 Tat (Bieniasz et al., 1998; Wei et al., 1998). However, several Tg mice lines that express human versions of CD4, either CXCR4 (hCD4/hCXCR4/hCycT1 Tg) or CCR5 (hCD4/hCCR5/hCycT1 Tg), and CycT1 did not efficiently support HIV-1 replication despite HIV-1 entry and reverse transcription proceeded normally (Browning et al., 1997; Sawada et al., 1998; Mariani et al., 2000). On the other hand, Tg mice carrying the *pol* gene-deleted HIV-1 proviral genome (HIV-Tg), which we previously generated, were able to not only express all viral mRNA species, including unspliced, singly spliced, and multiply spliced mRNAs, but also produce high levels of gag p24 antigen, after treatment with bacterial lipopolysaccharides (LPS) (Iwakura et al., 1992). Taken together, these results indicate that once the viral genome is efficiently integrated into the host chromosome, viral genes are expressed normally, and that unknown species barriers in mice are still present in the early phase of HIV-1 infection.

HIV-1 preintegration complex (PIC) is composed of newly synthesized viral cDNA and several host and viral proteins, the latter of which include integrase (IN), reverse transcriptase (RT), matrix (MA) and Vpr. The PIC is actively accumulated into the nucleus. We previously showed that the nuclear accumulation of GFP-fused IN (GFP-IN) was significantly reduced in mouse cells than in human cells (Tsurutani et al., 2007), suggesting that inefficient PIC nuclear accumulation in mouse cells might be attributed to the inability of IN to interact with host factors. Lens epithelium-derived growth factor (LEDGF) could be one of such candidates in that it can associate with IN and mediate HIV-1

nuclear accumulation and integration into the chromosome in human cells (Maertens et al., 2003). LEDGF is translated into two proteins, p75 and p52, as a result of alternative splicing (Ge et al., 1998a). LEDGF/p75, but not p52, can associate with IN through its IN-binding domain (IBD; Ge et al., 1998b). Moreover, IN and LEDGF/p75 co-localize in the nucleus of human cells, and recombinant LEDGF/p75 robustly enhances strand transfer activity of IN *in vitro* (Singh et al., 1999). It was also shown that RNAi-mediated knockdown of endogenous LEDGF/p75 abolished IN nuclear accumulation, HIV-1 integration and HIV-1 production (Maertens et al., 2003; Llano et al., 2004b; Ciuffi et al., 2005). LEDGF/p75 is also known as a critical factor for the selection of integration sites such as promoter regions and CpG islands. Thus, LEDGF/p75 has been suggested to be one of important host factors at the PIC nuclear accumulation and integration steps (Bukrinsky, 2004; Llano et al., 2004a, 2006a; Emiliani et al., 2005; Maillot et al., 2013).

In this study, we first created expression plasmids encoding human and mouse LEDGF/p75 (hLEDGF/p75 or mLEDGF/p75, respectively) to compare their ability to support HIV-1 infection in mouse embryonic fibroblast (MEF) cells, and found that the expression of hLEDGF/p75 rendered MEF cells more sensitive to HIV-1 infection than that of mLEDGF/p75. Moreover, we generated hLEDGF/p75 transgenic (hLEDGF/p75 Tg) mice and examined if the expression of hLEDGF/p75 could relieve the species barrier of HIV-1 infection in mouse cells. Transgenic expression of hLEDGF/p75 enhanced IN accumulation in the nucleus and HIV-1 infection in mouse cells, suggesting that LEDGF/p75 is one of the host factors that may determine a species barrier against HIV-1 in mouse cells. We therefore conclude that the transgenic introduction of hLEDGF/p75 would be helpful to generate a small animal model that could be more permissive to HIV-1 infection.

MATERIALS AND METHODS

CELLS

HeLa, 293T, and MT4 cells were obtained from ATCC (Rockville, USA), the RIKEN Cell Bank (Ibaraki, Japan), and the Health Science Research Resources Bank (Osaka, Japan), respectively. MEF cells from LEDGF/p75 Knockout mice were kindly provided by A. Engelman (Shun et al., 2007). HeLa, 293T, and MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS). MT4 cells were maintained in RPMI 1640 medium (Life Technologies, NY, USA) supplemented with 10% FBS.

PLASMIDS

Env-deficient HIV-1 proviral indicator construct pNL-Luc-E-R+, a vesicular stomatitis virus G (VSV-G)-expressing plasmid pHIT/G, and an HIV-1 NL-Env expression plasmid pNLnΔBS (Tokunaga et al., 1998) were described previously (Fouchier et al., 1997; Tokunaga et al., 2001). The expression vector for a codon-optimized HIV-1 IN fused N-terminally to GFP (GFP-IN) was previously constructed (Tsurutani et al., 2007). The hLEDGF/p75 transgene was constructed as follows. hLEDGF/p75 cDNA was obtained from HeLa cells using SuperScript First-Strand Synthesis System (Invitrogen, CA, USA). A 1.6 kb hLEDGF/p75 fragment was amplified by PCR with KOD-Plus- (TOYOBO, Osaka, Japan). The primer pairs used to amplify hLEDGF/p75 fragments were as follows: forward primer 5′-ACG AAT TCG CCA CCA TGA CTC GCG ATT TCA AAC CTG GAG ACC-3′, reverse primer 5′-CCG AAT TCT CAG TTA TCT AGT GTA GAA TCC TTC AGA GAT ATT TCA G-3′, that have *Eco*RI site, Kozak sequence respectively. PCR product was digested with *Eco*RI and inserted into pCAGGS mammalian expression vector (Niwa et al., 1991). Similarly, C-terminally HA-tagged versions of these plasmids were created by using pCAGGS-3HA expression vector (Iwabu et al., 2009). To generate CD4/CXCR4 expression plasmid, CD4, CXCR4, and ECMV IRES were PCR-amplified from pNL-CD4, pNL-CXCR4 (Tokunaga et al., 2001), and pIRESpuro2 (Clontech, CA, USA), and then digested with *Kpn*I/*Xho*I, *Xho*I/*Not*I, and *Not*I, respectively. Digested fragments were inserted into pCAGGS and the resultant expression plasmid was designated pCa-CD4/CXCR4.

TRANSFECTIONS AND PROTEIN ANALYSES

5×10^5 293T cells were transfected with 0.5 μg of either hLEDGF/p75 or mLEDGF/p75 expression plasmid by using FuGENE6 transfection reagent (Promega, Wisconsin, USA), and grown for 48 h. Cell extracts were subjected to gel electrophoresis and then transferred to a nitrocellulose membrane. The membranes were probed with an anti-HA antibody (BD biosciences, NJ, USA). Reacted proteins were visualized by chemiluminescence using an ECL Western blotting detection system (GE Healthcare, Little Chalfont, UK) and monitored using a LAS-3000 imaging system (FujiFilm, Tokyo, Japan).

INFECTION OF MEF CELLS TRANSIENTLY EXPRESSING LEDGF/p75 WITH HIV-1 REPORTER VIRUSES

2.5×10^5 MEF cells derived from LEDGF/p75 knockout mice were cotransfected with 0.5 μg of either hLEDGF/p75 or mLEDGF/p75 expression plasmid, 0.5 μg of pCa-CD4/CXCR4, and 5 ng of phRL-TK Renilla luciferase expression plasmid (Promega) by using Lipofectamine with Plus reagents (Life Technologies) and grown for 48 h. Infection experiments were performed as described below. Viruses were prepared by cotransfecting 293T cells with 1 μg of pNL-Luc-E-R+, 0.5 μg of pNLnΔBS, and 0.5 μg of an empty plasmid by using FuGENE6. After 48 h, the supernatants were treated with 37.5 units/ml DNase I (Roche Applied Science, MD, USA) for 37°C for 30 min and then harvested, and the amount of p24 antigen was measured by using an HIV-1 p24-antigen capture enzyme-linked immunosorbent assay (ELISA) (Advanced BioScience Laboratories, CA, USA). 1×10^4 MEF cells transiently expressing either hLEDGF/p75 or mLEDGF/p75 were infected with 1 ng of p24 antigen of HIV-1 reporter viruses. At 48 h after infection, cells were lysed with Passive Lysis Buffer (Promega). Cell lysates were subjected to the luciferase assay using the Dual Luciferase Reporter Assay Systems (Promega). Luciferase activity was measured by Centro LB 960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany). Values were normalized by Renilla luciferase activity.

GENERATION OF hLEDGF/p75 Tg MICE

The hLEDGF/p75 trangene was digested with *BamH*I-*Hind*III, purified from agarose gel with GeneClean Kit (MP-Biomedicals, CA, USA) and adjusted to a final concentration of 5×10^5 copies/μl (2.13 ng/μl). Purified fragments were injected into the male pronuclei of fertilized mouse embryos (C3H/HeN; CLEA Japan Inc, Tokyo, Japan). Mice were kept under specific-pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine and Systems Biology, the Institute of Medical Science, the University of Tokyo. All experiments were done according to the ethical guidelines for animal experimentation, which was approved by the Institutional Review Board of the Institute of Medical Science, the University of Tokyo.

SOUTHERN BLOT HYBRIDIZATION

Southern blot analyses were carried out as previously described (Smith and Murphy, 1993). Genomic DNA (10 μg) extracted from the mouse tails was digested with *Pst*I. Digested DNA was electrophoresed and blotted onto a membrane. Membrane was hybridized with ^{32}P labeled probe. To detect hLEDGF/p75, an *EcoR*I-*Pst*I (540 bp) fragment from pCAGGS was used as a β-globin probe. The autoradiograms were developed and the band intensity was quantified by a BAS 2000 Bio-Image analyzer (FujiFilm, Tokyo, Japan). Alpha-fetoprotein (AFP) was used as an internal control.

NORTHERN BLOT HYBRIDIZATION

Total RNA from the thymus, spleen, lymph nodes and thioglycolate (TGC)-elicited macrophages of hLEDGF/p75 Tg mice was prepared by the acid guanidium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). In order to obtain macrophages, mice were injected intraperitoneally with 2 ml of 4% TGC (Difco Laboratories, MI, USA). Three days later, peritoneal exudate cells were collected (Saijo et al., 2007). Northern blot analyses were carried out as previously described (Yasuda et al., 2001). hLEDGF/p75 probe (1.6 kb) was generated by PCR with KOD-Plus- by using the following primers: forward primer; 5′-ACG AAT TCG CCA CCA TGA CTC GCG ATT TCA AAC CTG GAG ACC-3′, reverse primer; 5′-CCG AAT TCT CAG TTA TCT AGT GTA GAA TCC TTC AGA GAT ATT TCA G-3′. The autoradiograms were developed, and the radioactivity of each the bands was quantified with the BAS 2000 Bio-Image analyzer (FujiFilm).

REAL-TIME RT-PCR

Total RNA from MEF cells was extracted using ReliaPrep RNA Cell Miniprep System (Promega) according to the manufacturer's instructions. Real-time RT-PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara, Shiga, Japan) with the ABI PRISM 7900HT Sequence Detection System. hLEDGF/p75 mRNA was detected by using the following primers: forward primer; 5′-GAG AAA CAT CAA TGG ATT CTC GAC-3′ and reverse primer; 5′-CTC AAT GCA TCT GTT CAC ATC AAG-3′. GAPDH was detected by using the following primers: forward primer; 5′-GAT GCT GGC GCT GAG TAC G-3′ and reverse primer; 5′-GCA GAG ATG ATG ACC CTT TTG-3′. Levels of hLEDGF/p75 mRNA were normalized with those of GAPDH mRNA.

PREPARATION OF MEF CELLS EXPRESSING hLEDGF/p75

Mouse embryonic fibroblast cells from hLEDGF/p75 Tg and wild-type (WT) mice were prepared as follows. Fetuses were harvested at 13.5 days of gestation. Embryonic internal organs were removed from the abdominal cavity using dissecting forceps. The embryos were transferred to a 10 cm dish containing 10 ml trypsin/EDTA solution and incubated 20 min at 37°C. After trypsinization, cells were washed and cultured in DMEM. Cell extracts were subjected to gel electrophoresis and then transferred to a nitrocellulose membrane. The membranes were probed with an anti-hLEDGF/p75 antibody (BD biosciences, NJ, USA). Reacted proteins were visualized by chemiluminescence using an ECL Western blotting detection system and monitored using a LAS-3000 imaging system.

FLUORESCENCE MICROSCOPY

One day prior to transfection, 2×10^5 HeLa, and MEF cells were plated in 8 well culture slides (Nalgene Nunc International, NY, USA) and transfected with 0.8 μg of DNA (GFP-IN) per well using Lipofectamine 2000 (Invitrogen). After 6 h, medium was replaced with fresh culture medium. Forty eight hours post-transfection, cells were washed once in PBS and fixed with acetone for 5 min. After washing with PBS, 1 μg/ml of Hoechst suspended in PBS was added. After 10 min incubation, cells were covered by a coverslip. Confocal microscopy was performed with a Nikon Optiphot-2 fluorescence microscope with a Bio-Rad MRC 1024 laser confocal imaging system (Nikon, Tokyo, Japan).

INFECTION OF hLEDGF/p75 Tg MEF CELLS WITH VSV-G-PSEUDOTYPED HIV-1 REPORTER VIRUSES

The VSV-G pseudotyped NL4-3-Luc viruses were produced by cotransfecting 293T cells with 1.9 μg of pNL-Luc-E-R+ and 0.1 μg of pHIT/G by using FuGENE6. After 48 h, the supernatants were treated with DNase I for 37°C for 30 min and then harvested, and the amount of p24 antigen was measured by using an HIV-1 p24-antigen capture ELISA. 1×10^4 hLEDGF/p75 Tg MEF cells (No. 089, 110, and 143) were infected with 200 pg of p24 antigen of VSV-G-pseudotyped HIV-1 reporter viruses. At 48 h after infection, cells were lysed with Passive Lysis Buffer and subjected to the luciferase assay. Luciferase activity was measured by Centro LB 960 Microplate Luminometer.

RESULTS

FUNCTIONAL DIFFERENCE BETWEEN HUMAN AND MURINE LEDGF/p75 PROTEINS

We previously showed that the nuclear accumulation of PIC was blocked in mouse cells (Tsurutani et al., 2007), suggesting that mLEDGF/p75 might be defective at this step. To test this hypothesis, we created HA-tagged hLEDGF/p75 and mLEDGF/p75 expression plasmids. Protein expressions in the cells transfected with each plasmid were confirmed by immunoblotting using anti-HA antibodies (**Figure 1A**). We cotransfected MEF cells derived from LEDGF/p75 knockout mice with either hLEDGF/p75 or mLEDGF/p75, together with CD4/CXCR4 expression plasmid. We then examined the efficiency of HIV-1 infection by using MEF cells transiently expressing hLEDGF/p75 or mLEDGF/p75. We

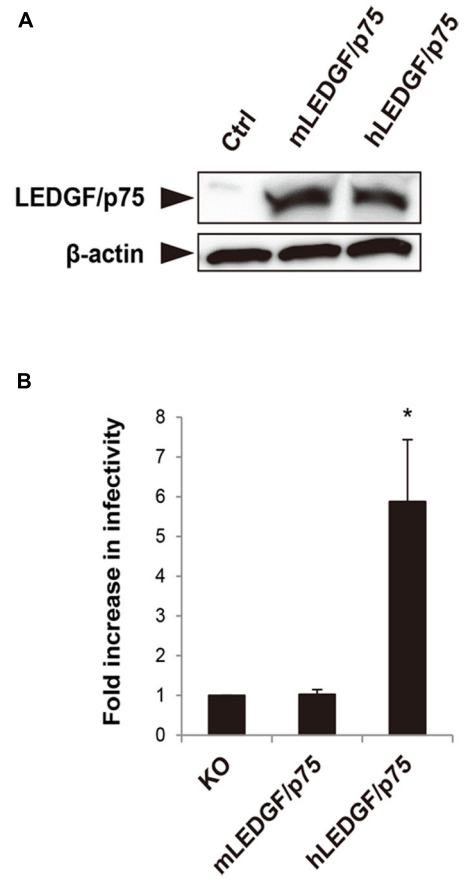


FIGURE 1 | Comparison of hLEDGF/p75 and mLEDGF/p75 in the ability to support HIV-1 infection. (A) Detection of hLEDGF/p75 and mLEDGF/p75 proteins. 293T cells were transfected with either hLEDGF/p75 or mLEDGF/p75 expression plasmid and grown for 48 h. Western blot was performed to detect LEDGF/p75 proteins by using an anti-HA antibody. β-actin was used as an internal control. **(B)** HIV-1 infectivity in cells expressing different LEDGF/p75 proteins. MEF cells derived from LEDGF/p75 knockout (KO) mice were cotransfected with either hLEDGF/p75 or mLEDGF/p75 expression plasmid, pCa-CD4/CXCR4, and phRL-TK Renilla luciferase expression plasmids, and grown for 48 h. MEF cells transiently expressing either hLEDGF/p75 or mLEDGF/p75 were infected with HIV-1 reporter viruses. At 48 h after infection, cells were subjected to the luciferase assay. Values were normalized with Renilla luciferase activity. $*P < 0.01$ (*t*-test).

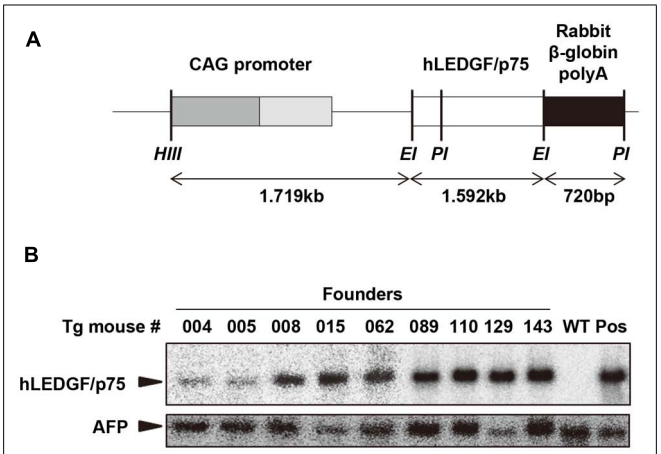


FIGURE 2 | Generation of hLEDGF/p75 Tg mice. (A) Construction of the transgene. hLEDGF/p75 gene (white box) was ligated downstream of the CMV-immediate-early enhancer/chicken β-actin promoter (CAG) promoter. Restriction enzyme sites are indicated as follows: *Hind*III (HIII), *Eco*RI (EI) and *Pst*I (PI). **(B)** Detection of the transgene in hLEDGF/p75 Tg mice by Southern blot hybridization analysis. Cellular DNA (10 μg) was extracted from the mouse tails, digested with *PstI*, and was electrophoresed in an agarose gel. β-globin poly A was used as a probe (black box). Cellular DNA from WT mouse was used as a negative control (WT), and WT DNA containing pCAGGS-hLEDGF/p75 plasmid copies was used as a positive control (Pos). Alpha-fetoprotein (AFP) was used as an internal control. The sizes expected for hLEDGF/p75 and AFP are indicated by arrowheads.

found that infection was more efficient in MEF cells expressing hLEDGF/p75 than those expressing mLEDGF/p75 (**Figure 1B**). These results suggest that hLEDGF/p75 expression renders MEF cells more susceptible to HIV-1 infection.

GENERATION OF TRANSGENIC MICE CARRYING THE hLEDGF/p75

We generated Tg mice carrying the hLEDGF/p75 to examine whether the host factor would be able to confer HIV-1 susceptibility to the animals. To obtain Tg mice expressing high levels of hLEDGF/p75 in multiple tissues, a transgene consisting of the cytomegalovirus immediate-early enhancer/chicken β-actin promoter, the hLEDGF/p75 cDNA and rabbit β-globin poly A, was constructed (**Figure 2A**). Transgenic founders were obtained by microinjecting the transgene into the pronuclei of fertilized embryos from WT parents (C3H/HeN). Southern blot hybridization was carried out to detect the transgene by using a probe specific for β-globin polyA. Out of 165 offspring, we obtained 9 transgenic founder mice in which the hLEDGF/p75 transgene was integrated (**Figure 2B**).

hLEDGF/p75 TRANSGENE EXPRESSION IN hLEDGF/p75 Tg MICE

In order to analyze the expression of hLEDGF/p75 mRNA in hLEDGF/p75 Tg F1 mice, Northern blot hybridization was performed by using total RNAs purified from thymus, spleen, lymph-nodes, and TGC-elicited macrophages. As shown in **Figure 3A**, 1.7 kb bands were detected in hLEDGF/p75 Tg mice, while the endogenous 2.1 kb bands were detected in HeLa and MT4 cells. hLEDGF/p75 mRNA from line No. 089 Tg mouse was detected in all the tissues, in which levels of hLEDGF/p75 expression was similar to those in HeLa or MT4 cells. The mRNA was also present in the thymus and spleen derived from the line No. 110 Tg mouse, in the thymus, spleen and lymph-nodes of the line No. 143 Tg mouse (**Figures 3A,B**). Either the line No. 004, 005, 008, 015, and 062 Tg mice or the line No. 129 Tg mice expressed only in the thymus or lymph-nodes, respectively (data not shown). For further studies, we thus selected 3 lines (line No. 089, 110, and 143 Tg mice) expressing relatively high hLEDGF/p75 mRNA in the thymus, spleen and lymph nodes.

ESTABLISHMENT OF MEF CELLS DERIVED FROM hLEDGF/p75 Tg MICE

To investigate whether hLEDGF/p75 promotes IN nuclear accumulation, we examined the subcellular localization of IN in hLEDGF/p75 Tg mouse cells. We generated MEF cells from hLEDGF/p75 Tg mice and the expression of hLEDGF/p75 was

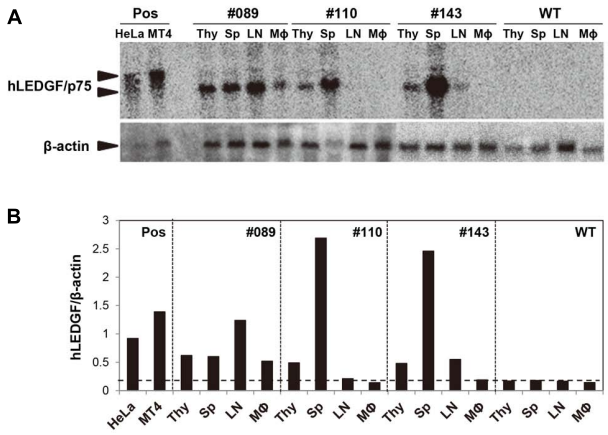


FIGURE 3 | hLEDGF/p75 expression in Tg mice. (A) Total RNA was extracted from the thymus (Thy), spleen (Sp), lymph-nodes (LN), and TGC-elicited macrophages from hLEDGF/p75 Tg mice. Northern blot hybridization was carried out using 10 μg of RNA prepared from tissues. hLEDGF/p75 cDNA was used as a probe, and β-actin was used as an internal control. RNA from the WT mouse and from HeLa and MT4 cells were used as a negative control (WT), and as positive controls (Pos), respectively. The sizes expected for hLEDGF/p75 and β-actin are indicated by arrowheads. **(B)** The band intensity in **(A)** was measured with a BAS 2000 Bio-Image analyzer. Expression levels of hLEDGF/p75 relative to those of β-actin are shown for each transgenic line. The threshold is indicated by a horizontal dotted line.

examined. As shown in **Figure 4A**, MEF cells from hLEDGF/p75 Tg mice were prepared from mice fetuses. Tg mice were mated with WT, and fetuses were harvested at 13.5 days of gestation and removed embryonic internal organs from the abdominal cavity. The embryos were dissociated and trypsinized to produce single-cell suspensions. After expansion, we confirmed that hLEDGF/p75 mRNA (**Figure 4B**) as well as hLEDGF/p75 protein (**Figure 4C**) was detected in MEF cells from the line No. 089, 110, and 143 Tg mice.

