535 **Figure legends**

536

537 **Fig. 1.** Genomic clone of ratHEV. (A) Schematic presentation (not in scale). The T7 RNA
538 polymerase promotor (pT7), ratHEV genome open reading frames 1-4 (ORF1-4), the
539 polyadenylation site (A_{17}), the flanking vector sequences (pUC19) and selected
540 restriction enzyme sites are indicated. (B) Restriction enzyme analysis of the genomic
541 clone of ratHEV. The plasmid DNA was digested with the indicated restriction enzymes
542 and subjected to electrophoresis on ethidium bromide-stained agarose gel. -: without
543 enzyme, M: molecular mass markers (Quick-Load 2-log DNA Ladder, New England
544 Biolabs; the sizes of selected bands are indicated).

545

546 **Fig. 2.** Kinetics of ratHEV excretion with stool after inoculation of nude rats with *in vitro*
547 transcribed and capped ratHEV RNA. Two nude rats (Long-Evans rnu/rnu) LR1 (○) and
548 LR2 (◇) were intrahepatically, two nude rats VR1 (△) and VR2 (▽) intravenously
549 inoculated with *in vitro*-transcribed and capped ratHEV RNA. The ratHEV RNA copy
550 numbers were determined by RT-qPCR.

551

552 **Fig. 3.** Infectivity testing of the ratHEV recovered from stool suspensions derived from
553 rats LR1 and LR2. Two nude rats (Long-Evans rnu/rnu) RR1 (○) and RR2 (△) and two
554 Wistar rats WR1 (□) and WR2 (◇) were intravenously inoculated with the stool
555 suspension from rats LR1 and LR2, respectively. Stool samples were collected weekly
556 from nude rats for detection of ratHEV RNA using RT-qPCR (A) and serum samples

were collected weekly from Wistar rats for detection of anti-ratHEV IgG using ELISA (B).

Fig. 4. Replication of ratHEV in a human hepatocarcinoma cell line. PLC/PRF/5 cells were inoculated with the rat (LR1) fecal specimen (○) or with the resulting ratHEV-containing culture supernatant (△). The culture supernatants were collected every 4 days and used for detection of ratHEV RNA by RT-qPCR (A) and ratHEV antigen by ratHEV-specific antigen ELISA (B).

Fig. 5. Infectivity testing of the ratHEV recovered from cell culture for rats. Two nude rats (Long-Evans rnu/rnu) CR1 (○) and CR2 (△) were intravenously inoculated with a ratHEV-containing cell culture supernatant. The ratHEV RNA copy numbers present in the faeces of the animals were determined by RT-qPCR.

571 **Supplementary Tables:**

572

573 **Supplementary Table S1:** Sequences of primers used for amplification and cloning of
574 the ratHEV genome.

575

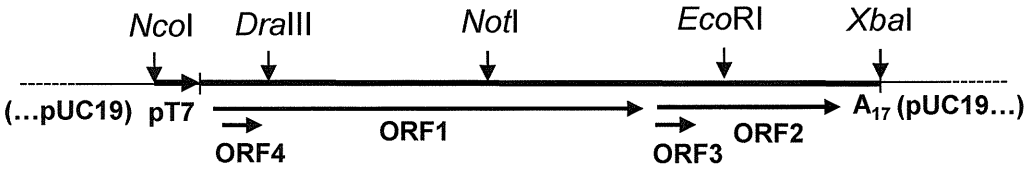
576 **Supplementary Table S2:** Nucleotide and amino acid exchanges present in the ratHEV
577 genomic clone as compared to the original sequence GU345042.

Figures

Click here to download Figure: Rat HEV infectious clone 150118.ppt

Fig. 1

A



B