ENHANCED NUCLEAR ACCUMULATION OF GFP-IN IN MEF CELLS FROM hLEDGF/p75 Tg MICE

HeLa cells, and MEF cells derived from either WT or hLEDGF/p75 Tg mice (line No. 089, 110, and 143) were transfected with a plasmid expressing codon-optimized IN that was N-terminally fused to GFP, and the subcellular localization was examined by fluorescence and confocal microscopy. As shown in **Figure 5A**, we observed the accumulation of GFP-IN in the nucleus of hLEDGF/p75 Tg-derived MEF cells, making a clear contrast to those from WT mice. To quantitatively evaluate the efficiency of nuclear accumulation of GFP-IN, we classified the cells as follows; (N) the fluorescence of GFP-IN is higher in the nucleus than in the cytoplasm; (N/C) the fluorescence of GFP-IN in the nucleus is similar to the cytoplasm; and (C) the fluorescence of GFP-IN is higher in the cytoplasm than in the nucleus (**Figure 5B**). As expected, GFP-IN preferentially localized mainly into the nucleus in HeLa cells (N: 93.1%), while GFP-IN did not localize exclusively in the nucleus in WT MEF cells (N: 14.2%) but mainly in both the cytoplasm and the nucleus in WT MEF cells (N/C: 56.7%). In contrast, GFP-IN significantly accumulated in the nucleus in MEF cells from line No. 089, 110, and 143 Tg mice (N: 70.8, 49.5, and 65.7, respectively; **Figure 5C**). These data suggest that hLEDGF/p75 enhances IN accumulation into the nucleus of

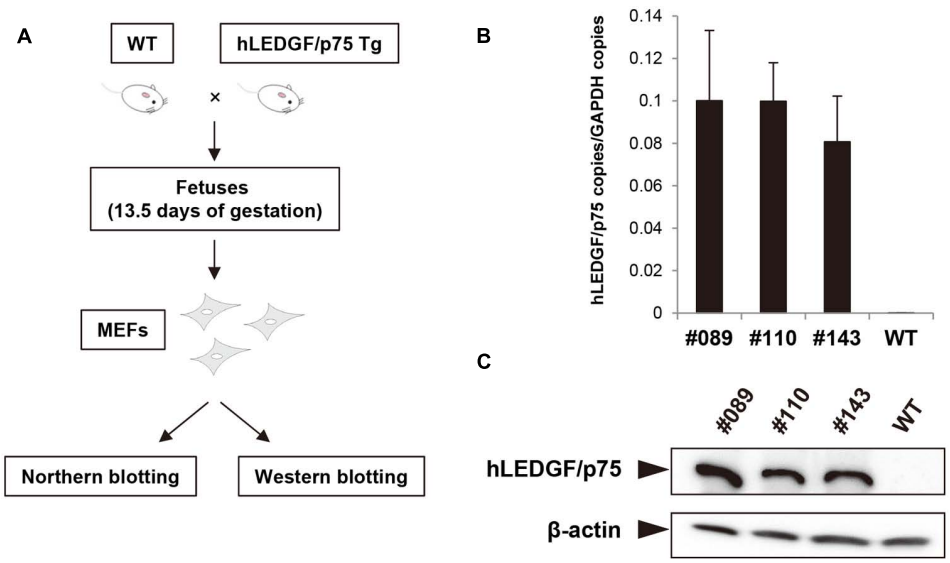


FIGURE 4 | Preparation of MEF cells and hLEDGF/p75 expression in MEF cells. (A) MEF cells from hLEDGF/p75 Tg and WT mice were prepared from mice fetuses. Mice were mated with WT mice, and the resulting fetuses were harvested at 13.5 days of gestation. Embryonic internal organs were removed from the abdominal cavity. The embryos were dissociated and trypsinized to produce single-cell suspensions. After expansion, the expression of hLEDGF/p75 in MEF cells was analyzed. **(B)** Real-time RT-PCR was performed to evaluate the hLEDGF/p75 mRNA expression in hLEDGF/p75 Tg MEF cells by using hLEDGF/p75 specific primers. The copy numbers were normalized with that of GAPDH. **(C)** Western blotting was carried out using proteins from Tg and WT MEF cells. An anti-hLEDGF/p75 antibody was used to detect the protein, and β-actin was detected as an internal control.

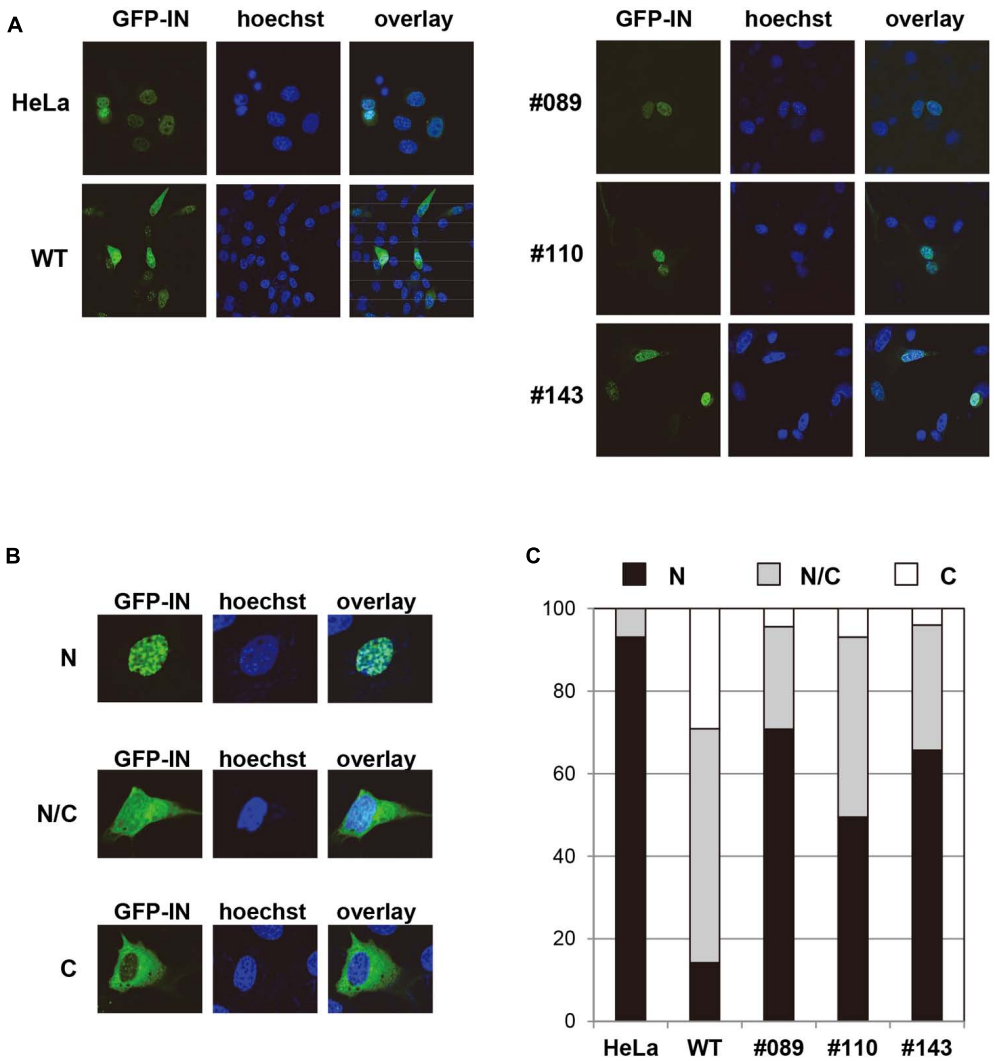


FIGURE 5 | Subcellular localization of GFP-IN in MEF cells. (A) HeLa and MEF cells were transfected with a plasmid expressing GFP-IN (green). After 48 h, cells were fixed and visualized by a confocal fluorescence microscopy. A typical distribution of GFP fluorescence is shown in MEF cells from different Tg lines. Hoechst (blue) was used to stain nuclear DNA. HeLa and WT MEF cells were used as a positive and negative control, respectively. **(B)** GFP positive cells were classified as follows; N, the fluorescence of GFP-IN is higher in the nucleus than in the cytoplasm; N/C, the fluorescence of GFP-IN in the nucleus is similar to the cytoplasm; and C, the fluorescence of GFP-IN is higher in the cytoplasm than in the nucleus. **(C)** GFP positive cells classified by (B) were quantified by counting approximately 100 cells.

mouse cells, probably by stably tethering the viral protein to chromatin as previously reported (Maertens et al., 2003; Llano et al., 2004b; Emiliani et al., 2005; Vanegas et al., 2005; Turlure et al., 2006).

THE SUSCEPTIBILITY OF hLEDGF/p75 Tg MEF CELLS TO HIV-1 INFECTION

Finally, we examined the susceptibility of MEF cells from hLEDGF/p75 Tg mice to HIV-1 infection. Tg (line No. 089, 110, and 143), WT, and LEDGF/p75 knockout MEF cells were infected with VSV-G-pseudotyped NL4-3 viruses, and then compared the efficiency of HIV-1 infection. We found that hLEDGF/p75 Tg MEF cells were significantly more susceptible to HIV-1 than WT and mLEDGF/p75 knockout MEF cells (**Figure 6**). It should be noted that the poor susceptibility of WT MEF cells to HIV-1 infection was almost equivalent to that of the mLEDGF/p75 knockout cells (**Figure 6**, left bar). Taken altogether, we conclude that hLEDGF/p75 is able to relieve the species barrier against HIV-1 infection in mouse cells by supporting HIV-1 PIC accumulation into the nucleus.

DISCUSSION

Pleiotropic functions of hLEDGF/p75 were suggested in HIV-1 infection including promotion of nuclear accumulation of PIC (Cherepanov et al., 2003, 2004; Maertens et al., 2003; Llano et al., 2004b; Ciuffi et al., 2005; Vanegas et al., 2005; Van Maele et al., 2006), selection of HIV-1 integration sites and enhancement of proviral integration (Bukrinsky, 2004; Llano et al., 2004a, 2006a; Emiliani et al., 2005). We previously showed that one of the species

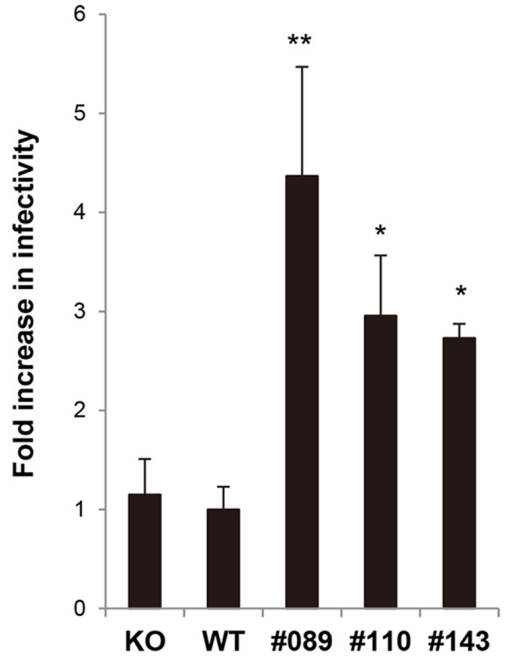


FIGURE 6 | Susceptibility of hLEDGF/p75 Tg MEF cells to HIV-1 infection. MEF cells derived from hLEDGF/p75 Tg, WT and LEDGF knockout (KO) mice were infected with an equivalent dose of VSV-G pseudotyped HIV-1 luciferase reporter virus. After 48 h, cells were lysed and subjected to luciferase assay. The data shown are the mean ± SD of triplicate experiments.*$P < 0.05$, **$P < 0.01$ (*t*-test).

barriers resides at the PIC nuclear accumulation step in mouse cells and that this restriction was caused by a dysfunction of the IN-dependent PIC accumulation system (Tsurutani et al., 2007).

In this study, we have generated 9 Tg mouse lines carrying hLEDGF/p75, to elucidate the role of LEDGF/p75 in the nuclear accumulation of IN. Among them, mice from line No. 089, 110, and 143 expressed hLEDGF/p75 in the thymus, spleen, lymph nodes and MEF cells. We found that the nuclear accumulation of HIV-1 IN in hLEDGF/p75 Tg MEF cells was greatly enhanced. Under these assay conditions, GFP-IN was less frequently localized to the nucleus in WT MEF cells. These results are consistent with the efficiency of luciferase expression after infection with HIV-1 pseudoviruses carrying a luciferase gene (**Figure 6**), suggesting that LEDGF/p75 is one of the host factors responsible for the HIV-1 species barrier.

The homology between hLEDGF/p75 and mLEDGF/p75 is high (92.3% amino acid identity). LEDGF/p75 is composed of four functional domains, the N-terminal Pro-Trp-Trp-Pro domain (residues 1–93), the C-terminal IN-binding domain (residues 347–429), the nuclear localization signal and the AT-hook DNA-binding motif (Maertens et al., 2003; Cherepanov et al., 2004, 2005; Vanegas et al., 2005; Llano et al., 2006a,b). The amino acid identity of these domains and motifs are completely matched between mouse and human LEDGF/p75. Therefore, it is likely that the species barrier in mouse cells might be caused by only small structural differences of previously underappreciated regions between mouse and human LEDGF/p75 proteins.

Shun et al. (2007) previously reported that HIV-1 integration was severely reduced in mLEDGF/p75 knockout mouse cells, suggesting that mLEDGF/p75 is able to support HIV-1 infection. In contrast, our data showed that the level of viral infectivity in WT MEF cells was almost equivalent to that in mLEDGF/p75 knockout cells used in the above report (Shun et al., 2007), rather suggesting that the mouse version might not contribute to the infection (**Figure 6**). This discrepancy might be due to the fact that our WT MEF cells are derived from the C3H/HeN mouse strain while mLEDGF/p75 knockout cells are derivatives of C57BL/6 mice. This needs to be elucidated with further experiments by comparison of different mouse strains. It should be noted that strain-specific epigenetic differences in mice, such as methylation patterns, have recently been reported (Schilling et al., 2009).

Small animal models for HIV-1 infection such as *Rag2*$^{-/-}$*/Il2rg*$^{-/-}$ mice (Traggiai et al., 2004) and BLT mice (Denton et al., 2008) have made significant contributions to our understanding of HIV/AIDS pathogenesis. However, the former mice show insufficient induction of an immune response against HIV-1 (Baenziger et al., 2006; An et al., 2007), while the use of latter mice has been influenced by some ethical restrictions and limited availability. On the other hand, immunologically intact transgenic mouse models are relatively straightforward and inexpensive, in which high levels of target gene expression can be easily achieved resulting in an obvious phenotype.

To generate humanized mouse models for HIV-1 infection, so far we have generated hCD4/hCXCR4/hCycT1 Tg mice and hCD4/hCCR5/hCycT1 Tg mice (Tsurutani et al., 2007). The addition of hLEDGF/p75 to these Tg mice should increase the susceptibility of these mice to HIV-1 infection, especially during the early phase of infection. However, we also need to pay attention to other host factors that restrict HIV-1 infection in mice, such as APOBEC3, which is an APOBEC-related cytidine deaminases (Kobayashi et al., 2004), because these inhibitors are also active in mouse cells (Yu et al., 2003; Kobayashi et al., 2004; Mous et al., 2012). Further characterization and identification of factors involved in host range barriers that are also present in the late phase of the viral replication cycle (transcription, RNA, export, and virion budding) should provide a new insight into the molecular mechanisms of HIV-1 replication and clues to the development of new therapeutics.

ACKNOWLEDGMENTS

We thank Ms. S. Kubo for her technical assistance and all of the members of our laboratory for their excellent animal care. We are grateful to A. Engelman (Harvard Medical School, Massachusetts, USA) for providing us with LEDGF knockout MEF cells. Kenzo Tokunaga is supported by grants from the Ministry of Health, Labor, and Welfare of Japan (Research on HIV/AIDS project no.H24-005, H24-008, and H25-010). Yoichiro Iwakura is supported by CREST program of the Japan Science and Technology Agency, the Promotion of Basic Research Activities for Innovative Biosciences, and Grants-in Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

Takuya Tada performed the experiments, analyzed the data and wrote the paper. Motohiko Kadoki analyzed the data. Yang Liu performed the experiments. Kenzo Tokunaga supervised the research, analyzed the data, and wrote the paper. Yoichiro Iwakura designed the study, supervised the work, analyzed data, and wrote the paper.

REFERENCES

An, D. S., Poon, B., Ho Tsong Fang, R., Weijer, K., Blom, B., Spits, H., et al. (2007). Use of a novel chimeric mouse model with a functionally active human immune system to study human immunodeficiency virus type 1 infection. *Clin. Vaccine Immunol.* 14, 391–396. doi: 10.1128/CVI.00403–406

Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M. O., et al. (2006). Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2-/- gamma c-/- mice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15951–15956. doi: 10.1073/pnas.0604493103

Berson, J. F., Long, D., Doranz, B. J., Rucker, J., Jirik, F. R., and Doms, R. W. (1996). A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. *J. Virol.* 70, 6288–6295.

Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1998). Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. *EMBO J.* 17, 7056–7065. doi: 10.1093/emboj/17.23.7056

Browning, J., Horner, J. W., Pettoello-Mantovani, M., Raker, C., Yurasov, S., Depinho, R. A., et al. (1997). Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14637–14641. doi: 10.1073/pnas.94.26.14637

Bukrinsky, M. (2004). A hard way to the nucleus. *Mol. Med.* 10, 1–5.

Cherepanov, P., Devroe, E., Silver, P. A., and Engelman, A. (2004). Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. *J. Biol. Chem.* 279, 48883–48892. doi: 10.1074/jbc.M406307200

Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., et al. (2003). HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. *J. Biol. Chem.* 278, 372–381. doi: 10.1074/jbc.M209278200

Cherepanov, P., Sun, Z. Y., Rahman, S., Maertens, G., Wagner, G., and Engelman, A. (2005). Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75. *Nat. Struct. Mol. Biol.* 12, 526–532. doi: 10.1038/nsmb937

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159. doi: 10.1006/abio.1987.9999

Ciuffi, A., Llano, M., Poeschla, E., Hoffmann, C., Leipzig, J., Shinn, P., et al. (2005). A role for LEDGF/p75 in targeting HIV DNA integration. *Nat. Med.* 11, 1287–1289. doi: 10.1038/nm1329

Denton, P. W., Estes, J. D., Sun, Z., Othieno, F. A., Wei, B. L., Wege, A. K., et al. (2008). Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. *PLoS Med.* 5:e16. doi: 10.1371/journal.pmed.0050016

Dragic, T., Charneau, P., Clavel, F., and Alizon, M. (1992). Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. *J. Virol.* 66, 4794–4802.

Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D., et al. (2005). Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. *J. Biol. Chem.* 280, 25517–25523. doi: 10.1074/jbc.M501378200

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872–877. doi: 10.1126/science.272.5263.872

Fouchier, R. A., Meyer, B. E., Simon, J. H., Fischer, U., and Malim, M. H. (1997). HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *EMBO J.* 16, 4531–4539. doi: 10.1093/emboj/16.15.4531

Ge, H., Si, Y., and Roeder, R. G. (1998a). Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. *EMBO J.* 17, 6723–6729. doi: 10.1093/emboj/17.22.6723

Ge, H., Si, Y., and Wolffe, A. P. (1998b). A novel transcriptional coactivator, p52, functionally interacts with the essential splicing factor ASF/SF2. *Mol. Cell* 2, 751–759. doi: 10.1016/S1097-2765(00)80290-7

Iwabu, Y., Fujita, H., Kinomoto, M., Kaneko, K., Ishizaka, Y., Tanaka, Y., et al. (2009). HIV-1 accessory protein Vpu internalizes cell-surface BST-2/tetherin through transmembrane interactions leading to lysosomes. *J. Biol. Chem.* 284, 35060–35072. doi: 10.1074/jbc.M109.058305

Iwakura, Y., Shioda, T., Tosu, M., Yoshida, E., Hayashi, M., Nagata, T., et al. (1992). The induction of cataracts by HIV-1 in transgenic mice. *AIDS* 6, 1069–1075. doi: 10.1097/00002030-199210000-00002

Kobayashi, M., Takaori-Kondo, A., Shindo, K., Abudu, A., Fukunaga, K., and Uchiyama, T. (2004). APOBEC3G targets specific virus species. *J. Virol.* 78, 8238–8244. doi: 10.1128/JVI.78.15.8238-8244.2004

Llano, M., Delgado, S., Vanegas, M., and Poeschla, E. M. (2004a). Lens epithelium-derived growth factor/p75 prevents proteasomal degradation of HIV-1 integrase. *J. Biol. Chem.* 279, 55570–55577. doi: 10.1074/jbc.M408508200

Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., et al. (2004b). LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. *J. Virol.* 78, 9524–9537. doi: 10.1128/JVI.78.17.9524-9537.2004

Llano, M., Saenz, D. T., Meehan, A., Wongthida, P., Peretz, M., Walker, W. H., et al. (2006a). An essential role for LEDGF/p75 in HIV integration. *Science* 314, 461–464. doi: 10.1126/science.1132319

Llano, M., Vanegas, M., Hutchins, N., Thompson, D., Delgado, S., and Poeschla, E. M. (2006b). Identification and characterization of the chromatin-binding domains of the HIV-1 integrase interactor LEDGF/p75. *J. Mol. Biol.* 360, 760–773. doi: 10.1016/j.jmb.2006.04.073

Lores, P., Boucher, V., Mackay, C., Pla, M., Von Boehmer, H., Jami, J., et al. (1992). Expression of human CD4 in transgenic mice does not confer sensitivity to human immunodeficiency virus infection. *AIDS Res. Hum. Retroviruses* 8, 2063–2071. doi: 10.1089/aid.1992.8.2063

Maddon, P. J., Littman, D. R., Godfrey, M., Maddon, D. E., Chess, L., and Axel, R. (1985). The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* 42, 93–104. doi: 10.1016/S0092-8674(85)80105-7

Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., et al. (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J. Biol. Chem.* 278, 33528–33539. doi: 10.1074/jbc.M303594200

Maillot, B., Levy, N., Eiler, S., Crucifix, C., Granger, F., Richert, L., et al. (2013). Structural and functional role of INI1 and LEDGF in the HIV-1 preintegration complex. *PLoS ONE* 8:e60734. doi: 10.1371/journal.pone.0060734

Mariani, R., Rasala, B. A., Rutter, G., Wiegers, K., Brandt, S. M., Krausslich, H. G., et al. (2001). Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. *J. Virol.* 75, 3141–3151. doi: 10.1128/JVI.75.7.3141-3151.2001

Mariani, R., Rutter, G., Harris, M. E., Hope, T. J., Krausslich, H. G., and Landau, N. R. (2000). A block to human immunodeficiency virus type 1 assembly in murine cells. *J. Virol.* 74, 3859–3870. doi: 10.1128/JVI.74.8.3859-3870.2000

Mous, K., Jennes, W., Camara, M., Seydi, M., Daneau, G., Mboup, S., et al. (2012). Expression analysis of LEDGF/p75, APOBEC3G, TRIM5alpha, and tetherin in a Senegalese cohort of HIV-1-exposed seronegative individuals. *PLoS ONE* 7:e33934. doi: 10.1371/journal.pone.0033934

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193–199. doi: 10.1016/0378-1119(91)90434-D

Saijo, S., Fujikado, N., Furuta, T., Chung, S. H., Kotaki, H., Seki, K., et al. (2007). Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat. Immunol.* 8, 39–46. doi: 10.1038/ni1425

Sawada, S., Gowrishankar, K., Kitamura, R., Suzuki, M., Suzuki, G., Tahara, S., et al. (1998). Disturbed CD4+ T cell homeostasis and in vitro HIV-1 susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. *J. Exp. Med.* 187, 1439–1449. doi: 10.1084/jem.187.9.1439

Schilling, E., El Chartouni, C., and Rehli, M. (2009). Allele-specific DNA methylation in mouse strains is mainly determined by cis-acting sequences. *Genome Res.* 19, 2028–2035. doi: 10.1101/gr.095562.109

Shun, M. C., Raghavendra, N. K., Vandegraaff, N., Daigle, J. E., Hughes, S., Kellam, P., et al. (2007). LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes Dev.* 21, 1767–1778. doi: 10.1101/gad.1565107

Singh, D. P., Ohguro, N., Chylack, L. T. Jr., and Shinohara, T. (1999). Lens epithelium-derived growth factor: increased resistance to thermal and oxidative stresses. *Invest. Ophthalmol. Vis. Sci.* 40, 1444–1451.

Smith, D. R., and Murphy, D. (1993). Genomic analysis of transgenic animals: southern blotting. *Methods Mol. Biol.* 18, 323–327. doi: 10.1385/0-89603-245-0:323

Tokunaga, K., Greenberg, M. L., Morse, M. A., Cumming, R. I., Lyerly, H. K., and Cullen, B. R. (2001). Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. *J. Virol.* 75, 6776–6785. doi: 10.1128/JVI.75.15.6776-6785.2001

Tokunaga, K., Kojima, A., Kurata, T., Ikuta, K., Akari, H., Koyama, A. H., et al. (1998). Enhancement of human immunodeficiency virus type 1 infectivity by Nef is producer cell-dependent. *J. Gen. Virol.* 79(Pt 10), 2447–2453.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J. C., Lanzavecchia, A., et al. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304, 104–107. doi: 10.1126/science.1093933

Tsurutani, N., Yasuda, J., Yamamoto, N., Choi, B. I., Kadoki, M., and Iwakura, Y. (2007). Nuclear import of the preintegration complex is blocked upon infection by human immunodeficiency virus type 1 in mouse cells. *J. Virol.* 81, 677–688. doi: 10.1128/JVI.00870–876

Turlure, F., Maertens, G., Rahman, S., Cherepanov, P., and Engelman, A. (2006). A tripartite DNA-binding element, comprised of the nuclear localization signal and two AT-hook motifs, mediates the association of LEDGF/p75 with chromatin in vivo. *Nucleic Acids Res.* 34, 1653–1665. doi: 10.1093/nar/gkl052

van Maanen, M., and Sutton, R. E. (2003). Rodent models for HIV-1 infection and disease. *Curr. HIV Res.* 1, 121–130. doi: 10.2174/1570162033352075

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F., and Debyser, Z. (2006). Cellular co-factors of HIV-1 integration. *Trends Biochem. Sci.* 31, 98–105. doi: 10.1016/j.tibs.2005.12.002

Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M., and Poeschla, E. (2005). Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering. *J. Cell Sci.* 118, 1733–1743. doi: 10.1242/jcs.02299

Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 92, 451–462. doi: 10.1016/S0092-8674(00)80939-3

Yasuda, J., Miyao, T., Kamata, M., Aida, Y., and Iwakura, Y. (2001). T cell apoptosis causes peripheral T cell depletion in mice transgenic for the HIV-1 vpr gene. *Virology* 285, 181–192. doi: 10.1006/viro.2001.0964

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., et al. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* 302, 1056–1060. doi: 10.1126/science.1089591

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 October 2013; accepted: 22 November 2013; published online: 17 December 2013.

Citation: Tada T, Kadoki M, Liu Y, Tokunaga K and Iwakura Y (2013) Transgenic expression of the human LEDGF/p75 gene relieves the species barrier against HIV-1 infection in mouse cells. Front. Microbiol. ***4****:377. doi: 10.3389/fmicb.2013.00377*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 14 May 2013
doi: 10.3389/fmicb.2013.00117

Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail

Takeo Kuwata[1], Kaori Takaki[1], Ikumi Enomoto[1], Kazuhisa Yoshimura[2] and Shuzo Matsushita[1]*

[1] Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[2] AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Tetsuro Matano, University of Tokyo, Japan
Tsutomu Murakami, National Institute of Infectious Diseases, Japan
Hirofumi Akari, Kyoto University, Japan

***Correspondence:**
Shuzo Matsushita, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan.
e-mail: shuzo@kumamoto-u.ac.jp

The role of antibodies in protecting the host from human immunodeficiency virus type 1 (HIV-1) infection is of considerable interest, particularly because the RV144 trial results suggest that antibodies contribute to protection. Although infection of non-human primates with simian immunodeficiency virus (SIV) is commonly used as an animal model of HIV-1 infection, the viral epitopes that elicit potent and broad neutralizing antibodies to SIV have not been identified. We isolated a monoclonal antibody (MAb) B404 that potently and broadly neutralizes various SIV strains. B404 targets a conformational epitope comprising the V3 and V4 loops of Env that intensely exposed when Env binds CD4. B404-resistant variants were obtained by passaging viruses in the presence of increasing concentration of B404 in PM1/CCR5 cells. Genetic analysis revealed that the Q733stop mutation, which truncates the cytoplasmic tail of gp41, was the first major substitution in Env during passage. The maximal inhibition by B404 and other MAbs were significantly decreased against a recombinant virus with a gp41 truncation compared with the parental SIVmac316. This indicates that the gp41 truncation was associated with resistance to antibody-mediated neutralization. The infectivities of the recombinant virus with the gp41 truncation were 7,900-, 1,000-, and 140-fold higher than those of SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. Immunoblotting analysis revealed that the gp41 truncation enhanced the incorporation of Env into virions. The effect of the gp41 truncation on infectivity was not obvious in the HSC-F macaque cell line, although the resistance of viruses harboring the gp41 truncation to neutralization was maintained. These results suggest that viruses with a truncated gp41 cytoplasmic tail were selected by increased infectivity in human cells and by acquiring resistance to neutralizing antibody.

Keywords: SIV, gp41, truncation, infectivity, resistance, neutralization, antibody

INTRODUCTION

The RV144 trial demonstrated 31% vaccine efficacy for preventing human immunodeficiency virus type 1 (HIV-1) infection (Rerks-Ngarm et al., 2009). Antibodies against the HIV-1, particularly against the V1/V2 loops, correlate inversely with infection risk (Haynes et al., 2012). Further recent isolation of monoclonal antibodies (MAbs) that neutralize a broad range of HIV-1 strains suggest the possibility for developing a vaccine that can induce cross-neutralizing antibodies effective for various HIV-1 strains (Kwong and Mascola, 2012). Although non-human primate models of simian immunodeficiency virus (SIV) infection can facilitate the evaluation of immunogens, epitopes and immune correlates, no potent and broad neutralizing MAb against SIV had been available.

To understand the mechanisms involved in neutralization of infectivity by antibodies in an SIV model, we recently isolated MAb B404 from a SIVsmH635FC-infected rhesus macaque, which potently and broadly neutralizes various SIV strains, such as SIVsmE543-3, SIVsmE660 and the neutralization-resistant variants, genetically diverse SIVmac316, and highly neutralization-resistant SIVmac239 (Kuwata et al., 2011). The B404 epitope, which comprises the V3 and V4 loops of Env and is intensely exposed by ligation of Env to CD4, is the target for potent and broad neutralization of SIV (Kuwata et al., 2013). Vigorous induction of B404-like neutralizing antibodies using the specific VH3 gene with a long complementarity-determining region 3 loop and λ light chain was observed in four SIVsmH635FC-infected macaques. The B404-resistant variants were induced by passaging viruses in the presence of increasing concentrations of B404. Genetical analysis of the gp120 region of B404-resistant variants revealed that the mutations in the C2 region of Env were important for the resistance to antibody-mediated neutralization (Kuwata et al., 2013).

In the present study, we further analyzed B404-resistant variants and determined the precise region responsible for the resistance to antibody-mediated neutralization. Genetic analysis of viruses during passage in the presence of B404 as well as phenotypic analysis using recombinant viruses revealed that a truncation of the gp41 cytoplasmic tail was the primary step leading to escape from neutralization.

MATERIALS AND METHODS

CELLS

PM1 (Lusso et al., 1995), PM1/CCR5 (Yusa et al., 2005), and HSC-F (Akari et al., 1999) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS). TZM-bl (Platt et al., 1998; Derdeyn et al., 2000; Wei et al., 2002; Takeuchi et al., 2008) and 293T (DuBridge et al., 1987) cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS.

GENETIC ANALYSIS OF B404-RESISTANT VARIANTS

The induction of variants resistant to Fab-B404 (Kuwata et al., 2011) from SIVmac316 (Mori et al., 1992) harboring full-length gp41 was performed as described previously (Yoshimura et al., 2006; Hatada et al., 2010; Kuwata et al., 2013). Briefly, 5,000 $TCID_{50}$ (50% tissue culture infectious dose) SIVmac316 was incubated with 5 ng/ml Fab-B404 for 30 min at 37°C. Then, 5×10^4 PM1/CCR5 cells were added to the virus–Fab mixture. After incubation for 5 h, cells were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% FBS without Fab-B404. The culture supernatant was harvested 7 days later and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of Fab-B404. Proviral DNA samples were extracted from cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) after 8, 17, 20, 23, and 26 passages as well as from P26C cells obtained after 26 passages in the absence of Fab-B404. The gp120 region was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) with primers SEnv-F (5′-ATG GGA TGT CTT GGG AAT CAG C-3′) and SER1 (5′-CCA AGA ACC CTA GCA CAA AGA CCC-3′). The whole *env* gene was amplified with primers SRev-F (5′-GGT TTG GGA ATA TGC TAT GAG-3′) and SEnv-R (5′-CCT ACT AAG TCA TCA TCT T-3′). The polymerase chain reaction (PCR) products were cloned using a TA cloning kit (Invitrogen), and subjected to sequencing. Nucleotide sequences were aligned and analyzed phylogenetically using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al., 2011).

CONSTRUCTION OF INFECTIOUS MOLECULAR CLONES WITH THE Env REGION FROM B404-RESISTANT VARIANTS

One of the clones from passage 26, P26B404 clone 26, was selected for construction of recombinant viruses, because this clone had mutations typical of the major population of P26B404 variants. Infectious molecular clones SS, SN, and NS were generated by replacing fragments *Sph*I–*Sac*I [nucleotides (nt) 6,446–9,226], *Sph*I–*Nhe*I (nt 6,446–8,742), and *Nhe*I–*Sac*I (nt 8,742–9,226) with the corresponding regions of SIVmac316, respectively. Mutants F277V and N295S, which have point mutations at amino acid residues 277 and 295 of Env, respectively, were constructed by PCR mutagenesis using the SIVmac316 plasmid as template. The changes from phenylalanine (TTC) to valine (GTC) in F277V and asparagine (AAT) to serine (AGT) in N295S were introduced using primers F277VFw (5′-TTG GTT TGG CGT CAA TGG TAC TAG GGC-3′), F277VRv (5′-GTA CCA TTG ACG CCA AAC CAA G-3′), N295SFw (5′-GGC AAT AGT AGT AGA ACC ATA ATT AG-3′), and N295SRv (5′-AAT TAT GGT TCT ACT ACT ATT GCC-3′). Mutant and parental SIVmac316 plasmids were transfected into 293T cells using X-tremeGENE 9 DNA Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany). After 2 days, the supernatants containing viruses were filtered (0.45 μm) and stored at −80°C.

ANALYSIS OF VIRAL INFECTIVITY

For determination of $TCID_{50}$ in PM1 and PM1/CCR5 cells, 5×10^4 cells in 50 μl were inoculated with 50 μl serially diluted virus stocks in a 96-well plate and cultured for 2 weeks. Virus replication was judged by observation of cytopathic effects (CPE) by light microscopy. The $TCID_{50}$ in TZM-bl cells was determined by measuring luciferase activities. Briefly, 100 μl medium, 50 μl serially diluted virus stock, and 50 μl 1×10^4 cells containing 37.5 μg/ml diethylaminoethyl (DEAE) dextran were added to the wells of a 96-well plate. The plate was then incubated at 37°C for 2 days. After washing with PBS, cells were lysed with 30 μl cell lysing buffer (Promega, Madison, WI, USA) for 15 min at room temperature (RT) and then 10 μl of cell lysate was transferred to a 96-well white solid plate (Coster, Cambridge, MA, USA). Luciferase activity was measured using a Centro XS3 LB960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and a luciferase assay system (Promega). The $TCID_{50}$ was calculated according to the formula of Reed and Muench (1938).

Infectivity of viruses in PM1, PM1/CCR5, and HSC-F cells was evaluated by detecting infected cells using flow cytometry as described previously (Kuwata et al., 2011). Briefly, PM1 and PM1/CCR5 cells were adjusted to 1×10^6 cells/ml and HSC-F cells were adjusted to 5×10^6 cells/ml. Aliquots of 100 μl cells per well in a 24-well plate were inoculated with 100 μl of diluted virus stocks. After incubation for 6 h, 800 μl fresh medium was added to wells. One-half of the cells in each well were collected at 4, 7, and 10 days post-inoculation. Cells were washed with PBS and fixed with IC Fixation Buffer (eBioscience, San Diego, CA, USA). After washing with Permeabilization Buffer (eBioscience) twice, the cells were intracellularly stained with 4 μg/ml (50 μl) anti-p27 Fab, B450 (Kuwata et al., 2011) by incubation for 20 min at RT. The cells were then incubated with 50 μl anti-HA antibody (1:200; 3F10, Roche Molecular Biochemicals) for 20 min at RT followed by incubation with 50 μl of anti-rat-FITC (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min at RT. The stained cells were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Frequencies of infected cells were determined by comparison with an uninfected control. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA, USA).

All infectivity experiments were performed at least twice and the representative results are shown.

ANALYSIS OF NEUTRALIZING ACTIVITIES

The Fab clones B404 and K8, isolated from an SIV-infected macaque (Kuwata et al., 2011), and murine MAb M318T (Matsumi et al., 1995) were used to examine the sensitivity of viruses to antibody-mediated neutralization in TZM-bl cells as described previously (Kuwata et al., 2011). Briefly, 100 μl serially diluted antibodies in duplicate were incubated with 200 $TCID_{50}$ (50 μl) of virus in a 96-well plate. After incubation for 1 h at 37°C, 100 μl

of 1×10^5 TZM-bl cells/ml containing 37.5 μg/ml DEAE dextran were added. After incubation for 2 days, luciferase activities were measured as described above for the analysis of viral infectivity. The 50% inhibitory concentrations (IC_{50}) and maximal percent of inhibition (MPI) were calculated from the average values by non-linear regression using Prism5 (GraphPad Software, San Diego, CA, USA).

Sensitivity to neutralization by B404 in macaque cells was analyzed using HSC-F cells, a cynomolgus macaque cell line immortalized by infection with *Herpesvirus saimiri* (Akari et al., 1999). Fab-B404 was serially diluted and 50 μl aliquots were mixed with 50 μl undiluted or 10-fold diluted virus in a 96-well plate. After 1 h incubation at 37°C, 2×10^5 cells in 100 μl were added to each well and cultured for 1 day. The infected cells were washed twice with PBS, resuspended in 200 μl fresh medium, and cultured in a new 96-well plate. Viral infection was examined 4 days post-inoculation by intracellular staining of p27, as described above for the analysis of viral infectivity. Infectivity was determined in duplicate and the average value was used for the analysis of neutralization.

All neutralizing assays were performed at least twice and the representative results are shown.

WESTERN BLOTTING ANALYSIS OF VIRAL PROTEINS

Cells and supernatants were collected from six-well plate 2 days after transfection of 293T cells with infectious molecular clones, as previously described (Yuste et al., 2005). Supernatants were filtered (0.45 μm) and clarified by centrifugation for 10 min at 3,000 rpm. The clarified supernatants were centrifuged at 13,200 rpm for 90 min at 4°C, and the viral pellets were resuspended in 1 ml PBS and centrifuged again. Pellets were then dissolved in 80 μl sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue]. Cells were washed with PBS and lysed in 300 μl sample buffer. Samples of virions and cell lysates were boiled for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis using SuperSep Ace 5–20% (Wako Pure Chemical Industries, Osaka, Japan). Proteins were transferred to an Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skim milk TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at RT, and then washed three times with TBS-T. For the detection of gp120, the membrane was incubated overnight at 4°C with 1 μg/ml M318T (Matsumi et al., 1995) in 5% skim milk TBS-T. After washing three times with TBS-T, the membrane was incubated with anti-mouse immunoglobulin G (IgG) peroxidase (1:4,000, Santa Cruz Biotechnology) for 1 h at RT. The membrane was washed three times with TBS-T and once with TBS, and then TMB solution (KPL, Gaithersburg, MD, USA) was added to develop color. Viral proteins gp41 and p26 were similarly examined using crude supernatants from bacterial culture producing B408 and B450 (Kuwata et al., 2011), which were mixed with the same amount of 5% skim milk TBS-T. The membrane was incubated with anti-HA-HRP antibody (1:1,000; Roche Molecular Biochemicals) and Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan), and viral proteins were visualized using ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA)

RESULTS

EVOLUTION OF VIRUSES DURING PASSAGE UNDER THE PRESSURE OF Fab-B404

To select for variants resistant to MAb B404, an antibody that targets a conformational epitope comprising the gp120 V3 and V4 loops, we passaged SIVmac316 that possesses a full-length gp41 in PM1/CCR5 cells in the presence of increasing concentrations of Fab-B404. The virus recovered at passage 26 (P26B404) was resistant to neutralization by B404 (V3/V4) and other antibodies, MAbs K8 (CD4i) and M318T (V2), that target epitopes other than that recognized by B404 (Kuwata et al., 2013). The region covering the whole *env* gene were amplified by PCR and cloned from viruses at passage 8, 17, 20, 23, and 26. The nucleotide sequences were phylogenetically analyzed to show the evolution of B404-resistant variants (**Figure 1**). The first major mutation was a change from glutamine (CAG) to a stop codon (TAG) at 733rd amino acid residue of Env. The Q733stop substitution in the gp41 cytoplasmic domain was observed in 12 of 14 clones at passage 8 and in all clones thereafter. Another stop codon (W782stop) was the second

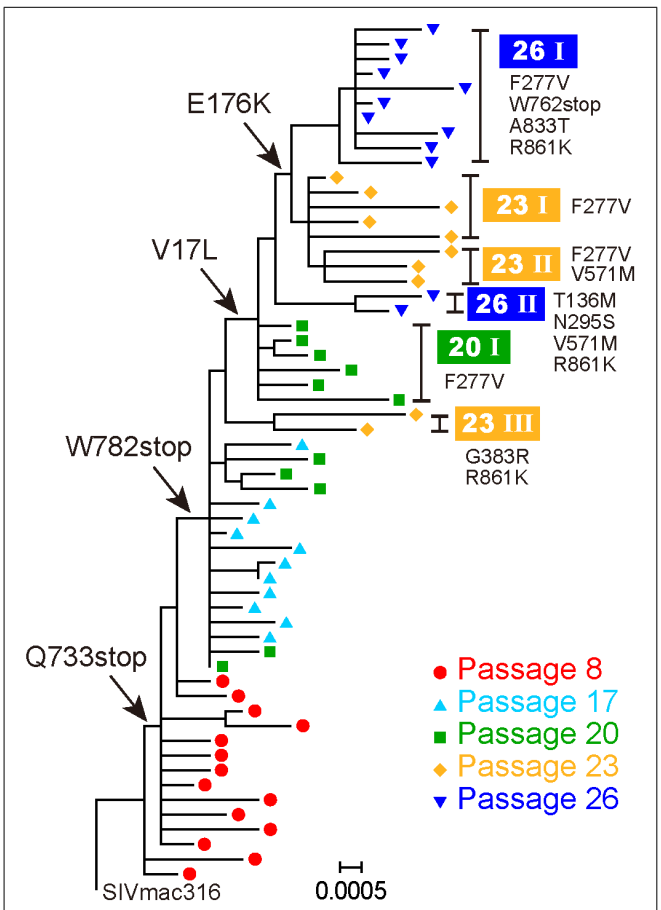


FIGURE 1 | Evolution of B404-resistant variants. Nucleotide sequences of the *env* gene, which were derived from proviruses at passages 8, 17, 20, 23, and 26, were phylogenetically analyzed using MEGA5. Arrows indicate major substitutions as follows: Q733stop, W782stop, V17L, and E176K. Clones with representative amino acid substitutions were separated into groups, which are shown on the right side along with the corresponding substitutions.

major mutation, which was detected after 17 passages. Substitutions V17L in the signal peptide and E176K in the V2 loop emerged after 20 and 23 passages, respectively, although the E176K substitution was also observed in P26C, control viruses after 26 passages in the absence of B404 (**Table 1**). In addition to these substitutions, most of clones acquired the F277V substitution in the late stage of evolution, except for one group at passage 26 which has the N295S substitution (see **Figure 1**, group 26II). Group 26II was clearly distinguished from group 26I by amino acid substitutions, such as T136M, N295S, and D571M/E (**Table 1**), suggesting two lineages of variants in P26B404.

These results demonstrated that the first step in acquiring resistance to B404 was the truncation of gp41. Although substitutions in gp120, represented by F277V, might contribute to the resistance to a high concentration of B404, 20 passages were required for the emergence of these substitutions.

Table 1 | Frequency* of amino acid substitutions in Env clones from B404-resistant variants after 26 passages.

Substitution	Region	P26B404		P26C
		I	II	
	gp120	**($n = 22$)**	**($n = 8$)**	**($n = 14$)**
V17L	Signal peptide	**100%**	**100%**	0.0%
G62S	C1	0.0%	0.0%	21.4%
M67V/L/T	C1	4.5%	0.0%	21.4%
A68T	C1	0.0%	0.0%	**92.9%**
T136M	V1	4.5%	**87.5%**	0.0%
T137I	V1	0.0%	0.0%	14.3%
K141E/R	V1	0.0%	12.5%	7.1%
E176K	V2	**90.9%**	12.5%	35.7%
F277V	C2	**100%**	0.0%	0.0%
N295S	C2	0.0%	**100%**	0.0%
Q341H	V3	13.6%	12.5%	14.3%
D374N	C3	0.0%	0.0%	28.6%
K403R	V4	0.0%	12.5%	7.1%
W441R	C4	4.5%	0.0%	7.1%
	gp41	**($n = 10$)**	**($n = 2$)**	**($n = 7$)**
F528S/L	Extracellular	20.0%	0.0%	0.0%
D571M/E	Extracellular	10.0%	**100%**	0.0%
Q733stop	Cytoplasmic	**100%**	**100%**	0.0%
W762stop	Cytoplasmic	**100%**	0.0%	0.0%
W782stop	Cytoplasmic	**100%**	0.0%	0.0%
A833T	Cytoplasmic	**90.0%**	0.0%	0.0%
R839K	Cytoplasmic	0.0%	0.0%	**57.1%**
R861K	Cytoplasmic	**100%**	**100%**	0.0%

Percentages of substitutions in populations P26B404 and P26C, which were obtained after 26 passages in the presence and absence of B404, respectively, are shown. The P26B404 population is separated into two subpopulations according to the phylogenetic analysis in **Figure 1. All the substitutions that are observed in more than one clone are shown here. Boldface indicates substitutions dominant (>50%) in each population. The numbers of clones analyzed for the gp120 and gp41 regions are shown in parentheses.*

TRUNCATION OF gp41 CONFERRED RESISTANCE TO ANTIBODY-MEDIATED NEUTRALIZATION

To analyze effect of substitutions in B404-resistant variants on resistance to neutralization, recombinant viruses were constructed (**Figure 2**). The *env* region of SIVmac316 was replaced by that of P26B404 clone 26, which had substitutions typical to the P26I group. The resultant molecular clones SS, SN, and NS had substitutions in the entire *env* region, gp120 and gp41 from P26B404I, respectively. SS and NS were predicted to have a truncated gp41 with no other mutation in gp41, because the Q733stop substitution was the first substitution in gp41. Point mutants with substitutions F277V and N295S, which were representative mutations at late passages, were also constructed by PCR mutagenesis.

These mutant viruses were examined for their sensitivity to neutralization by three MAbs B404 (V3/V4 conformational), K8 (CD4i), and M318T (V2). The neutralization of SS that contain the entire *env* region from P26B404I was similar to those of P26B404, indicating that the *env* region is responsible for the

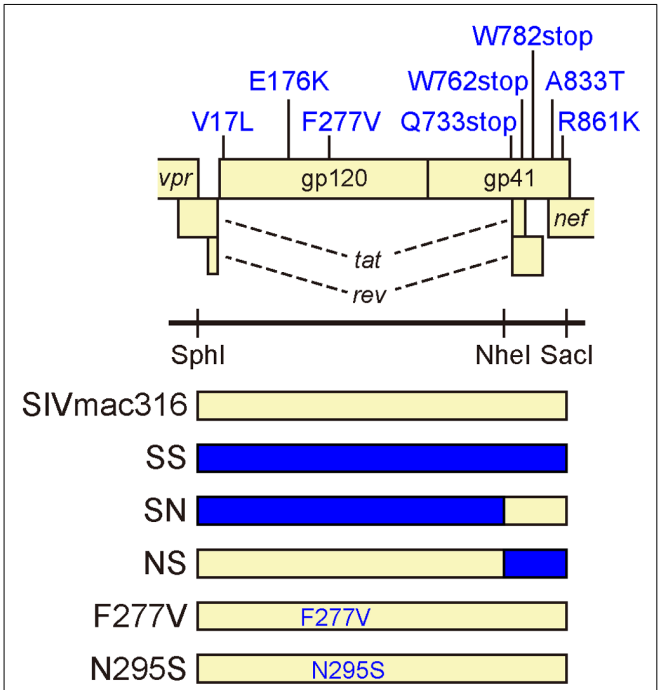
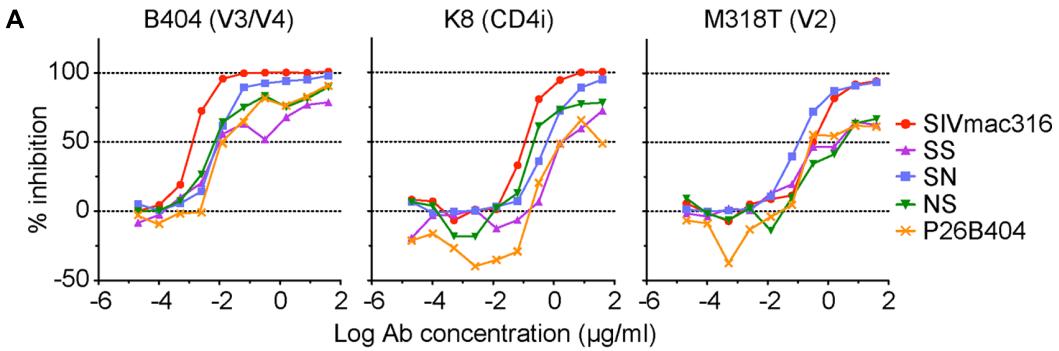


FIGURE 2 | Construction of infectious SIV clones with B404-resistant mutations. The open reading frames of the SIV genome are shown along with the Env substitutions in the B404-resistant variant typical to P26B404I. Full-length SIV clones were constructed from parental SIVmac316 by replacing the *env* region with that of the B404-resistant variant (blue) using *Sph*I, *Nhe*I, and *Sac*I sites. The resultant virus SS contains all of the Env substitutions present in P26B404I. Viruses SN and NS contain substitutions in gp120 and gp41, respectively. Point mutants F277V and N295S were constructed by inserting the substitutions F277V and N295S into SIVmac316, respectively.

resistance to neutralization (**Figure 3A**). Recombinants SN and NS, which have substitutions in gp120 and gp41 from P26B404I, respectively, showed varying degrees of resistance. The IC_{50} values of SN and NS against B404 were intermediates between the parental SIVmac316 and the neutralization-resistant P26B404. Maximal inhibition reached a plateau at 73.8, 82.3, and 81.9% in SS, NS, and P26B404, respectively, but the MPI value of SN (94.9%) was close to that of SIVmac316 (100%; **Figure 3B**). Neutralization resistance to anti-CD4i MAb K8 was characterized by decreases in the IC_{50} value of SN and the MPI of NS. Neutralization by anti-V2 MAb M318T was even enhanced in SN, although NS showed the resistance comparable to those of SS and P26B404. The decreases in MPI values were commonly observed for the neutralization of NS by the three MAbs (**Figure 3B**). Resistance to neutralization was not significantly detected by the point mutants F277V and N295S, except for the neutralization of F277V by K8 (4.3-fold decrease of IC_{50} value). These results indicated that the entire *env* region, including substitutions in both gp120 and gp41, was responsible for the full-resistance of P26B404 to neutralization. The decrease of MPI values for NS suggested that truncation of gp41 by the Q733stop substitution, the first major substitution in viral evolution, was important to escape from the neutralizing antibodies.

INCREASED INFECTIVITY FOR HUMAN CELLS BY SIV WITH A TRUNCATED gp41

Truncation of gp41 in SIV is associated with the adaptation to human cells (Hirsch et al., 1989; Kodama et al., 1989), which may partially contribute to neutralization resistance (Yuste et al., 2005). To explore the mechanism of neutralization resistance of P26B404, the infectivity of recombinant viruses was analyzed by determining the $TCID_{50}$ values of virus stocks prepared by transfection of 293T cells (**Table 2**). The $TCID_{50}$ values in all the human cells tested were significantly higher for SS and NS viruses with truncated gp41 than



B

Neutralization profile of mutant viruses

Virus	B404 (V3/V4)		K8 (CD4i)		M318T (V2)	
	IC_{50}	MPI	IC_{50}	MPI	IC_{50}	MPI
SIVmac316	1.3E-03 (1.0)	100 (0.0)	1.1E-01 (1.0)	99.0 (0.0)	3.3E-01 (1.0)	94.3 (0.0)
SS	1.9E-02 (14.5)	73.8 (-26.2)	1.8E+00 (16.3)	67.9 (-31.1)	9.4E-01 (2.8)	65.5 (-28.8)
SN	9.1E-03 (6.8)	94.9 (-5.1)	5.9E-01 (5.3)	94.7 (-4.4)	1.0E-01 (0.3)	92.5 (-1.8)
NS	6.8E-03 (5.1)	82.3 (-17.7)	2.1E-01 (1.9)	77.0 (-22.0)	1.7E+00 (5.2)	67.6 (-26.7)
F277V	3.8E-03 (2.8)	100 (0.0)	4.8E-01 (4.3)	99.6 (0.5)	3.0E-01 (0.9)	94.8 (0.5)
N295S	2.3E-03 (1.7)	99.6 (-0.4)	1.4E-01 (1.2)	99.9 (0.8)	3.1E-01 (0.9)	97.0 (2.7)
P26B404	1.7E-02 (12.5)	81.9 (-18.1)	8.2E-01 (7.3)	54.6 (-44.5)	2.5E-01 (0.8)	60.0 (-34.3)

Fold change of IC_{50}	% change of MPI
> 10	< -20
3-10	-10- to -20
< 3	> -10

FIGURE 3 | Sensitivities of viruses with B404-resistant mutations to neutralization by MAbs. (A) Sensitivities of mutant viruses SS, SN, and NS to neutralization by MAbs Fab-B404 (anti-V3/V4), Fab-K8 (anti-CD4i), and M318T (anti-V2) are shown. Neutralization of parental SIVmac316 and B404-resistant P26B404 is also shown as controls sensitive and resistant to neutralization, respectively. **(B)** The sensitivities to neutralization are represented by values for IC_{50} (μg/ml) and MPI (%). Viruses were examined for their sensitivities to neutralization by MAbs Fab-B404, Fab-K8, and M318T in TZM-bl cells. The IC_{50} and MPI values were determined using Prism 5. The fold-change of IC_{50} was calculated by dividing the IC_{50} value by that of the parental SIVmac316. Percent change of MPI was calculated by subtracting the MPI value from that of SIVmac316. These changes are shown in parentheses and significant changes are indicated by magenta (IC_{50}: >10; MPI: < −20) and orange (IC_{50}: 3–10; MPI: −20 to −10) highlighting.

parental SIVmac316 and SN, in which gp41 is intact. In particular, NS showed a striking increase in $TCID_{50}$ values, which were 7,100-, 1,000-, and 140-fold higher than those of parental SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. These results indicate that truncation of gp41 caused by the Q733stop substitution increases viral infectivity for human cells.

To compare viral infectivity in human and macaque cells, viral infection was monitored after inoculation of PM1 and PM1/CCR5 human cells and the HSC-F cynomolgus macaque cell line with varying dilutions of virus stocks (**Figure 4**). Consistent with the $TCID_{50}$ analysis, a higher frequency of infected cells was detected earlier in PM1 and PM1/CCR5 cells inoculated with NS than the parental SIVmac316. In contrast, SN showed decreased infectivity in PM1 and PM1/CCR5 cells, apparently because PM1 cells were not infected by a 1,000-fold diluted SN stock. Although the $TCID_{50}$ values of SS were much higher than those of SIVmac316, the replication kinetics of SS were similar to those of SIVmac316 in PM1 and PM1/CCR5 cells. These results suggest that gp41 truncation increases infectivity for human cells and that the substitutions in gp120 of P26B404I are associated with slow and poor replication compared with that of SIVmac316.

Infectivity for macaque cells was more significantly affected than that for human cells by the substitutions in gp120 of P26B404I (**Figure 4**, lower panels). Infected cells were detected in HSC-F cells inoculated with 1,000-fold diluted virus stocks of SIVmac316 and NS, but viral infection in HSC-F cells was limited to a low frequency even by inoculation with 10-fold diluted virus stocks of SS and SN. Truncation of gp41 did not significantly affect replication in HSC-F macaque cells, although truncation of gp41 was disadvantageous for replication in primary T cell cultures from macaques (Hirsch et al., 1989; Kodama et al., 1989).

These results demonstrate that gp41 truncation strikingly increases infectivity for human cells, but not for macaque cells, and that the substitutions in gp120 decrease infectivity in human and macaque cells. Truncation of gp41, which conferred extremely high infectivity for PM1/CCR5 cells, may be the first step to escape from neutralization and the substitutions in gp120 may be the second step to replicate in the presence of high concentration of B404.

Table 2 | Infectivity* of viruses with substitutions from P26B404.

Viruses	PM1	PM1/CCR5	TZM-bl
SIVmac316	4.2E+02 (1.0)	1.4E+03 (1.0)	9.6E+04 (1.0)
SS	2.9E+05 (710)	4.7E+05 (350)	6.3E+06 (66)
SN	2.0E+03 (4.8)	8.4E+03 (6.2)	2.9E+05 (3.1)
NS	2.9E+06 (7,100)	1.4E+06 (1,000)	1.4E+07 (140)

**Infectivity is shown by the $TCID_{50}$/ml values of the viruses, which were prepared by transfection of 293T cells, in PM1, PM1/CCR5, and TZM-bl cells. The fold-change, which was calculated by dividing the mutant $TCID_{50}$/ml value by that of the parental SIVmac316, is shown in the parentheses.*

INCREASED INCORPORATION OF Env INTO VIRIONS IN SIV WITH TRUNCATED gp41

Incorporation of Env into virions was examined using these recombinant viruses, because increased infectivity by gp41 truncation was suggested to be associated with the Env content of virions (Manrique et al., 2001; Zhu et al., 2003, 2006; Yuste et al., 2004, 2005). Analysis of viral proteins in cells and supernatants from transfected 293T cells revealed that incorporation of Env into virions was significantly high in SS and NS viruses with the Q733stop substitution (**Figure 5**). MAb to gp120 showed a higher amount

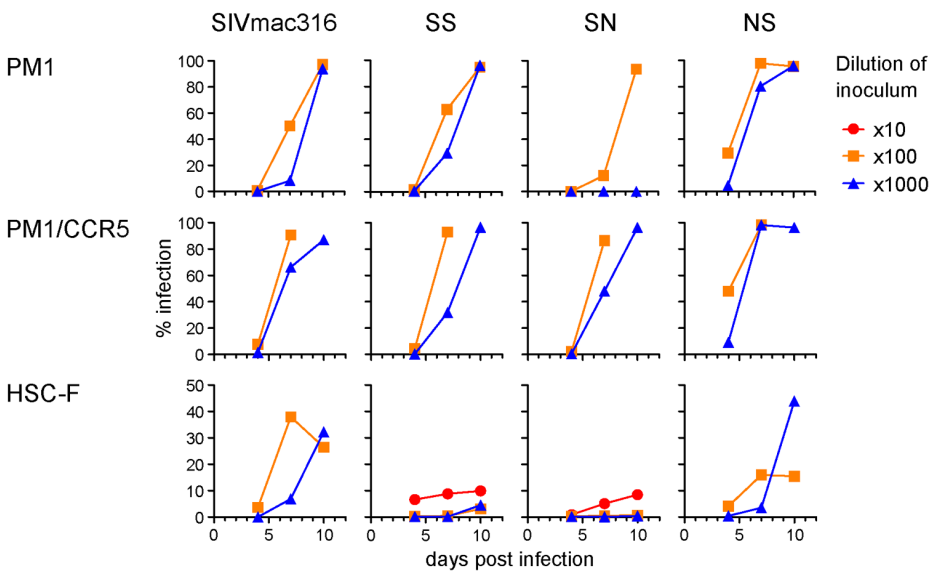


FIGURE 4 | Replication of B404-resistant viruses in human and macaque cells. Frequencies of infected cells at 4, 7, and 10 days post-infection are shown. PM1 and PM1/CCR5 human T cells and HSC-F cynomolgus macaque T cells were infected with the various dilutions of SIVmac316, SS, SN, and NS. Infected cells were detected using flow cytometry after intracellular staining by B450 (anti-p27 Fab).

of gp120 and gp160 in virions from SS and NS than those from SN and the parental SIVmac316, although the production of Env proteins in the transfected cells was at the same level among all the viruses (**Figure 5A**). MAb to gp41 also demonstrated that truncated gp41 was more abundant in virions compared with full-length gp41 (**Figure 5B**). Semi-quantification by densitometric scanning of gp41 and p26 images suggested that the levels of gp41 amount per virion in SS and NS were 12- and 44-fold higher than that of SIVmac316, respectively, after adjusting virion numbers using the p26 amounts. In contrast to the increased amount of Env proteins in virions from viruses with truncated gp41, the level of Gag p27 in virions was low in SS and NS compared with those in SN and SIVmac316 (**Figure 5C**). This indicates that the Env content per virion, which was normalized by the amount of p27, was significantly high in viruses with truncated gp41. These results suggest that truncation of gp41 by the Q733stop substitution enhances incorporation of Env into virions.

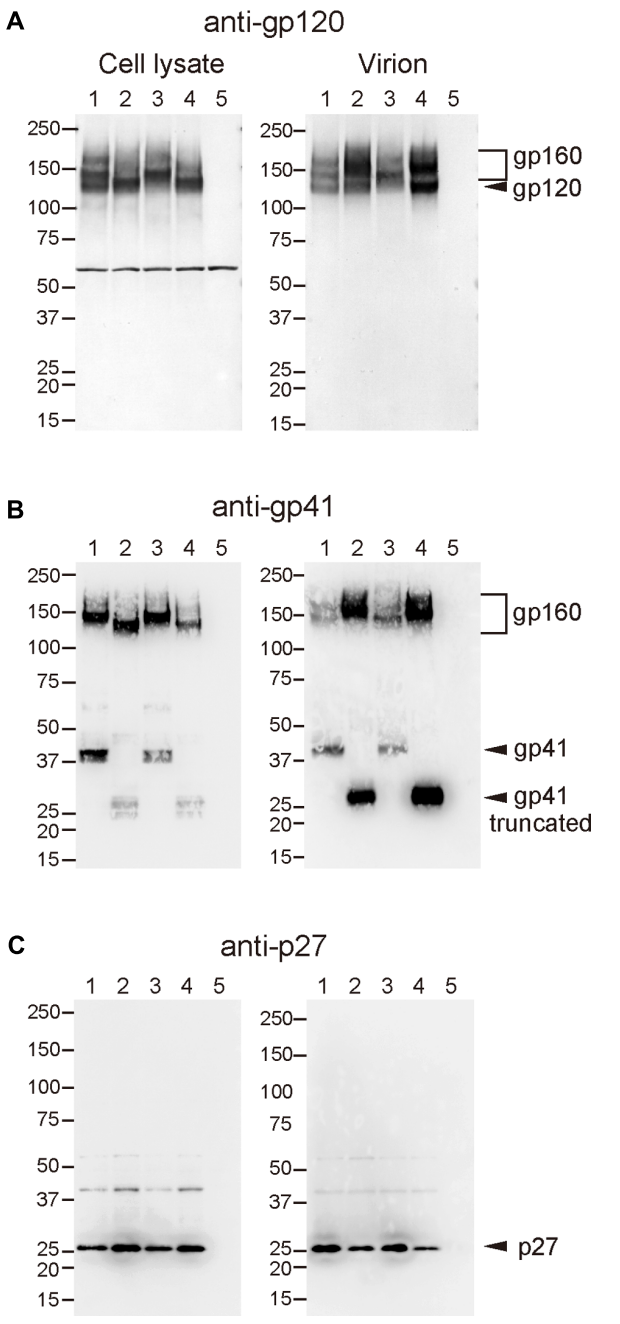


FIGURE 5 | Effect of B404-resistant mutations on the incorporation of Env into virions. Cell lysates (left panels) and virions (right panels) of SIVmac316 (lane 1), SS (lane 2), SN (lane 3), NS (lane 4), and mock-infected control (lane 5), which were prepared from transfected 293T cells, were analyzed by Western blotting. Viral proteins gp120 **(A)**, gp41 **(B)**, and p27 **(C)** were detected using MAbs M318T (anti-gp120 V2), B408 (anti-gp41 cluster I), and B450 (anti-p27), respectively.

NEUTRALIZATION RESISTANCE OF SIV WITH TRUNCATED gp41 IN MACAQUE CELLS

The analysis of infectivity of recombinant viruses suggested that the resistance to neutralization by truncation of gp41 might be due to adaptation to human cells. To examine this hypothesis, sensitivity to neutralization by B404 was determined in HSC-F macaque cells using SIVmac316 and NS, which showed similar infectivity for HSC-F cells (**Figure 4**). In flow cytometric analysis, infection in the presence or absence of B404 demonstrated that the high sensitivity of SIVmac316 and resistance of NS to neutralization were maintained in HSC-F cells (**Figure 6**). The frequency of infected cells decreased from 41.5% to the background level (2.03%) in inoculation with the undiluted stock of SIVmac316. In contrast, infection with NS, even with a 10-fold diluted virus stock, was significant in HSC-F cells in the presence of B404 (**Figure 6A**). Neutralization of NS in HSC-F cells was characterized by a decrease in maximal inhibition (**Figure 6B**), which was also observed in TZM-bl cells (**Figure 3A**). The magnitude of resistance of NS to B404 was greater when infection was performed using the undiluted stock compared with the 10-fold diluted stock, raising the possibility that B404 did not inhibit infection with a high titer of viruses. However, the resistance of NS was shown by infection with a low titer of NS, in which the frequency of infected cells in the absence of B404 (23.8%) was lower than infection with undiluted SIVmac316 (41.5%). Further, immunoblotting analysis revealed that the amount of virions was higher in the virus stock of SIVmac316 than that of NS (**Figure 5**).

These results indicate that gp41 truncation by the Q733stop substitution contributes to neutralization resistance of viruses in macaque cells. This suggests that the resistance to neutralization by truncation of gp41 is not due to the adaptation to human cells. The Q733stop substitution, the first major mutation during passages in the presence of B404, might be selected because it facilitates adaptation of virus to human cells and imparts resistance to antibody.

DISCUSSION

In the present study, truncation of the cytoplasmic tail of gp41, which was caused by the Q733stop substitution in Env, was the first major mutation detected during passage of SIV in the presence of the neutralizing antibody B404. Analysis of recombinant viruses suggested that the gp41 truncation was selected by their resistance to neutralizing antibody, which was characterized by the decrease of maximal inhibition compared with viruses with intact gp41, and

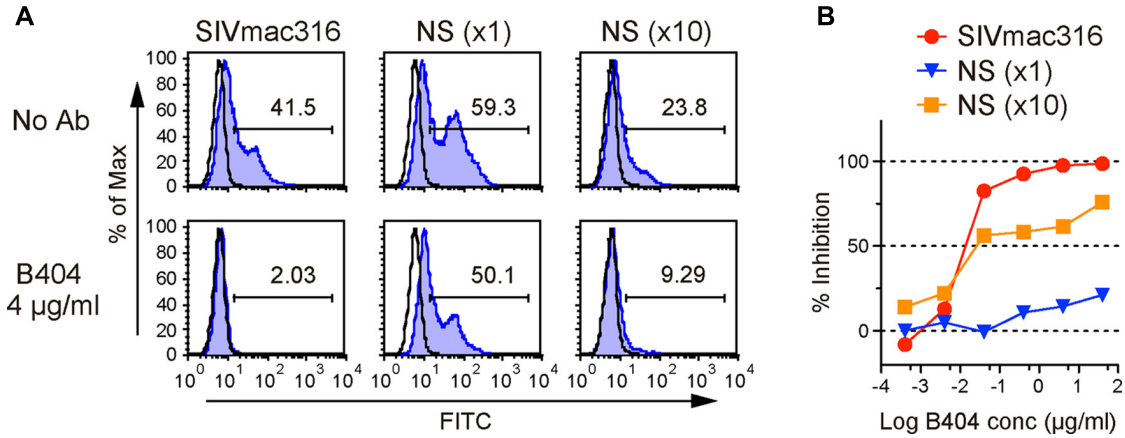


FIGURE 6 | Contribution of gp41 truncation to neutralization resistance in infection of macaque cells. (A) Infection of HSC-F cells in the presence (4 μg/ml) or absence of Fab-B404 is shown. HSC-F cells were inoculated with undiluted SIVmac316 and NS, and 10-fold diluted NS. Viral infection was monitored 4 days post-infection using flow cytometry after intracellular staining by B450 (anti-p27 Fab). **(B)** Neutralization of SIVmac316 and NS by B404 in HSC-F cells are shown. Percent inhibition was calculated by comparison with the infected culture in the absence of B404.

increased infectivity for human cells. The premature stop codon in the gp41 cytoplasmic region was frequently detected in SIV strains propagated in human cell culture *in vitro*, such as the original SIVmac316 clone, SIVmac1A11 and 17E-Fr (Hirsch et al., 1989; Kodama et al., 1989; Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005). The truncation of gp41 is considered as an adaptation of SIV to replication in human cell culture, because the premature stop codon rapidly reverted to express full-length gp41 after infection of rhesus primary cell culture *in vitro* and rhesus macaques *in vivo* (Hirsch et al., 1989; Kodama et al., 1989). Mutant viruses harboring the gp41 truncation showed increased infectivity for human cells, although the effects on infectivity varied depending on the SIV strain and the length of the gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005; Vzorov et al., 2005, 2007). The enhancement effect of gp41 truncation on incorporation of Env into virions, which were demonstrated by quantification of viral proteins in virions (Yuste et al., 2004) and electron tomography analysis of Env trimers on virions (Zhu et al., 2003, 2006), was partly associated with the increased infectivity caused by gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005). Because expression of Env on the cell surface is regulated by the cytoplasmic domain of gp41, truncation of gp41 may increase Env density on both cells and virions (LaBranche et al., 1995; Berlioz-Torrent et al., 1999; Postler and Desrosiers, 2013). Consistent with these studies, infectivity for human cells and Env incorporation into virions was enhanced by gp41 truncation in the present study. Although the mechanism responsible for increasing viral infectivity caused by gp41 truncation remains unclear, the high virion Env content may contribute to the efficient replication of viruses with truncated gp41 in human cells.

The effect of gp41 truncation on susceptibility to antibody-mediated neutralization is controversial, perhaps due to the SIV strains used for the analyses. Because most of prototypic SIV clones with truncated gp41 were macrophage-tropic, CD4-independent, and neutralization-sensitive (Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005), the truncation of gp41 was assumed responsible for the high sensitivity to neutralization. However, the resistance to neutralization by gp41 truncation was shown using the E767stop mutant of SIVmac316 (Yuste et al., 2005). This is consistent with our results using SIVmac316 harboring the Q733stop substitution, indicating that gp41 truncation contributes to resistance of SIVmac316 to neutralization. The increased infectivity of viruses with gp41 truncation in human cells may partially play a role in resistance by overcoming antibody-mediated neutralization via efficient attachment and entry of viruses to cells. However, we showed that gp41 truncation was also associated with neutralization resistance in macaque cells, in which gp41 truncation did not significantly affect infectivity. This suggests that the increased infectivity in human cells does not significantly affect the neutralization resistance of viruses with truncated gp41. As shown by provision of excess Env in trans, high Env content in virions may be critical for antibody-mediated neutralization (Yuste et al., 2005). Further studies will be required to understand the mechanism of resistance to neutralization conferred by gp41 truncation.

In the present study, we demonstrated that truncation of the cytoplasmic tail of gp41 contributes to resistance to antibody-mediated neutralization. Although non-human primate models of SIV infection are commonly used to estimate vaccine efficacy, the lack of broadly neutralizing MAbs has hampered development of antibody-based vaccine candidates in an SIV-macaque model. The broadly neutralizing MAb B404, which neutralizes multiple, diverse SIV isolates (Kuwata et al., 2013), is a useful tool for understanding the mechanism of neutralization in an SIV-macaque model and will contribute to the development of HIV-1 vaccines.

ACKNOWLEDGMENTS

We thank Dr. Hirofumi Akari for providing HSC-F cells. TZM-bl cells were obtained from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work

was supported by MEXT KAKENHI Grant Number 10839786, the Program of Founding Research Centres for Emerging and Re-emerging Infectious Diseases, the Global COE program Global Education and Research Centre Aiming at the Control of AIDS and a grant-in-aid for scientific research (C-24591484) from the Ministry of Education, Culture, Sport, Science and Technology, Japan and a grant from the Ministry of Health, Welfare and Labour of Japan (H24-AIDS-007).

REFERENCES

Akari, H., Nam, K. H., Mori, K., Otani, I., Shibata, H., Adachi, A., et al. (1999). Effects of SIVmac infection on peripheral blood CD4+CD8+ T lymphocytes in cynomolgus macaques. *Clin. Immunol.* 91, 321–329.

Berlioz-Torrent, C., Shacklett, B. L., Erdtmann, L., Delamarre, L., Bouchaert, I., Sonigo, P., et al. (1999). Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. *J. Virol.* 73, 1350–1361.

Bonavia, A., Bullock, B. T., Gisselman, K. M., Margulies, B. J., and Clements, J. E. (2005). A single amino acid change and truncated TM are sufficient for simian immunodeficiency virus to enter cells using CCR5 in a CD4-independent pathway. *Virology* 341, 12–23.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W. A., Ratner, L., et al. (2000). Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* 74, 8358–8367.

DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., and Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein–Barr virus shuttle system. *Mol. Cell. Biol.* 7, 379–387.

Hatada, M., Yoshimura, K., Harada, S., Kawanami, Y., Shibata, J., and Matsushita, S. (2010). Human immunodeficiency virus type 1 evasion of a neutralizing anti-V3 antibody involves acquisition of a potential glycosylation site in V2. *J. Gen. Virol.* 91, 1335–1345.

Haynes, B. F., Gilbert, P. B., Mcelrath, M. J., Zolla-Pazner, S., Tomaras, G. D., Alam, S. M., et al. (2012). Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N. Engl. J. Med.* 366, 1275–1286.

Hirsch, V. M., Edmondson, P., Murphey-Corb, M., Arbeille, B., Johnson, P. R., and Mullins, J. I. (1989). SIV adaptation to human cells. *Nature* 341, 573–574.

Kodama, T., Wooley, D. P., Naidu, Y. M., Kestler, H. W. III, Daniel, M. D., Li, Y., et al. (1989). Significance of premature stop codons in env of simian immunodeficiency virus. *J. Virol.* 63, 4709–4714.

Kuwata, T., Katsumata, Y., Takaki, K., Miura, T., and Igarashi, T. (2011). Isolation of potent neutralizing monoclonal antibodies from an SIV-Infected rhesus macaque by phage display. *AIDS Res. Hum. Retroviruses* 27, 487–500.

Kuwata, T., Takaki, K., Yoshimura, K., Enomoto, I., Wu, F., Ourmanov, I., et al. (2013). Conformational epitope consisting of the V3 and V4 loops as a target for potent and broad neutralization of simian immunodeficiency viruses. *J. Virol.* 87, 5424–5436.

Kwong, P. D., and Mascola, J. R. (2012). Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. *Immunity* 37, 412–425.

LaBranche, C. C., Sauter, M. M., Haggarty, B. S., Vance, P. J., Romano, J., Hart, T. K., et al. (1995). A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. *J. Virol.* 69, 5217–5227.

Lusso, P., Cocchi, F., Balotta, C., Markham, P. D., Louie, A., Farci, P., et al. (1995). Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. *J. Virol.* 69, 3712–3720.

Manrique, J. M., Celma, C. C., Affranchino, J. L., Hunter, E., and Gonzalez, S. A. (2001). Small variations in the length of the cytoplasmic domain of the simian immunodeficiency virus transmembrane protein drastically affect envelope incorporation and virus entry. *AIDS Res. Hum. Retroviruses* 17, 1615–1624.

Matsumi, S., Matsushita, S., Yoshimura, K., Javaherian, K., and Takatsuki, K. (1995). Neutralizing monoclonal antibody against a external envelope glycoprotein (gp110) of SIVmac251. *AIDS Res. Hum. Retroviruses* 11, 501–508.

Mori, K., Ringler, D. J., Kodama, T., and Desrosiers, R. C. (1992). Complex determinants of macrophage tropism in env of simian immunodeficiency virus. *J. Virol.* 66, 2067–2075.

Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* 72, 2855–2864.

Postler, T. S., and Desrosiers, R. C. (2013). The tale of the long tail: the cytoplasmic domain of HIV-1 gp41. *J. Virol.* 87, 2–15.

Reed, L. J., and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 27, 493–497.

Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., et al. (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* 361, 2209–2220.

Takeuchi, Y., Mcclure, M. O., and Pizzato, M. (2008). Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. *J. Virol.* 82, 12585–12588.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.

Vzorov, A. N., Gernert, K. M., and Compans, R. W. (2005). Multiple domains of the SIV Env protein determine virus replication efficiency and neutralization sensitivity. *Virology* 332, 89–101.

Vzorov, A. N., Weidmann, A., Kozyr, N. L., Khaoustov, V., Yoffe, B., and Compans, R. W. (2007). Role of the long cytoplasmic domain of the SIV Env glycoprotein in early and late stages of infection. *Retrovirology* 4, 94.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., et al. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* 46, 1896–1905.

Yoshimura, K., Shibata, J., Kimura, T., Honda, A., Maeda, Y., Koito, A., et al. (2006). Resistance profile of a neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable synergism with anti-CCR5 inhibitors. *AIDS* 20, 2065–2073.

Yusa, K., Maeda, Y., Fujioka, A., Monde, K., and Harada, S. (2005). Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. *J. Biol. Chem.* 280, 30083–30090.

Yuste, E., Johnson, W., Pavlakis, G. N., and Desrosiers, R. C. (2005). Virion envelope content, infectivity, and neutralization sensitivity of simian immunodeficiency virus. *J. Virol.* 79, 12455–12463.

Yuste, E., Reeves, J. D., Doms, R. W., and Desrosiers, R. C. (2004). Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J. Virol.* 78, 6775–6785.

Zhu, P., Chertova, E., Bess, J., Lifson, J. D., Arthur, L. O., Liu, J., et al. (2003). Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15812–15817.

Zhu, P., Liu, J., Bess, J., Chertova, E., Lifson, J. D., Grisé, H., et al. (2006). Distribution and three-dimensional structure of AIDS virus envelope spikes. *Nature* 441, 847–852.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 March 2013; accepted: 25 April 2013; published online: 14 May 2013.

*Citation: Kuwata T, Takaki K, Enomoto I, Yoshimura K and Matsushita S (2013) Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail. Front. Microbiol. **4**:117. doi: 10.3389/fmicb.2013.00117*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 30 January 2013
doi: 10.3389/fmicb.2013.00012

Natural infection of murine norovirus in conventional and specific pathogen-free laboratory mice

Takeo Ohsugi[1]*, Kumi Matsuura[1], Satomi Kawabe[1], Naoko Nakamura[1], Jerald M. Kumar[1,2], Makoto Wakamiya[1], Saki Morikawa[1] and Toru Urano[1]

[1] Division of Microbiology and Genetics, Institute of Resource Development and Analysis, Kumamoto University, Kumamoto, Japan
[2] Centre for Cellular and Molecular Biology, Hyderabad, India

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Akio Adachi, The University of Tokushima Graduate School, Japan
Hironori Sato, National Institute of Infectious Diseases, Japan

***Correspondence:**
Takeo Ohsugi, Division of Microbiology and Genetics, Institute of Resource Development and Analysis, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan.
e-mail: ohsugi@gpo.kumamoto-u.ac.jp

Noroviruses cause most cases of acute viral gastroenteritis worldwide. The lack of a cell culture infection model for human norovirus necessitates the use of molecular methods and/or viral surrogate models amenable to cell culture to predict norovirus inactivation. Murine norovirus (MNV) may be used to construct a small animal model for studying the biology and pathogenesis of noroviruses because MNV is the only norovirus that replicates in cell culture and a small animal model. However, recent studies have shown that natural MNV infection is widespread in laboratory mouse colonies. We investigated MNV infection in both conventional and specific pathogen-free (SPF) genetically modified mice from Japan and the US, and commercial mice from several animal breeders in Japan, using serological and molecular techniques. MNV antibodies were detected in 67.3% of conventional mice and 39.1% of SPF mice from Japan and 62.5% of conventional mice from the US. MNV antibodies were also found in 20% of commercial SPF C57BL/6 mice from one of three breeders. Partial gene amplification of fecal isolates from infected animals showed that the isolates were homologous to reported MNV sequences. These results suggest that both conventional and SPF laboratory mice, including commercial mice, are widely infected with MNV, which might require considerable attention as an animal model of human disease.

Keywords: genetically modified mice, mice, microbiological monitoring, MNV, mouse norovirus, specific pathogen-free

INTRODUCTION

Human noroviruses are the major cause of non-bacterial epidemic gastroenteritis worldwide (Patel et al., 2009). The study of human norovirus has been hampered by the lack of a cell culture system. Murine norovirus (MNV) was first isolated and characterized as a sporadic and lethal pathogen in immunocompromised knockout mice (Karst et al., 2003). MNV is the only norovirus that replicates in cell culture and in a small animal model, though several studies have reported norovirus infection in humans, cattle, swine, dogs, and mice (Wobus et al., 2006; Patel et al., 2009). Thus, MNV is expected to be a surrogate for evaluating the resistance of human norovirus to disinfectants and can be used in animal model studies of human norovirus infection.

Natural MNV infection is prevalent in animal facilities around the world (Hsu et al., 2005; Perdue et al., 2007; Pritchett-Corning et al., 2009). These infections might influence not only the results of a mouse model for studying the biology and pathogenesis of noroviruses, but also those of other biological studies (Lencioni et al., 2008; Cadwell et al., 2010). Until recently, no MNV infection had been reported in laboratory mice in Japan. However, the first reports of MNV detected in conventional mouse colonies in Japan were published in 2009 (Goto et al., 2009b; Kitajima et al., 2009). Recent serological analysis of MNV found that MNV infection is also prevalent in conventional animal colonies in Japan (Kitagawa et al., 2010). However, information on the prevalence of MNV infection in specific pathogen-free (SPF) mice colonies and commercial SPF mice in Japan is not currently available. Most researchers in Japan think that MNV infection is absent among SPF mice facilities.

The Institute of Resource Development and Analysis at Kumamoto University collects, preserves, and distributes experimental animals; thus, numerous mice are transferred to the institute from across Japan and from the other countries for embryo freezing or microbiological cleaning (Nakagata and Yamamura, 2009). Our division in the institute checks the microbiological status of genetically modified mice before or after embryo transfer. In the present study, we examined the prevalence of MNV infection in conventional and SPF genetically modified mice and commercial SPF mice using both serological and molecular biological methods.

MATERIALS AND METHODS

MICE

Ninety-six genetically modified mice derived from 29 facilities in 2011, including 18 mice derived from five different animal facilities in the US which were to be converted to SPF grade by *in vitro* fertilization, were used in this study. Thirty commercial SPF C57BL/6 mice derived from three breeders in Japan were tested for MNV infection. After arriving at our facility, the mice were kept in the laminar flow racks (negative air pressure) in our quarantine animal rooms, which are separate from the SPF animal rooms, until the collection of embryos and sperm. The quarantine period was less than 1 week, except for five mice used in an experiment for the frequency of detection of MNV. All

procedures involving animals and their care were approved by the Animal Care Committee of Kumamoto University in accordance with the Regulations for Animal Experiments at Kumamoto University.

MICROBIOLOGICAL MONITORING

The animals were checked for the presence of specific pathogens using routine methods described previously (Goto et al., 2009a). Briefly, the analysis included a range of viruses, bacteria, mycoplasmas, and protozoans, which are listed in full in **Table 1**. The absence of all tested pathogens indicated that an animal was SPF grade.

SEROLOGICAL TEST FOR MNV

Serological analysis of MNV infection was performed using an enzyme-linked immunosorbent assay (ELISA) kit (Mouse Norovirus; Biotech Trading Partners, Encinitas, CA, USA) according to the manufacturer's protocol.

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was extracted from fresh feces as described previously (Nakamura et al., 2005). Primers and reverse transcriptase-polymerase chain reaction (RT-PCR) conditions were described elsewhere (Kitajima et al., 2009). PCR products were separated by 2% agarose gel electrophoresis.

SEQUENCE ANALYSIS

Polymerase chain reaction products were sequenced as described previously (Ohsugi et al., 2004). Nucleotide sequences were aligned using ClustalW. A phylogenetic tree was generated from a bootstrap analysis of 1,000 replicates using the neighbor joining method. Evolutionary distances were computed by the p-distance method using MEGA 5.05 software (Tamura et al., 2011).

RESULTS

IDENTIFICATION OF MICROBIOLOGICAL STATUS IN MICE REARED UNDER SPF OR CONVENTIONAL CONDITIONS

Microbiological analysis was performed in 96 mice from 29 animal facilities, including 18 mice from five US facilities. In addition, 10 of each SPF female C57BL/6 mouse were purchased from breeders A, B, and C (**Figure 1**). Most of the tested mice were negative for viruses and bacteria (**Table 1**). *Mycoplasma pulmonis* was isolated from a single mouse from a Japanese conventional facility. *Trichomonas* spp. contamination was common in US facilities,

Table 1 | Microbiological status in tested facilities.

Pathogens	Methods*	U.S. facilities		Domestic facilities		Breeders		
		SPF†	Conv†	SPF	Conv	A	B	C
Viruses								
Mouse hepatitis virus	E, I	0/2‡	0/16	0/23	0/55	0/10	0/10	0/10
Sendai virus	E, I	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Bacteria and mycoplasma								
Citrobacter rodentium	C	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Clostridium piliforme	E, I	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Corynebacterium	C	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Helicobacter hepaticus	RT-PCR	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Mycoplasma pulmonis	C, E, I	0/2	0/16	0/23	**1/55**	0/10	0/10	0/10
Pasteurella	C	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Salmonella spp.	C	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Parasites and protozoa								
Aspiculuris tetraptera	M	0/2	0/16	0/23	**23/55**	0/10	0/10	0/10
Syphacia spp.	M	0/2	0/16	0/23	**6/55**	0/10	0/10	0/10
Giardia muris	M	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Spironucleus muris	M	0/2	0/16	0/23	0/55	0/10	0/10	0/10
Trichomonas spp.	M	0/2	**16/16**	0/23	**12/55**	0/10	0/10	0/10
Octomitus pulcher	M	0/2	0/16	0/23	**1/55**	0/10	0/10	0/10
Entamoeba spp.	M	0/2	0/16	0/23	**6/55**	0/10	0/10	0/10
Chilomastix spp.	M	0/2	0/16	0/23	**3/55**	0/10	0/10	0/10
Ectoparasite	M	0/2	0/16	0/23	**5/55**	0/10	0/10	0/10

**C, culture; E, ELISA; I, indirect fluorescent antibody method; M, microscopic examination; RT-PCR, reverse transcriptase-polymerase chain reaction.*
† SPF, specific pathogen-free; Conv, conventional.
‡ Number of positive/number of tested. Positive results are indicated in bold.

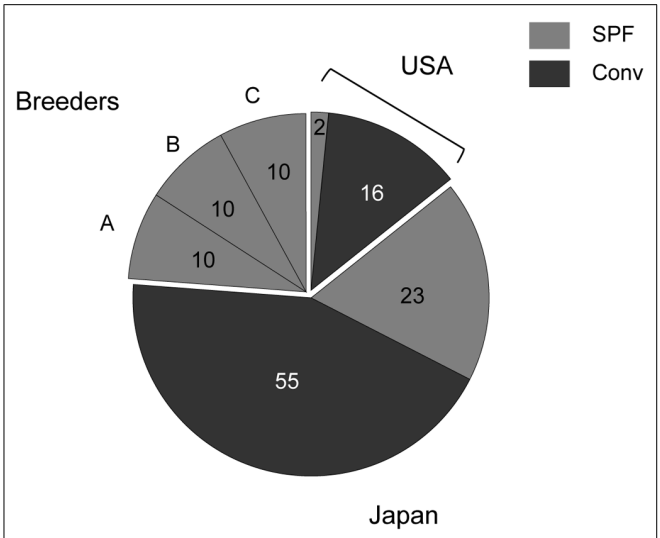


FIGURE 1 | Numbers of tested mice. Two SPF and 16 conventional mice derived from US animal facilities, 23 SPF and 55 conventional mice derived from domestic facilities, and 10 of each SPF female C57BL/6 mouse obtained from breeders A, B, and C were tested in this study.

despite their disparate geographic locations across the country. Almost all of the mice reared under conventional conditions were positive for parasites and protozoa.

ANTIBODIES TO MNV IN ANIMAL FACILITIES IN JAPAN AND THE US AND COMMERCIAL MICE IN JAPAN

Specific pathogen-free mice from the US were negative for MNV, whereas 62.5% (10/16) of conventional mice from US animal facilities were positive for MNV (**Figure 2A**). MNV infection was found in 39.1% (9/23) of SPF and 67.3% (37/55) of conventional mice in Japan. Surprisingly, antibodies were found in 20% (2/10) of commercial SPF mice derived from breeder C.

DETECTION OF MNV IN FECES FROM SPF OR CONVENTIONAL MICE

Stool specimens were collected from 126 animals from 32 separate laboratory colonies in Japan and the US and screened for MNV (**Figure 2B**). No MNV genome segments were isolated from SPF mice from the US, similar to the serological study, whereas MNV was isolated from 25% (4/16) of conventional mice. MNV was isolated from 26% (6/23) of SPF mice and 27.3% (15/55) of conventional mice from Japan. The frequency of MNV isolation was similar between SPF and conventional mice from Japan and the US. In commercial SPF mice, MNV was isolated from 20% (2/10) of the mice from breeder C. The mice positive by both serological and molecular biological methods are shown in **Figure 2C**. These results suggest that MNV infection is widespread in both conventional and SPF mice, including commercial mice in Japan.

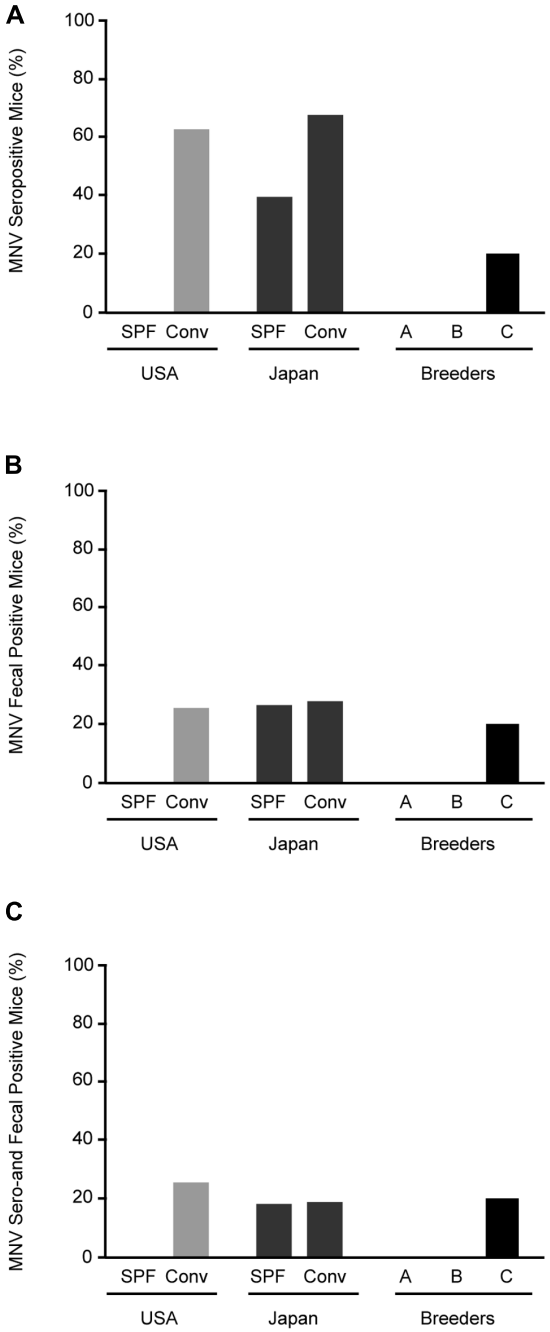


FIGURE 2 | Prevalence of MNV infection in conventional and SPF mice. **(A)** MNV seropositive mice from animal facilities in Japan and the US and breeders in Japan. **(B)** RT-PCR detection of MNV sequences in feces from tested mice. **(C)** Mice positive for both MNV antibodies by ELISA and MNV-specific sequences in feces by RT-PCR. SPF, specific pathogen-free; Conv, conventional.

FREQUENCY OF DETECTION OF MNV

Five mice were autopsied 4 weeks after MNV-specific sequence detection and tested for antibodies against MNV (**Table 2**). All tested mice had antibodies for MNV. The MNV-specific sequence was not always detected in the feces of the mice except mice D and E during tested 4 weeks. The sensitivity of RT-PCR did not improve with different PCR conditions, PCR reagents, or primers. Thus, we speculated that the amount of MNV particles in the feces might vary in each mouse due to, for example, host defenses or intestinal circumstances.

Table 2 | Change of MNV detection from feces after the first isolation.

Mice	Week after detection of MNV					ELISA
	0	1	2	3	4	
A	+*	+	−	−	−	+
B	+	−	−	+	−	+
C	+	−	−	−	−	+
D	+	+	+	+	+	+
E	+	+	+	+	+	+

RT-PCR positive.

PHYLOGENETIC ANALYSIS

The partial nucleotide sequence was determined for the 5′ terminus of the capsid gene (**Figure 3**). Phylogenetic analysis of the partial capsid sequences of MNV showed widespread expression in various facilities throughout Japan and the US, and that these virus strains comprise reported MNV subgroups. Reference strains MNV-1 and Ku29 derived from an SPF facility in Japan were divergent from the other strain group including reference stains MNV-2, -3, and -4. Ku02 and Ku04 derived from the same facilities in Japan exhibited moderate similarity, but the sequences of Ku22 and Ku23 derived from different conventional US facilities were closely related despite their disparate geographic locations across the country.

DISCUSSION

In this study, we analyzed MNV infection in mice derived from animal facilities in Japan, the US, and three breeders (Japan) using serological and molecular techniques. We show that MNV infection is widespread among both conventional and SPF mice in Japan, as well as commercial SPF mice. In a previous study, serological analysis revealed that 53.4% of conventional mice are positive for MNV, 48% positive for mouse hepatitis virus, and 31.2% positive for *M. pulmonis* (Kitagawa et al., 2010). Consistent with this report, we show that MNV is the most prevalent infection in mouse laboratory colonies, and the mice we tested, with the exception of one case, were negative for the antibodies for hepatitis virus and *M. pulmonis* (**Table 1**). This report is

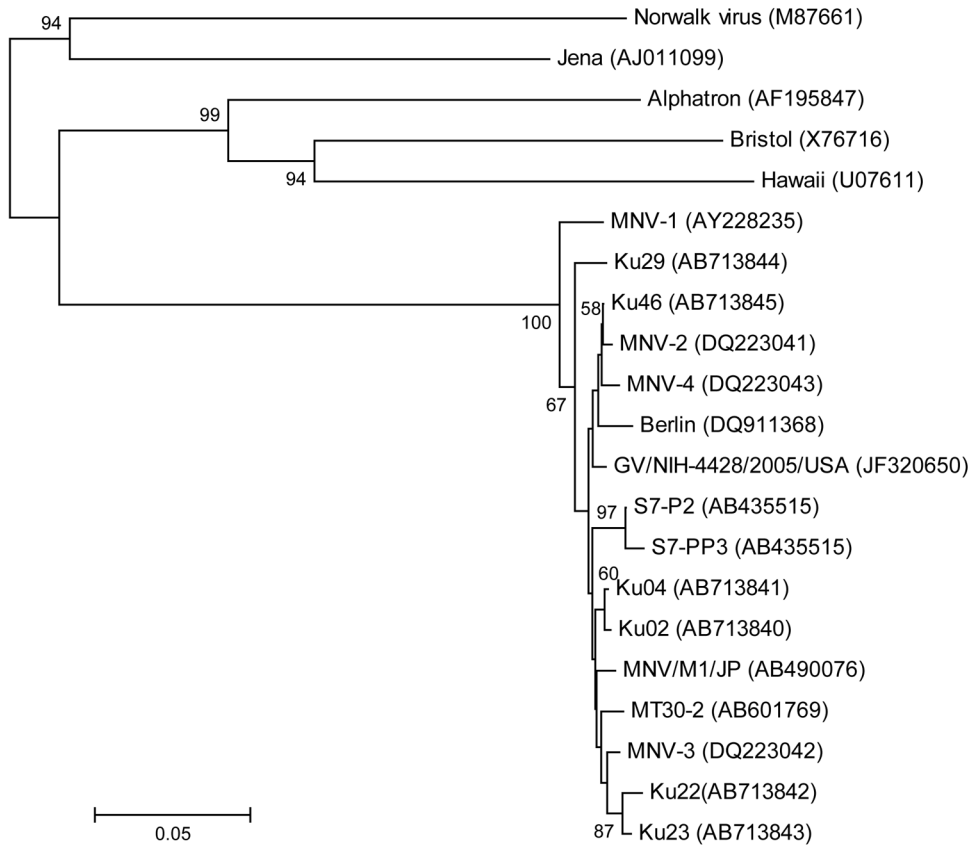


FIGURE 3 | Phylogenetic analysis of the partial capsid gene of norovirus (approximately 350 nucleotides). Phylogenetic relationships were inferred using the neighbor joining method, with the evolutionary distances computed using the p-distance model. The statistical support for tree nodes was evaluated by bootstrap analysis (1,000 replicates). Bootstrap values (>50%) are indicated at branch nodes. Scale bar indicates nucleotide substitutions per site. Numbers in parentheses indicate GenBank accession numbers. Human isolates, Norwalk, Alphatron, Bristol, and Hawaii; bovine isolate, Jena; North American isolates, MNV-1, -2, -3, and -4, GV/NIH-4428/2005/USA, Ku22, and Ku 23; European isolate, Berlin; Japanese isolates, S7-P2, S7-PP3, MNV/M1/JP, MT30-2, Ku02, Ku04, Ku46, and Ku29 (SPF mice).

the first on MNV infection in SPF mice in Japan and commercial mice.

The frequency of MNV infection as detected by RT-PCR was lower than that detected by serological study using ELISA. We detected MNV antibodies in all five mice tested 4 weeks after the first MNV sequences were detected by RT-PCR Using RT-PCR, MNV sequences were detected consistently in the feces of only two mice. Thus, a survey for MNV infection might require the use of serological methods, such as ELISA. The first norovirus to infect mice, MNV-1, caused death in severely immunocompromised mice lacking recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT-1; RAG2/STAT1$^{-/-}$) and mice lacking both the alpha/beta interferon (IFN-α/β) and the IFN-γ receptors (IFN-αβγR$^{-/-}$; Karst et al., 2003; Mumphrey et al., 2007). In this study, the mice infected with MNV exhibited no clinical signs, though the tested mice, with the exception of the mice from breeders, were genetically modified, including compromised immune systems. Recently, MNVs (except MNV-1) were associated with asymptomatic infection and shedding in both normal and genetically modified mice (Hsu et al., 2006; Muller et al., 2007). The origin of MNV in laboratory mouse strains is unknown. Phylogenetic analysis of the partial capsid sequences of MNV in this study showed that the isolated virus strains comprised the reported MNV subgroups, though MNV-1 and Ku29 isolated from the SPF facility were divergent form the main group. However, bootstrap values for the position of Ku29 were not high (<70%). Thus, further study is needed to address whether the Ku29 isolate belongs to a new MNV genotype. These results indicate that attenuated (persistent) strains of MNV, such as MNV-2, -3, and -4, are spread around the world, making the viruses difficult to detect in animals without active screening (Muller et al., 2007; Barron et al., 2011). Using ELISA, we did not detect MNV antibodies in stocked serum from mice transported from domestic animal facilities before 2009 (data not shown). These results suggest that MNV has been spreading through 50% of animal facilities in Japan in only 2 years.

Finally, our results suggest that MNV infections have spread widely throughout the animal facilities in Japan, not only conventional mice colonies, but also SPF mice colonies. The findings warrant further studies to elucidate the spread of MNV infection in SPF colonies, including breeders, in Japan. These results also suggest that SPF laboratory mice, including commercial mice, which might require considerable attention as an animal model for human norovirus infection.

ACKNOWLEDGMENTS

We thank Yoshiteru Tanaka and Keiko Honda for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Japan Leukaemia Research Fund, and Kumamoto University's Centers of Excellence (COE) Programs.

REFERENCES

Barron, E. L., Sosnovtsev, S. V., Bok, K., Prikhodko, V., Sandoval-Jaime, C., Rhodes, C. R., et al. (2011). Diversity of murine norovirus strains isolated from asymptomatic mice of different genetic backgrounds within a single U.S. research institute. *PLoS ONE* 6:e21435. doi: 10.1371/journal.pone.0021435

Cadwell, K., Patel, K. K., Maloney, N. S., Liu, T. C., Ng, A. C., Storer, C. E., et al. (2010). Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell* 141, 1135–1145.

Goto, K., Hayashimoto, N., Ishida, T., Takakura, A., and Kagiyama, N. (2009a). First trial in the developmental phase of the "performance evaluation program" based on the ICLAS animal quality network program: self-assessment of microbiological monitoring methods using test samples supplied by ICLAS. *Exp. Anim.* 58, 47–52.

Goto, K., Hayashimoto, N., Yasuda, M., Ishida, T., Kameda, S., Takakura, A., et al. (2009b). Molecular detection of murine norovirus from experimentally and spontaneously infected mice. *Exp. Anim.* 58, 135–140.

Hsu, C. C., Riley, L. K., Wills, H. M., and Livingston, R. S. (2006). Persistent infection with and serologic cross-reactivity of three novel murine noroviruses. *Comp. Med.* 56, 247–251.

Hsu, C. C., Wobus, C. E., Steffen, E. K., Riley, L. K., and Livingston, R. S. (2005). Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. *Clin. Diagn. Lab. Immunol.* 12, 1145–1151.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., and Virgin, H. W. T. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 299, 1575–1578.

Kitagawa, Y., Tohya, Y., Ike, F., Kajita, A., Park, S. J., Ishii, Y., et al. (2010). Indirect ELISA and indirect immunofluorescent antibody assay for detecting the antibody against murine norovirus S7 in mice. *Exp. Anim.* 59, 47–55.

Kitajima, M., Oka, T., Tohya, Y., Katayama, H., Takeda, N., and Katayama, K. (2009). Development of a broadly reactive nested reverse transcription-PCR assay to detect murine noroviruses, and investigation of the prevalence of murine noroviruses in laboratory mice in Japan. *Microbiol. Immunol.* 53, 531–534.

Lencioni, K. C., Seamons, A., Treuting, P. M., Maggio-Price, L., and Brabb, T. (2008). Murine norovirus: an intercurrent variable in a mouse model of bacteria-induced inflammatory bowel disease. *Comp. Med.* 58, 522–533.

Muller, B., Klemm, U., Mas Marques, A., and Schreier, E. (2007). Genetic diversity and recombination of murine noroviruses in immunocompromised mice. *Arch. Virol.* 152, 1709–1719.

Mumphrey, S. M., Changotra, H., Moore, T. N., Heimann-Nichols, E. R., Wobus, C. E., Reilly, M. J., et al. (2007). Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. *J. Virol.* 81, 3251–3263.

Nakagata, N., and Yamamura, K. (2009). Current activities of CARD as an international core center for mouse resources. *Exp. Anim.* 58, 343–350.

Nakamura, N., Yoshizumi, S., Urano, T., and Ohsugi, T. (2005). Differentiation of mouse hepatitis virus genotypes by restriction fragment length polymorphism analysis. *Lab. Anim.* 39, 107–110.

Ohsugi, T., Kumasaka, T., and Urano, T. (2004). Construction of a full-length human T cell leukemia virus type I genome from MT-2 cells containing multiple defective proviruses using overlapping polymerase chain reaction. *Anal. Biochem.* 329, 281–288.

Patel, M. M., Hall, A. J., Vinje, J., and Parashar, U. D. (2009). Noroviruses: a comprehensive review. *J. Clin. Virol.* 44, 1–8.

Perdue, K. A., Green, K. Y., Copeland, M., Barron, E., Mandel, M., Faucette, L. J., et al. (2007). Naturally occurring murine norovirus infection in a large research institution. *J. Am. Assoc. Lab. Anim. Sci.* 46, 39–45.

Pritchett-Corning, K. R., Cosentino, J., and Clifford, C. B. (2009). Contemporary prevalence of infectious agents in laboratory mice and rats. *Lab. Anim.* 43, 165–173.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.

Wobus, C. E., Thackray, L. B., and Virgin, H. W. T. (2006). Murine norovirus: a model system to study norovirus biology and pathogenesis. *J. Virol.* 80, 5104–5112.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any

commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 December 2012; accepted: 14 January 2013; published online: 30 January 2013.

Citation: Ohsugi T, Matsuura K, Kawabe S, Nakamura N, Kumar JM, Wakamiya M, Morikawa S and Urano T (2013) Natural infection of murine norovirus in conventional and specific pathogen-free laboratory mice. Front. Microbio. ***4****:12. doi: 10.3389/fmicb.2013.00012*

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 August 2013
doi: 10.3389/fmicb.2013.00251

HPV18 E1^E4 is assembled into aggresome-like compartment and involved in sequestration of viral oncoproteins

Naoko Kajitani[1,2], Ayano Satsuka[1,2†], Satoshi Yoshida[2†] and Hiroyuki Sakai[2,3]*

[1] Laboratory of Mammalian Molecular Biology, Department of Molecular and Cellular Biology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan
[2] Laboratory of Gene Analysis, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan
[3] Department of Viral Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Akio Adachi, The University of Tokushima Graduate School, Japan
Masako Nomaguchi, The University of Tokushima Graduate School, Japan

***Correspondence:**
Hiroyuki Sakai, Laboratory of Gene Analysis, Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: hsakai@virus.kyoto-u.ac.jp

†Present address:
Ayano Satsuka, Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA; Satoshi Yoshida, Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA

Papillomavirus is the etiological agent for warts and several squamous carcinomas. Skin cancer induced by cottontail rabbit papillomavirus was the first animal model for virus-induced carcinogenesis. The target organ of the virus infection is stratified epithelium and virus replication is tightly regulated by the differentiation program of the host cell. E1^E4 protein is a viral gene product, and although it is considered to be involved in the control of virus replication, little is known about the biological role. We found that HPV18 E1^E4 was assembled into an aggresome-like compartment and was involved in sequestration of virus oncoproteins, which might contribute to the differentiation-dependent lifecycle of papillomavirus.

Keywords: HPV, E1^E4, aggresome, HPV oncoproteins, HPV replication

INTRODUCTION

Papillomavirus is a small virus containing a double-stranded circular DNA as its genome (zur Hausen, 2002). Genomic DNA of typical papillomavirus, human papillomavirus type 16 (HPV16) or HPV18 is ca. 8 kb long and coding six regulatory genes (E1, E2, E4, E5, E6, E7) and two structural genes (L1, L2). Papillomaviruses are found in almost all mammals and also in amniotes. The virus infects to stratified epithelium organ, such as cutaneous or mucosal membrane, and the infection causes various types of hyperplasia. It is known that the infections of some types of papillomaviruses occasionally induce malignant tumors. The cancer formation by the infection of cottontail rabbit papillomavirus (CRPV) was the first animal model of virus-induced carcinogenesis (Campo, 2002).

The replication of papillomavirus is regulated by the differentiation program of the host cell (Doorbar, 2005). The target cell of the virus infection is basal cell of stratified epithelium, in which the virus replication maintains latent status. Cell division of the infected basal cell produces a daughter cell, and the daughter cell is moved to the surface region of the epithelium and proceeds to differentiate. Virus gene expression and genome replication are enhanced in accordance with the cell differentiation, and the productive replication occurs in fully differentiated cells (Sakakibara et al., 2013). The regulatory mechanism of the differentiation-dependent viral replication remains largely unknown.

A variety of mRNAs are produced by alternative splicing in HPV (Schwartz, 2013). About E4 gene, 5′ region of E1 is jointed to E4 coding sequence by RNA splicing, then the gene product contains five amino acid residues of E1 at the N-terminus of the protein coded by E4 ORF, which is called "E1^E4". By the analysis of the specimens obtained from infected individuals and animals, the expression level of E1^E4 appeared to be intense in differentiated layers of the infected lesions (Sterling et al., 1993; Doorbar et al., 1997), suggesting that E1^E4 is involved in the productive stage of viral replication. It was reported on CRPV that the E1^E4 was required for the viral DNA amplification and the late protein expressions (Peh et al., 2004). E1^E4s of HPV16 and HPV31 were reported to be involved in viral genome amplification and cell cycle maintenance in S-phase of differentiated cells (Nakahara

et al., 2005; Wilson et al., 2005). HPV16 E1^E4 was also reported to be required for viral genome maintenance in undifferentiated basal cells (Nakahara et al., 2005). There was a paper describing that HPV18 E1^E4 was participated in viral genome amplification and the late gene expression in differentiated cells, although it was not involved in the viral genome maintenance or the S-phase maintenance of differentiated cells (Wilson et al., 2007). With these findings, E1^E4 could be considered to play a role in productive phase of virus replication.

Several biological and biochemical properties of E1^E4 were reported previously. HPV16 E1^E4 interacts with cytokeratins and collapses the cytokeratin networks spreading in the cytoplasm (Doorbar et al., 1991). Phosphorylation of HPV16 E1^E4 by extracellular signal-regulated kinase (ERK) was reported to cause conformational change of E1^E4 and promote the interaction with cytokeratins (Wang et al., 2009).

The expression of E1^E4 of HPV16 orHPV18 induces G2/M cell cycle arrest (Davy et al., 2002; Nakahara et al., 2002) and the interaction between the E1^E4 and Cyclin A/B has been proposed to be involved in the cell cycle arrest (Davy et al., 2005, 2006). HPV16 E1^E4 was also reported to be involved in RNA processing through its association with E4-DEAD box protein (E4-DBP), a putative RNA helicase (Doorbar et al., 2000), in RNA metabolism (Bell et al., 2007), and in mitochondrial function (Raj et al., 2004). There was a report that HPV1 E4 induced the redistribution of nuclear domain 10 (ND10) body, which is a candidate site of the HPV genome replication (Roberts et al., 2003). These biological properties of E1^E4 might be involved in the HPV lifecycle, however, their precise roles in virus replication remain to be elucidated.

There is a self-association motif in the C-terminal region of E1^E4, and E1^E4s form aggregates in the cytoplasm through the motifs (Bryan et al., 1998). It was reported that the aggregate had amyloid-like structure (McIntosh et al., 2008). Several viruses were reported to utilize cytoplasmic aggregates called as "aggresome" for their replication (Wileman, 2007). Although the biological significance of the aggregate formed by E1^E4 was unknown, it might contribute to HPV lifecycle.

"Aggresome" was originally defined as a cytoplasmic compartment in which misfolded proteins are assembled (Johnston et al., 1998). Accumulation of misfolded proteins is toxic for cell viability as in the cases of neurological disorders including Parkinson's, Alzheimer's, and Huntchington's diseases. To counteract the toxicity, misfolded proteins are refolded into native structure or eliminated by molecular chaperones or proteasomes, respectively. However, aggregated proteins exhibit resistance to proteolysis. The aggregates are assembled at microtubule organizing center (MTOC) region and form "aggresome", for which the dynein-dependent retrograde transport along microtubules is involved. Aggresomes contain polyubiquitinated proteins, molecular chaperones, and histone deacetylase 6 (HDAC6), and are wrapped in vimentin cage. It is considered that aggresomes activate autophagy pathway and they are processed in autophagy-dependent manner (Kopito, 2000).

In order to investigate E1^E4 function, we searched for cellular factors that interact with 18E1^E4 protein, and vimentin was identified as a candidate. We also found the 18E1^E4 aggregates were wrapped with vimentin as "aggresomes." In this report, we present the structure of 18E1^E4 aggregate and its possible role in HPV replication.

MATERIALS AND METHODS

CELL CULTURE, TRANSFECTION

HeLa, CV1 and 293T cells were maintained with Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. The cells were transfected with plasmid DNA (5 μg) and herring sperm DNA (5 μg; Roche Diagnostics, GmbH, Mannheim, Germany) by a standard calcium phosphate coprecipitation method.

DNA CONSTRUCTION

HPV18 and HPV11 genomic DNAs were provided by Dr. Peter M. Howley (Harvard Medical School, Boston, USA). 18E1^E4, 11E1^E4, 18E5, 18E6, and 18E7 cDNAs were obtained by a polymerase chain reaction (PCR). 18E1^E4 and 11E1^E4 cDNAs were cloned into pPC86 vector (Invitrogen^{TM}, Life Technologies, Corp., Carlsbad, CA, USA), pGEX-5X (Promega Corp., Madison, WI, USA), $pCMV_4$ (Nakahara et al., 2002), and pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA). 18E5, 18E6, and 18E7 cDNAs were cloned into pCMV7.1 (Sigma-Aldrich Corp., St. Louis, MO, USA) in order to express 3xFLAG-tagged proteins.

YEAST TWO-HYBRID SYSTEM

We used ProQuest^{TM} Two-Hybrid System (Invitrogen^{TM}, Life Technologies, Corp, Carlsbad, CA, USA). 18E1^E4 cDNA was cloned into pPC86 vector. For cDNA library, we used ProQuest^{TM} Human Fetal Brain cDNA Library (Invitrogen^{TM}, Life Technologies, Corp., Carlsbad, CA, USA). Screening was performed by following manufacturer's instruction.

GST PULL DOWN ASSAY

Glutathione *S*-transferase (GST)-tagged 18E1^E4 and 11E1^E4 were expressed by using pGEX-5X vector (Promega Corp., Madison, WI, USA). The fusion proteins were expressed in *E. coli* (BL21 strain), and purified with Glutathione Sepharose 4B beads (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK). ^{35}S-methionine labeled protein was synthesized with TNT Quick Coupled Transcription/Translation Systems (Promega Corp., Madison, WI, USA). Vimentin cDNA was obtained by PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) with mRNAs obtained from HeLa cells. The cDNA was cloned into pGEM-3Zf(+) (Promega Corp., Madison, WI, USA) for *in vitro* transcription/translation.

Purified GST-fusion proteins and ^{35}S-Met labeled vimentin were incubated in a binding buffer [20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 4 mM $MgCl_2$, 0.5% Nonidet P-40, 2% skim milk, 2 mM dithiothreitol (DTT)] at 4°C for 2 h. The complex was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), and the vimentin bound to GST-fusion protein was detected with BAS5000 (FUJIFILM Corp., Tokyo, Japan).

IMMUNOPRECIPITATION AND IMMUNOBLOT

Total cell lysates were prepared with triple detergent lysis buffer [150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate] supplemented with protease

inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and 1 mM DTT. The cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were used for immunoprecipitation and immunoblot. The supernatants were used as soluble fractions in several experiments. The pellets were resuspended in 2× SDS sample buffer [0.125 M Tris–HCl (pH6.8), 4% SDS, 0.2 M DTT, 20% glycerol, 0.001% bromophenol blue] and used as insoluble fractions. In our experiment, 10 μg of protein could be obtained from ca. 1×10^4 cells as soluble fraction. For immunoblot analysis, 10 μg of soluble fraction was loaded into each lane. It was not feasible to measure the protein concentration of insoluble fraction, therefore the portion equivalent to 1×10^4 cells was loaded into each lane.

For immunoprecipitation, the cell lysates, Protein-G agarose (Invitrogen Corp., Carlsbad, CA, USA) and an appropriate antibody were incubated in NET-Gel Buffer [150 mM NaCl, 50 mM Tris–HCl (pH7.5), 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin] at 4°C for ≥ 4 h. The complex bound to Protein-G agarose beads was washed six times, and then suspended in 6× SDS sample buffer [0.35 M Tris–HCl (pH6.8), 10% SDS, 0.6 M DTT, 30% glycerol, 0.012% bromophenol blue].

The immunoprecipitation samples or the cell lysates were subjected to SDS-PAGE, and blotted to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK). The immunoblot with anti-β-actin antibody (Clone AC-15; Sigma-Aldrich Corp., St. Louis, MO, USA) was used for checking the protein amount loaded on the gel. Following antibodies were used for immunoblot and immunofluorescence analyses; anti-FLAG polyclonal antibody (F7425), anti-FLAG monoclonal antibody (F3165; Sigma-Aldrich Corp., St. Louis, MO, USA), anti-vimentin antibody (sc-6260), anti-DnaJB6 (Hsp40) antibody (sc-100710), anti-HDAC6 antibody (sc-11420; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-γ-tubulin antibody (ab11316), anti-ubiquitin antibody (ab7780; Abcam plc., Cambridge, UK), and anti-p62 antibody (PM045; Medical & Biological Laboratory Co., Ltd, Nagoya, Japan). Horseradish peroxidase (HRP)-conjugated secondary antibodies and a luminal reagent (ECL-prime) were purchased commercially (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK). The chemiluminescent signal was visualized with a chemiluminescent image analyzer (LAS-3000; FUJIFILM Corp., Tokyo, Japan).

IMMUNOFLUORESCENCE ANALYSIS

For IFA, the cells on cover glasses were fixed with 4% paraformaldehyde (PFA) at room temperature for 5 min or cold methanol (for γ-tubulin staining) at −20°C for 20 min, permeabilized with 0.1% Nonidet P-40/phosphate buffered saline (PBS) followed by blocking with 5% non-fat dry milk. The samples were incubated with each primary antibodies diluted as manufacturer's instruction. Alexa Fluor® 488 or 546 labeled secondary antibodies were purchased commercially (Molecular Probes®, Life Technologies Corp., Carlsbad, CA, USA). Fluorescence microscope (Axiovert200 and AxioVision; Carl Zeiss Microscopy GmbH, Jena, Germany) and confocal laser microscope (TCS SP2 AOBS, Leica Microsystems GmbH, Wetzlar, Germany) were used for analysis.

CHEMICAL INHIBITORS

At 24 h after transfection, chemical inhibitors were added into the culture medium. After incubation for 24 h, the cells were harvested to obtain cell lysates, or fixed for IFA. Nocodazole (Sigma-Aldrich Co., St. Louis, MO, USA), MG132 (Wako Pure Chemicals Industries, Ltd, Osaka, Japan), ciliobrevin D (Merck KGaA, Darmstadt, Germany), and tubacin (Santa Cruz Biotechnologies, Inc., Dallas, TX, USA) were purchased commercially, solubilized in DMSO, and used at 10, 10, 20, and 10 μM, respectively, as working concentration.

RESULTS

INTERACTION BETWEEN HPV18 E1^E4 AND VIMENTIN PROTEINS

To investigate the biological function of HPV E1^E4, we searched for cellular factors that interact with HPV18 E1^E4 protein (18E1^E4). For screening, we used the yeast two-hybrid assay with 18E1^E4 as the bait. Among several factors identified from screening, we focused on vimentin, a cytoskeletal protein categorized as a type III intermediate filament. It is known that vimentin is involved in various cellular events, including cell division and signal transduction (Ivaska et al., 2007); therefore, we considered that the interaction between 18E1^E4 and vimentin might induce a modification of the cellular structure or function to adapt it in favor of virus replication.

The interaction between 18E1^E4 and vimentin was confirmed by the *in vitro* binding assay (**Figure 1A**). We could detect weak but significant interaction between GST-tagged 18E1^E4 and vimentin obtained by *in vitro* translation, indicating the direct binding of 18E1^E4 to vimentin. Similar binding activity was also detected between HPV11 E1^E4 (11E1^E4) and vimentin (**Figure 1A**).

Next, we examined the interaction between endogenous vimentin and ectopically expressed 18E1^E4 in 293T cells. For the experiment, a FLAG epitope-tag was added at the N-terminus of 18E1^E4. The FLAG-18E1^E4 was immunoprecipitated with anti-FLAG antibody, and then co-precipitated vimentin was detected by immunoblotting analysis. As shown in **Figure 1B**, 18E1^E4 could interact with endogenous vimentin.

Intracellular localizations of 18E1^E4 and vimentin were analyzed with CV1 cells, monkey kidney epithelial cells negative for papillomavirus infection. In control cells, vimentin showed filamentous distribution throughout the cytoplasm (**Figure 1C**). The ectopically expressed 18E1^E4 formed aggregates in cytoplasm, as reported previously (**Figure 1C**; Nakahara et al., 2002). In 18E1^E4-expressing cells, vimentin was co-localized at the E1^E4 aggregates. 11E1^E4 could also form aggregates with vimentin (**Figure 1C**). The fine localization of 18E1^E4 and vimentin was examined with confocal microscopic analysis, and it was found that the aggregate was wrapped by vimentin (**Figure 1D**). These results indicated that 18E1^E4 and vimentin were associated *in vivo*, and suggested that 18E1^E4 recruited vimentin to its aggregates through this interaction.

E1^E4 WAS ASSEMBLED INTO AGGRESOME-LIKE COMPARTMENT

It is known that cytoplasmic aggregates are organized in cells infected with several viruses; the aggregate is called an "aggresome" (Wileman, 2007). Aggresomes are structures assembled close to the MTOC. They contain molecular chaperones, ubiquitinated

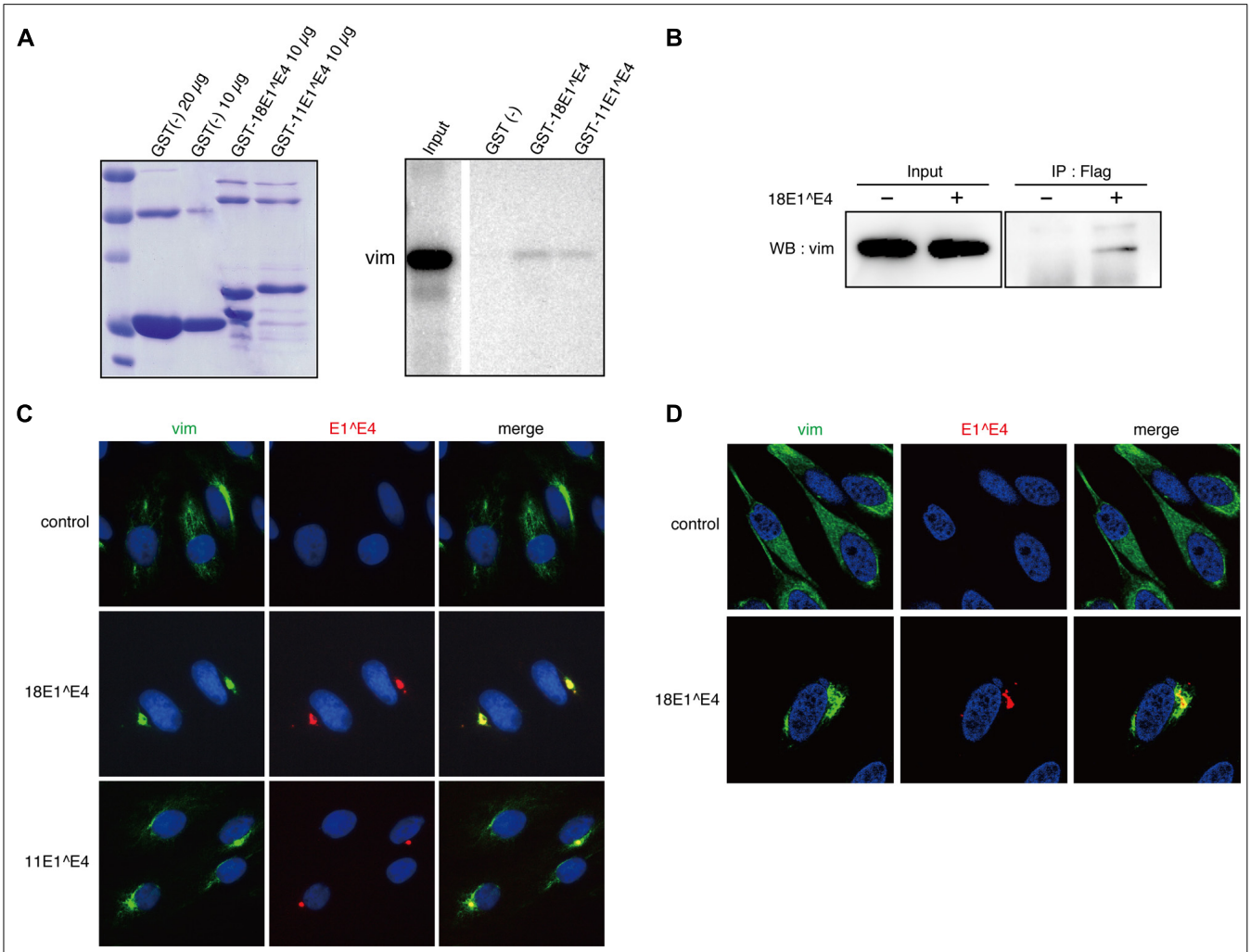


FIGURE 1 | Interaction between 18E1^E4 and vimentin. (A) GST-pull down experiment was performed with GST-E1^E4 and *in vitro* translated vimentin. The GST-fusion proteins used for the experiment were checked by Coomassie Brilliant Blue staining (left panel). *In vitro* translated vimentin bound to GST protein (10 μg) was shown in right panel. "Input" indicates the *in vitro* translated vimentin used for each binding assay. **(B)** Binding of ectopically expressed FLAG-18E1^E4 to endogenous vimentin. 293T cells were transfected with FLAG-18E1^E4 expression plasmid, and the endogenous vimentin bound to FLAG-18E1^E4 was detected by immunoprecipitation with anti-FLAG antibody followed by immunoblot with anti-vimentin antibody. **(C)** Co-localization of endogenous vimentin (vim, green) and FLAG-18E1^E4 (E1^E4, red) in CV1 cells. Nuclei were stained with DAPI. Note that the co-localization could be observed in majority of the cells (≥70%) in that FLAG-18E1^E4 aggregates were detected. **(D)** Fine analysis of intracellular localization with confocal microscopic analysis. FLAG-18E1^E4 aggregate (red) was wrapped with vimentin (vim, green) in CV1 cells. Control shows mock transfected cells.

proteins, proteasomes, and HDAC6, and are wrapped with a vimentin cage (Rodriguez-Gonzalez et al., 2008). 18E1^E4 formed aggregates on the periphery of a nucleus and was associated with vimentin as shown in **Figures 1C,D**, raising the possibility that the E1^E4 proteins were assembled in an aggresome-like compartment.

We examined the intracellular localizations of 40 kDa heat shock protein (Hsp40), HDAC6, and p62, all of which were known to be assembled in the aggresome (Johnston, 2006). From immunofluorescence analysis of CV1 cells, it appeared that these factors were co-localized with the 18E1^E4-containing aggregates (**Figure 2A**). Because ubiquitinated proteins have been known to be recruited to the aggresome (Johnston, 2006), their localizations were also analyzed using anti-ubiquitin antibody. As shown in **Figure 2B**, ubiquitinated proteins were accumulated in the 18E1^E4 aggregates. These observations indicated that the 18E1^E4 aggregate had an aggresome-like composition. These results suggested that 18E1^E4 formed an aggresome-like compartment, called "18E1^E4-aggresome" hereafter.

It is considered that aggresomes are assembled by recruiting their components by retrograde transport through microtubules and are located close to MTOC. We analyzed the localization of γ-tubulin, a component of MTOC (**Figure 2C**). In control cells, γ-tubulin appeared at the centrosome as small dots in the perinuclear region. In the cells expressing 18E1^E4, γ-tubulin was

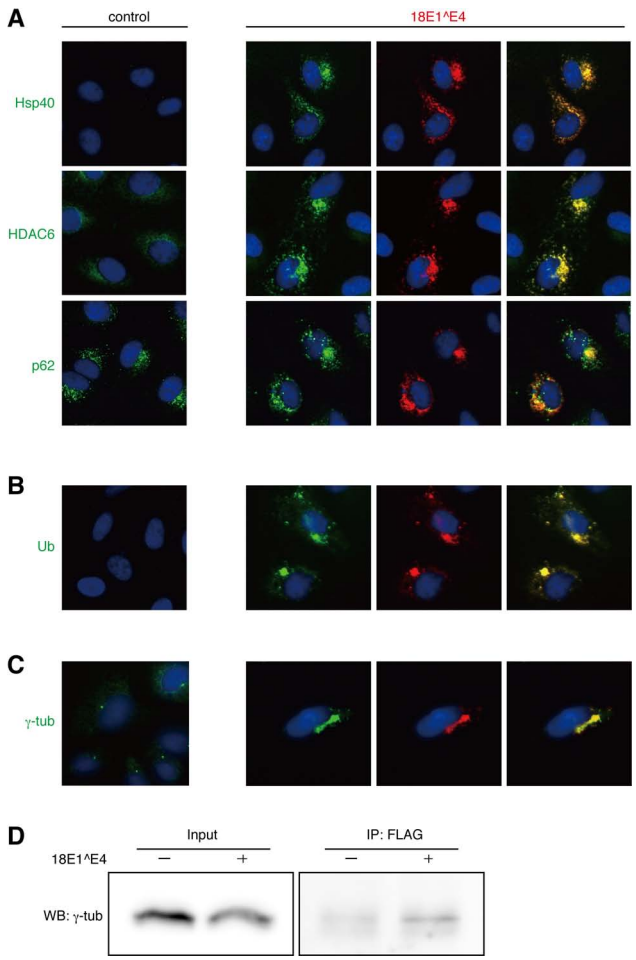


FIGURE 2 | Intracellular localization of "aggresome"-associated factors. (A) Co-localization of endogenous Hsp40, HDAC6, or p62 (green) with FLAG-18E1^E4 (red) in CV1 cells. Nuclei were stained with DAPI. Control shows mock transfected cells. **(B)** Ubiquitinated proteins (Ub, green) were accumulated in FLAG-18E1^E4 aggregates (red) in CV1 cells. **(C)** γ-tubulin was localized at centrosomes in control cells (control). In FLAG-18E1^E4 expressing CV1 cells, γ-tubulin (γ-tub, green) was co-localized at the E1^E4 (red). For **(A–C)**, the co-localizations could be observed in most of the cells (≥90%) in that 18E1^E4 aggregates were detected. **(D)** The cell lysates were obtained from the 293T cells transfected with FLAG-18E1^E4 expression plasmid. The lysates were used for immunoprecipitation with anti-FLAG antibody, and then the precipitates were analyzed by immunoblot with anti-γ-tubulin antibody.

co-localized at the 18E1^E4-aggresome, and the normal centrosome could not be detected in those cells, suggesting that 18E1^E4-aggresome formation disrupted the normal centrosome or MTOC structure.

The finding that γ-tubulin was co-localized at the 18E1^E4-aggresome urged us to investigate the interaction between γ-tubulin and 18E1^E4. 18E1^E4 with a FLAG-epitope tag at its N-terminus was expressed in 293T cells, and anti-FLAG antibody was used for immunoprecipitation of 18E1^E4-containing complexes. The complexes were analyzed by immunoblot detection with anti-γ-tubulin (**Figure 2D**). The result indicated the interaction between 18E1^E4 and γ-tubulin, which might be involved in the co-localization of γ-tubulin at the 18E1^E4-aggresome as observed in **Figure 2C**.

DYNEIN-DEPENDENT FORMATION OF 18E1^E4 AGGRESOME

Misfolded/ubiquitinated proteins are connected to dynein, a motor protein, the association of which is mediated by HDAC6 as a linker molecule (Johnston, 2006). This complex is transported along microtubule filaments to the proximate region of MTOC and forms an aggresome (Kawaguchi et al., 2003). Nocodazole treatment interferes with the polymerization of microtubules and prevents aggresome formation.

Nocodazole treatment of normal HeLa cells induced early M-phase cell cycle arrest and the cells were round (control, **Figure 3A**). In contrast, 18E1^E4-expressing cells were flat (18E1^E4, **Figure 3A**). We reported that 18E^E4 expression induced G2/M cell cycle arrest and accumulation of aneuploid cells (≥4N; Nakahara et al., 2002), suggesting that the cells were maintained in S and G2 phases of the cell cycle. By nocodazole treatment, the formation of 18E1^E4-aggresome was significantly inhibited and small aggregates of 18E1^E4 were broadly distributed in the cytoplasm, indicating that the assembly of 18E1^E4-aggresome required functional microtubule networks. We could detect γ-tubulin in 18E1^E4 small aggregates in nocodazole-treated cells (**Figure 3B**), suggesting that 18E1^E4 associated with γ-tubulin in cytoplasm and assembled it to an 18E1^E4-aggresome in a microtubule-dependent manner.

A similar experiment was performed with a dynein inhibitor, ciliobrevin D (**Figure 3C**). Ciliobrevin D treatment strongly suppressed E1^E4-aggresome formation, indicating that dynein-dependent transport was involved in E1^E4-aggresome formation.

The effect of an HDAC6 inhibitor, tubacin, was also tested (**Figure 3D**). HDAC6 is important for aggresome formation by loading the cargo containing misfolded/ubiquitinated proteins onto a dynein motor (Kawaguchi et al., 2003). Tubacin treatment disrupted the E1^E4-aggresome and small aggregates containing 18E1^E4 were detected in the cytoplasm, as in the cases of nocodazole and ciliobrevin D treatments.

These results suggested that the 18E1^E4-aggresome was assembled by dynein-dependent retrograde transport along microtubule filaments.

PROTEASOME INHIBITOR AUGMENTED E1^E4-AGGRESOME FORMATION

In the cytoplasmic region, proteasomes are located around the centrosome, close to cytoskeletal networks and on the surface of the endoplasmic reticulum (ER), and the centrosome region is considered as the major site for proteasome-dependent proteolysis, called the proteolysis center (Wójcik and DeMartino, 2003). It was reported that inhibition of proteasome function accelerated aggresome formation in the centrosome region (Johnston et al., 1998), which is considered as one of the hallmarks of aggresomes.

We examined the effect of MG132, a proteasome inhibitor, on cells expressing 18E1^E4, and found that MG132 treatment augmented 18E1^E4-aggresome formation (**Figure 4A**). This observation was consistent with the idea that 18E1^E4 formed aggresome-like compartment.

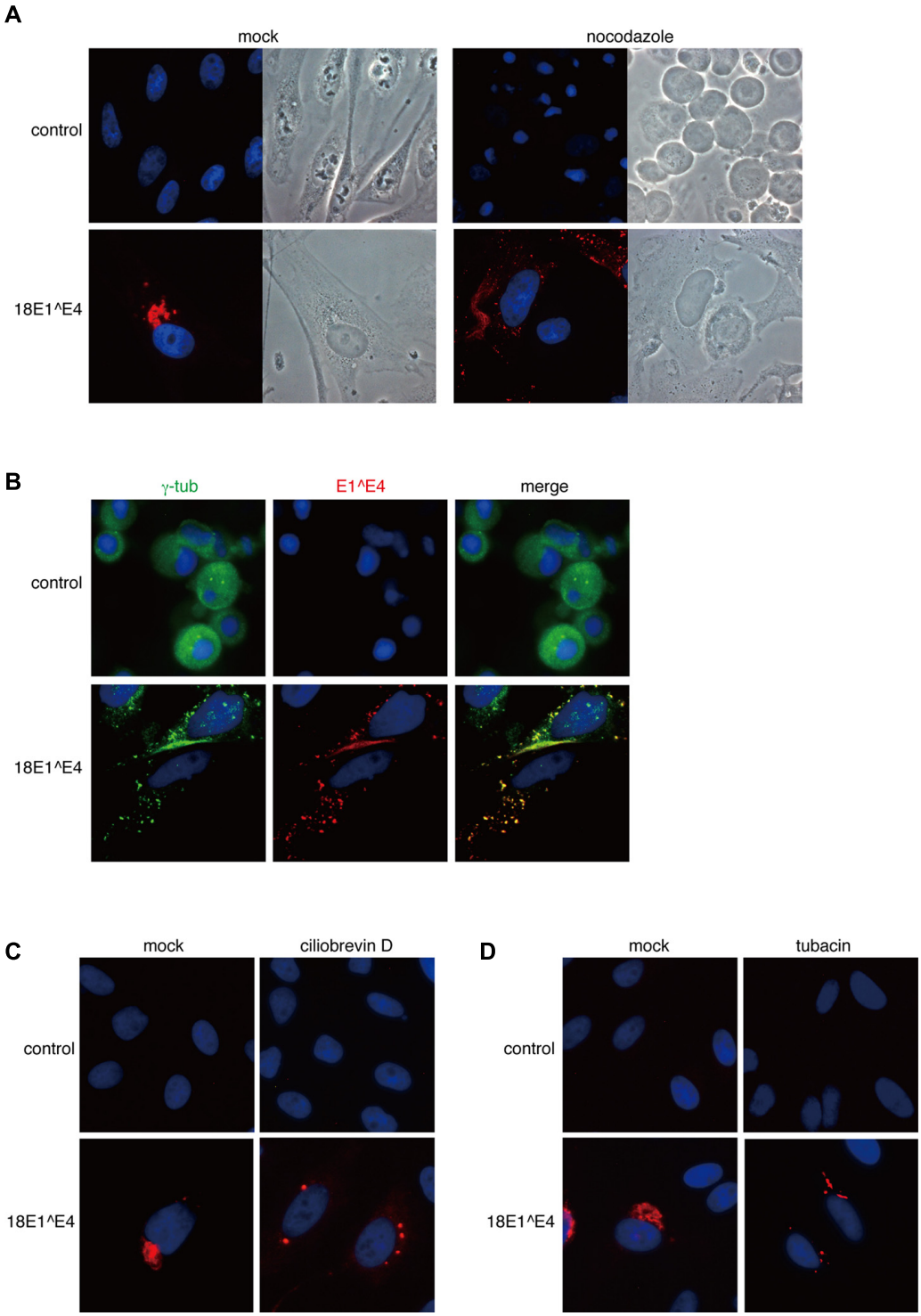


FIGURE 3 | Dynein-dependent transport through microtubule filaments was required for the assembly of 18E1^E4-aggresome. (A) Nocodazole treatment disrupted the 18E1^E4-aggresome assembly. HeLa cells were transfected with FLAG-18E1^E4 expression plasmids, treated by nocodazole (10 mM) at 24 h after transfection. At 24 h after the treatment, the cells were fixed by 4% PFA, then stained with anti-FLAG antibody (red). Control shows untransfected cells, and "mock" indicates mock-treated cells. **(B)** γ-tubulin (green) was associated with small aggregates of FLAG-18E1^E4 (red) in the nocodazole-treated cells. The association could be detected in most of the cells (≥90%) that were positive for FLAG-18E1^E4 expression. Cells were prepared and treated as in **(A)**, except for fixation by cold methanol. **(C,D)** Ciliobrevin D (a dynein inhibitor) and tubacin (an HDAC6 inhibitor) treatments prevented 18E1^E4-aggresome assembly. Cells were prepared, fixed, and stained as in **(A)**, except for the inhibitors.

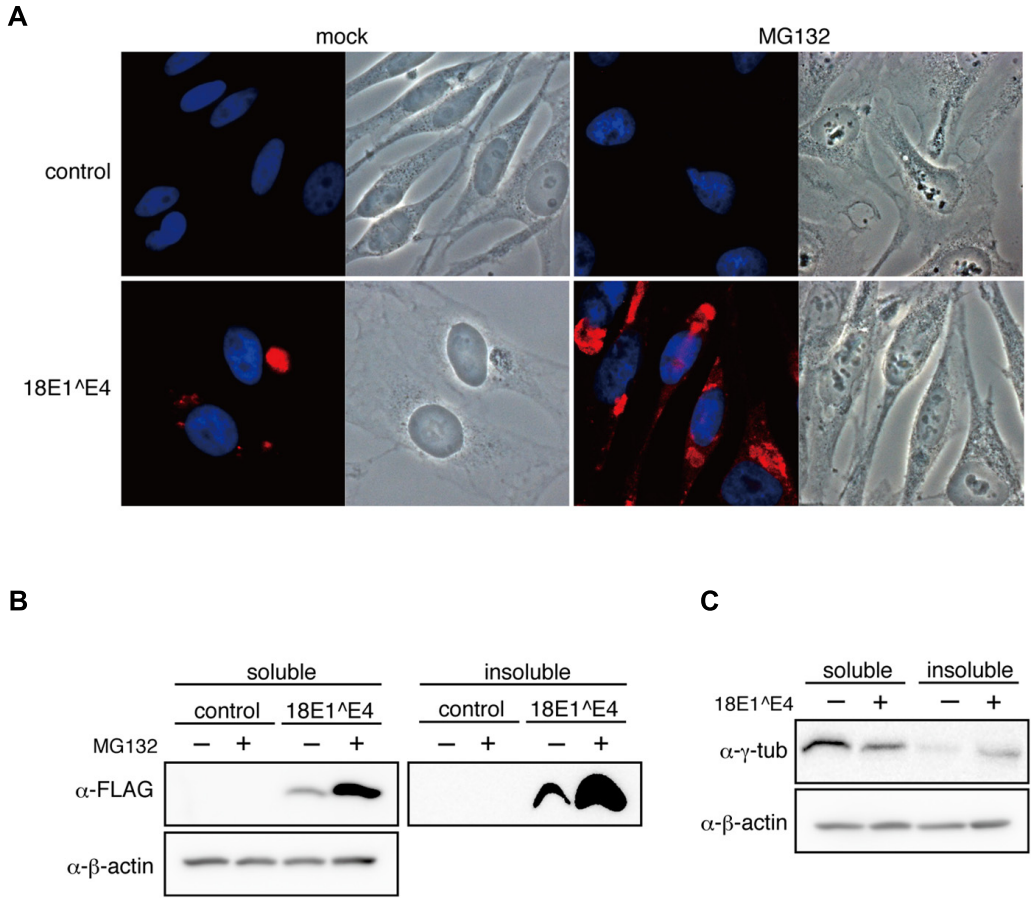


FIGURE 4 | Effect of proteasome inhibitor on 18E1^E4-aggresome assembly. (A) MG132 treatment augmented the aggresome assembly. HeLa cells were transfected with FLAG-18E1^E4 expression plasmid, treated with MG132 (10 mM) at 24 h after transfection. "mock" indicates mock-transfected cells. Cells were fixed by 4% PFA at 24 h after treatment, then stained with anti-FLAG antibody (red). Control shows mock transfected cells. **(B)** Cells prepared as shown in **(A)** were used to obtain cell lysates. The soluble and insoluble fractions were immunoblotted with anti-FLAG antibody. Most part of 18E1^E4 protein was found in the insoluble fraction of cell lysate. MG132 treatment increased the amounts of 18E1^E4 in both fractions. **(C)** Immunoblot analysis was performed as shown in **(B)** with anti-γ-tubulin antibody. γ-tubulin was partially sequestrated in the insoluble fraction by 18E1^E4.

The expression levels of 18E1^E4 were examined in MG132-treated cells. As reported previously (Nakahara et al., 2002), most of 18E1^E4 was found in the insoluble fraction of cell lysate (**Figure 4B**), which was corresponding to 18E1^E4-aggresome formation. With MG132, 18E1^E4 in the insoluble fraction was increased significantly, reflecting the augmentation of aggresome formation. Surprisingly, 18E1^E4 in the soluble fraction was also increased, suggesting that some portion of 18E1^E4 was processed in proteasome-dependent manner (**Figure 4B**).

18E1^E4 proteins were assembled into aggresomes as insoluble fraction of cell lysate, indicating that the factors recruited to 18E1^E4-aggresomes might be sequestrated as insoluble materials. As shown in **Figures 2C,D**, γ-tubulin was associated with 18E1^E4 and recruited to the aggresomes. We examined the effect of 18E1^E4 expression on the protein levels of γ-tubulin (**Figure 4C**). The amounts of soluble γ-tubulin were reduced by 18E1^E4 expression. On the contrary, those in the insoluble fraction were increased, suggesting that γ-tubulin was sequestrated into the 18E1^E4-aggresome as insoluble material, which might reduce active fraction of γ-tubulin and disturb normal centrosome/MTOC formation as shown in **Figure 2C**.

18E1^E4 AGGRESOME WAS INVOLVED IN THE TURN OVER OF HPV ONCOPROTEINS

As described above, 18E1^E4 could sequestrate γ-tubulin in the aggresome. In considering the involvement of 18E1^E4-aggresome in HPV replication, we examined the possibility that the aggresome contributed to sequestration of other viral proteins.

In CV1 cells, FLAG-epitope tagged 18E5, 18E6, or 18E7 was expressed with or without 18E1^E4, and then the expression level was monitored by immunoblotting analysis (**Figure 5A**). Although the expression of E5 was not affected, those of E6 and E7 in the soluble fraction were significantly reduced by 18E1^E4 and accumulation of those proteins in insoluble material was observed. This result suggested that E6 and E7 were sequestrated in 18E1^E4-aggresomes. Nocodazole treatment blocked the effect of 18E1^E4 (**Figure 5B**), suggesting that the 18E1^E4-aggresome formation was involved in sequestration of 18E6 and18E7.

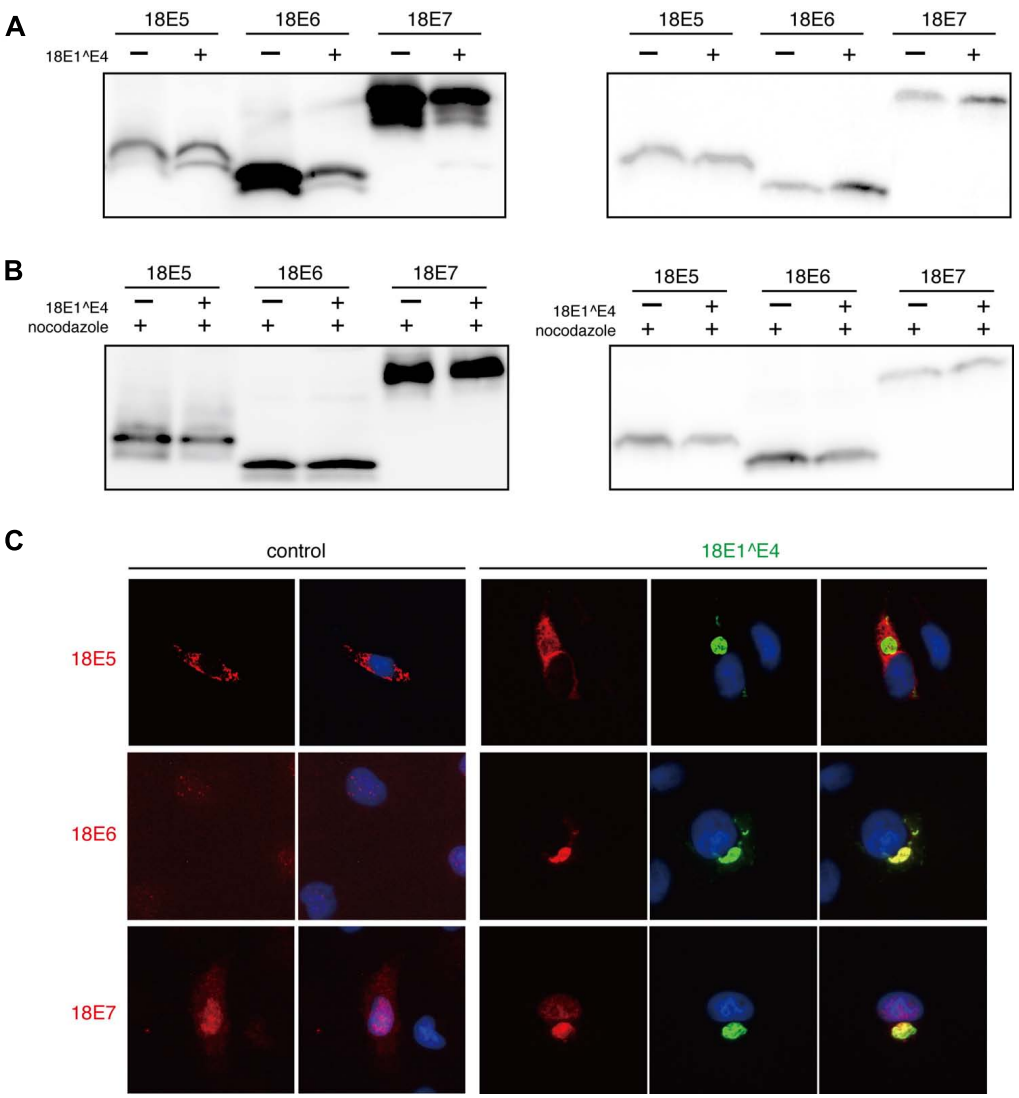


FIGURE 5 | Major viral oncoproteins were sequestrated in 18E1^E4-aggresome. (A) CV1 cells were co-transfected with 18E1^E4 and FLAG-tagged 18E5, 18E6, or 18E7 expression plasmids. At 48 h after transfection, cells were lysed by triple detergent buffer. The expression levels of HPV18 E5, E6, and E7 were analyzed both in soluble (left panel) and insoluble fractions (right panel) of cell lysates. **(B)** The effect of nocodazole treatment (10 mM) was examined by a similar experiment as shown in **(A)**. **(C)** Intracellular localization of FLAG-tagged 18E5, 18E6, or 18E7 (red) with EGFP-tagged 18E1^E4 (green) in CV1 cells. Nuclei were stained with DAPI. The colocalization could be detected in most of the cells (≥90%) that were positive for EGFP-tagged 18E1^E4 expression.

In the cells expressing 18E1^E4, E6 and E7 were co-localized at 18E1^E4-aggresomes (**Figure 5C**). The localization of 18E5 was not altered by 18E1^E4 expression. These observations indicated that major viral oncoproteins, E6 and E7, were recruited to the 18E1^E4-aggresome and sequestrated in insoluble materials.

DISCUSSION

It was reported that ectopically expressed HPV E1^E4 formed aggregates in cytoplasm (Doorbar et al., 1991), although the function of the aggregate remained to be clarified. In this paper, we described that 18E1^E4 was assembled into an aggresome-like compartment (18E1^E4-aggresome) and was involved in the sequestration of viral oncoproteins.

AGGRESOME-LIKE COMPARTMENT FORMATION BY 18E1^E4.

We found that 18E1^E4 interacted with vimentin and recruited it to the 18E1^E4 aggregates (**Figure 1**), which inspired us to consider that 18E1^E4 was assembled into an aggresome-like compartment because aggresomes are known to be wrapped by vimentin.

There is a report that 16E1^E4 could interact with cytokeratins 8/18 (CK8/18) but not with vimentin (Wang et al., 2004). We therefore analyzed the interaction between 18E1^E4 and endogenous vimentin both *in vivo* and *in vitro* (**Figures 1B,C**), although they used an *in vitro* binding assay with recombinant vimentin and 16E1^E4. The different experimental condition could be the cause of the controversial observations.

Aggresomes are assembled to process misfolded/ubiquitinated proteins that are not well handled by the ubiquitin-proteasome pathway or the chaperone-dependent refolding system (Goldberg, 2003). It is known that aggresomes incorporate molecular chaperones, ubiquitinated proteins, p62 and HDAC6 (Rodriguez-Gonzalez et al., 2008). We confirmed that these molecules were recruited to the 18E1^E4-aggregate (**Figures 2A,B**). This observation strongly suggested that 18E1^E4 aggregate had an aggresome-like structure.

Aggresome formation is dependent on microtubules and dynein. Microaggregates of misfolded proteins are transported to MTOC along microtubules in a dynein-dependent manner (Johnston, 2006). Dynein is a motor protein and microaggregates are linked to dynein through HDAC6 (Kawaguchi et al., 2003). We examined the effects of nocodazole, an inhibitor of microtubule polymerization; ciliobrevin D, a dynein inhibitor; and tubacin, a HDAC6 inhibitor, on 18E1^E4 aggregate formation, and found that all of the inhibitors could efficiently interfere with aggregate formation (**Figure 3**). This result supported the possibility that 18E1^E4 was assembled in the aggresome-like compartment, 18E1^E4-aggresome. We are currently investigating a role of the interaction between 18E1^E4 and vimentin in aggresome formation.

DISRUPTION OF MTOC BY 18E1^E4

Aggresomes are known to be assembled close to MTOC (Johnston et al., 1998). We examined the localization of γ-tubulin, a component of MTOC, in 18E1^E4-expressing cells, and found that it was co-localized at the 18E1^E4-aggresome (**Figure 2C**). Direct interaction was found between 18E1^E4 and γ-tubulin (**Figure 2D**), by which γ-tubulin might be recruited to the E1^E4-aggresome. Even though nocodazole treatment inhibited E1^E4-aggresome formation, colocalization of 18E1^E4 and γ-tubulin could be detected (**Figure 3B**). It was also found that regular centrosome or MTOC formation was disrupted in 18E1^E4 expressing cells (**Figure 2C**). Proper assembly of MTOC is essential for mitotic events (Bettencourt-Dias and Glover, 2007), and the disturbance of MTOC formation by 18E1^E4 might contribute to the G2/M cell cycle arrest induced by 18E1^E4.

POSSIBLE ROLE OF 18E1^E4 AGGRESOME IN VIRUS REPLICATION

Although it is known that aggresome formation has a protective role against bacterial and protozoal infections (Wileman, 2007), several viruses are reported to utilize aggresomes for their replication processes (Wileman, 2007). Nucleocytoplasmic large DNA viruses (NCLDV), including poxviruses, African swine fever virus (ASFV), iridoviruses and phycodnaviruses, have been reported to utilize aggresomes as compartments for the accumulation of host and viral proteins, where virus replication and virion assembly are accelerated. It has been proposed that infection with a retrovirus or herpes virus produces an aggresome-like structure in the perinuclear region, which is utilized as a virus assembly site (Wileman, 2007). These findings suggested that the 18E1^E4-aggresome had a functional role in virus replication.

As shown in **Figures 2C,D**, 18E1^E4 bound to γ-tubulin and recruited it to aggresome-like compartment. This sequestration of γ-tubulin might cause disruption of normal centrosome/MTOC organization. We considered that the 18E1^E4-aggresome might be involved in sequestration of other viral proteins, especially of the viral oncoproteins. We examined the effect of 18E1^E4 on the expression levels of 18E5, 18E6, and 18E7 (**Figure 5A**). Although the expression level of E5 did not altered by 18E1^E4, those of E6 and E7 in soluble fraction were severely reduced. E6 and E7 were found in insoluble fraction and co-localized at 18E1^E4-aggresomes (**Figures 5A,C**). These observations suggested that 18E1^E4 sequestrated E6 and E7 into the inactive aggregate and reduced active fractions of them. We could not detect direct binding activity of 18E1^E4 to 18E6 or 18E7 (data not shown), and therefore it will be necessary to clarify the mechanism by which E6 and E7 are recruited to the aggresome.

Most 18E6 and 18E7 are partitioned in soluble fraction as shown in **Figure 5A**. The amounts of these proteins in soluble fraction were significantly reduced by 18E1^E4 expression, although those in insoluble fraction were increased modestly. The result suggested that 18E1^E4 expression reduced the total amounts of these oncoproteins in the cells possibly by accelerating their turn over. We are now investigating the effect of 18E1^E4 expression on total amounts of the viral oncoproteins.

In lesions infected with cutaneous-type HPVs, HPV1, HPV4, and HPV63, E1^E4 aggregate could be detected in upper layers of the warts as intracytoplasmic inclusion bodies (Egawa, 1994). In the case of HPV16 infection, it was reported that inclusion bodies of E1^E4 were found in differentiated layers of cervical intraepithelial neoplasia grade 1 (CIN1) lesions (Doorbar et al., 1997; Doorbar, 2005). These observations suggest that the E1^E4-aggresome functions in the upper layers of the infected lesion.

Here we propose a model of E1^E4 function in viral replication. In basal and parabasal cells of HPV-infected lesions, viral oncoproteins, E6 and E7, are expressed from the viral early promoter. This suppresses cell differentiation and promotes cell proliferation (Nguyen et al., 2003; Ueno et al., 2006), which is required for expanding the population of infected cells. As cellular differentiation progresses, the viral late promoter is activated and directs the expression of E1^E4. E1^E4 causes G2/M cell cycle arrest and activates endoreduplication (Nakahara et al., 2005). This cellular condition favors genome amplification and gene expression of the virus. Then the high-level expression of E1^E4 induces aggregate formation in upper layers of the lesion, where the E1^E4-aggresome sequestrates E6 and E7, suppresses their inhibitory effect on cellular differentiation and induces terminal differentiation. Terminal differentiation is required for capsid protein expression and virion assembly, although the underlying mechanism remains unknown (Sakakibara et al., 2013).

It was reported that the formation of E1^E4 aggregates disrupted cytokeratin networks and might be helpful for virion egress from keratinized cells (Doorbar et al., 1991). This idea is very attractive for an E1^E4 function, and it is important to verify these E1^E4 functions in an animal infection model, histological analysis of human samples, or an organotypic raft culture system.

ACKNOWLEDGMENTS

The research was supported in part by Grant-in-Aid for Cancer Research from Ministry of Health Labor and Welfare of Japan and Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan (C) (No. 23590540) (Hiroyuki Sakai). Full-length clones for HPV18 and HPV11 were kindly provided by Dr. Peter M. Howley. We greatly appreciate scientific advice from Dr. Gen Matsumoto (RIKEN, Wako, Japan).

REFERENCES

Bell, I., Martin, A., and Roberts, S. (2007). The E1^E4 protein of human papillomavirus interacts with the serine-arginine-specific protein kinase SRPK1. *J. Virol.* 81, 5437–5448. doi: 10.1128/JVI.02609-06

Bettencourt-Dias, M., and Glover, D. M. (2007). Centrosome biogenesis and function: centrosomics brings new understanding. *Nat. Rev. Mol. Cell Biol.* 8, 451–463. doi: 10.1038/nrm2180

Bryan, J. T., Fife, K. H., and Brown, D. R. (1998). The intracellular expression pattern of the human papillomavirus type 11 E1^E4 protein correlates with its ability to self associate. *Virology* 241, 49–60. doi: 10.1006/viro.1997.8965

Campo, M. S. (2002). Animal models of papillomavirus pathogenesis. *Virus Res.* 89, 249–261. doi: 10.1016/S0168-1702(02)00193-4

Davy, C. E., Ayub, M., Jackson, D. J., Das, P., McIntosh, P., and Doorbar, J. (2006). HPV16 E1^E4 protein is phosphorylated by Cdk2/cyclin A and relocalizes this complex to the cytoplasm. *Virology* 349, 230–244. doi: 10.1016/j.virol.2006.02.024

Davy, C. E., Jackson, D. J., Raj, K., Peh, W. L., Southern, S. A., Das, P., et al. (2005). Human papillomavirus type 16 E1^E4-induced G2 arrest is associated with cytoplasmic retention of active Cdk1/cyclin B1 complexes. *J. Virol.* 79, 3998–4011. doi: 10.1128/JVI.79.7.3998-4011.2005

Davy, C. E., Jackson, D. J., Wang, Q., Raj, K., Masterson, P. J., Fenner, N. F., et al. (2002). Identification of a G(2) arrest domain in the E1^E4 protein of human papillomavirus type 16. *J. Virol.* 76, 9806–9818. doi: 10.1128/JVI.76.19.9806-9818.2002

Doorbar, J. (2005). The papillomavirus life cycle. *J. Clin. Virol.* 32(Suppl. 1), S7–S15. doi: 10.1016/j.jcv.2004.12.006

Doorbar, J., Elston, R. C., Napthine, S., Raj, K., Medcalf, E., Jackson, D., et al. (2000). The E1^E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus. *J. Virol.* 74, 10081–10095. doi: 10.1128/JVI.74.21.10081-10095.2000

Doorbar, J., Ely, S., Sterling, J., McLean, C., and Crawford, L. (1991). Specific interaction between HPV-16 E1^E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* 352, 824–827. doi: 10.1038/352824a0

Doorbar, J., Foo, C., Coleman, N., Medcalf, L., Hartley, O., Prospero, T., et al. (1997). Characterization of events during the late stages of HPV16 infection *in vivo* using high-affinity synthetic Fabs to E4. *Virology* 238, 40–52. doi: 10.1006/viro.1997.8768

Egawa, K. (1994). New types of human papillomaviruses and intracytoplasmic inclusion bodies: a classification of inclusion warts according to clinical features, histology, and associated HPV types. *Br. J. Dermatol.* 130, 158–166. doi: 10.1111/j.1365-2133.1994.tb02894.x

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* 426, 895–899. doi: 10.1038/nature02263

Ivaska, J., Pallari, H. M., Nevo, J., and Eriksson, J. E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp. Cell Res.* 313, 2050–2062. doi: 10.1016/j.yexcr.2007.03.040

Johnston, J. A. (2006). "The aggresome: proteasomes, inclusion boies, and protein aggregation," in *Protein Misfolding, Aggregation, and Conformational Diseases*, eds V. N. Uversky and A. L. Fink (New York: Springer), 175–222. doi: 10.1007/0-387-25919-8_10

Johnston, J. A., Ward, C. L., and Kopito, R. R. (1998). Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* 143, 1883–1898. doi: 10.1083/jcb.143.7.1883

Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., and Yao, T. P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* 115, 727–738. doi: 10.1016/S0092-8674(03)00939-5

Kopito, R. R. (2000). Aggresomes, inclusion bodies, and protein aggregation. *Trends Cell Biol.* 10, 524–530. doi: 10.1016/S0962-8924(00)01852-3

McIntosh, P. B., Martin, S. R., Jackson, D. J., Khan, J., Isaacson, E. R., Calder, L., et al. (2008). Structural analysis reveals an amyloid form of the human papillomavirus type 16 E1^E4 protein and provides a molecular basis for its accumulation. *J. Virol.* 82, 8196–8203. doi: 10.1128/JVI.00509-08

Nakahara, T., Nishimura, A., Tanaka, M., Ueno, T., Ishimoto, A., and Sakai, H. (2002). Modulation of the cell division cycle by human papillomavirus type 18 E4. *J. Virol.* 76, 10914–10920. doi: 10.1128/JVI.76.21.10914-10920.2002

Nakahara, T., Peh, W. L., Doorbar, J., Lee, D., and Lambert, P. F. (2005). Human papillomavirus type 16 E1^E4 contributes to multiple facets of the papillomavirus life cycle. *J. Virol.* 79, 13150–13165. doi: 10.1128/JVI.79.20.13150-13165.2005

Nguyen, M. L., Nguyen, M. M., Lee, D., Griep, A. E., and Lambert, P. F. (2003). The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia *in vivo*. *J. Virol.* 77, 6957–6964. doi: 10.1128/JVI.77.12.6957-6964.2003

Peh, W. L., Brandsma, J. L., Christensen, N. D., Cladel, N. M., Wu, X., and Doorbar, J. (2004). The viral E4 protein is required for the completion of the cottontail rabbit papillomavirus productive cycle *in vivo*. *J. Virol.* 78, 2142–2151. doi: 10.1128/JVI.78.4.2142-2151.2004

Raj, K., Berguerand, S., Southern, S., Doorbar, J., and Beard, P. (2004). E1^E4 protein of human papillomavirus type 16 associates with mitochondria. *J. Virol.* 78, 7199–7207. doi: 10.1128/JVI.78.13.7199-7207.2004

Roberts, S., Hillman, M. L., Knight, G. L., and Gallimore, P. H. (2003). The ND10 component promyelocytic leukemia protein relocates to human papillomavirus type 1 E4 intranuclear inclusion bodies in cultured keratinocytes and in warts. *J. Virol.* 77, 673–684. doi: 10.1128/JVI.77.1.673-684.2003

Rodriguez-Gonzalez, A., Lin, T., Ikeda, A. K., Simms-Waldrip, T., Fu, C., and Sakamoto, K. M. (2008). Role of the aggresome pathway in cancer: targeting histone deacetylase 6-dependent protein degradation. *Cancer Res.* 68, 2557–2560. doi: 10.1158/0008-5472.CAN-07-5989

Sakakibara, N., Chen, D., and McBride, A. A. (2013). Papillomaviruses use recombination-dependent replication to vegetatively amplify their genomes in differentiated cells. *PLoS Pathog.* 9:e1003321. doi: 10.1371/journal.ppat.1003321

Schwartz, S. (2013). Papillomavirus transcripts and posttranscriptional regulation. *Virology*. doi: 10.1016/j.virol.2013.04.034 [Epub ahead of print].

Sterling, J. C., Skepper, J. N., and Stanley, M. A. (1993). Immunoelectron microscopical localization of human papillomavirus type 16 L1 and E4 proteins in cervical keratinocytes cultured *in vivo*. *J. Invest. Dermatol.* 100, 154–158. doi: 10.1111/1523-1747.ep12462790

Ueno, T., Sasaki, K., Yoshida, S., Kajitani, N., Satsuka, A., Nakamura, H., et al. (2006). Molecular mechanisms of hyperplasia induction by human papillomavirus E7. *Oncogene* 25, 4155–4164. doi: 10.1038/sj.onc.1209445

Wang, Q., Griffin, H., Southern, S., Jackson, D., Martin, A., McIntosh, P., et al. (2004). Functional analysis of the human papillomavirus type 16 E1^E4 protein provides a mechanism for *in vivo* and *in vitro* keratin filament reorganization. *J. Virol.* 78, 821–833. doi: 10.1128/JVI.78.2.821-833.2004

Wang, Q., Kennedy, A., Das, P., McIntosh, P. B., Howell, S. A., Isaacson, E. R., et al. (2009). Phosphorylation of the human papillomavirus type 16 E1^E4 protein at T57 by ERK triggers a structural change that enhances keratin binding and protein stability. *J. Virol.* 83, 3668–3683. doi: 10.1128/JVI.02063-08

Wileman, T. (2007). Aggresomes and pericentriolar sites of virus assembly: cellular defense or viral design? *Annu. Rev. Microbiol.* 61, 149–167. doi: 10.1146/annurev.micro.57.030502.090836

Wilson, R., Fehrmann, F., and Laimins, L. A. (2005). Role of the E1^E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31. *J. Virol.* 79, 6732–6740. doi: 10.1128/JVI.79.11.6732-6740.2005

Wilson, R., Ryan, G. B., Knight, G. L., Laimins, L. A., and Roberts, S. (2007). The full-length E1^E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression. *Virology* 362, 453–460. doi: 10.1016/j.virol.2007.01.005

Wójcik, C., and DeMartino, G. N. (2003). Intracellular localization of

proteasomes. *Int. J. Biochem. Cell Biol.* 35, 579–589. doi: 10.1016/S1357-2725(02)00380-1

zur Hausen, H. (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* 2, 342–350. doi: 10.1038/nrc798

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 July 2013; accepted: 07 August 2013; published online: 27 August 2013.

Citation: Kajitani N, Satsuka A, Yoshida S and Sakai H (2013) HPV18 E1^E4 is assembled into aggresome-like compartment and involved in sequestration of viral oncoproteins. Front. Microbiol. ***4****:251. doi: 10.3389/fmicb.2013.00251*

This article was submitted to Virology, a section of the journal Frontiers in Microbiology.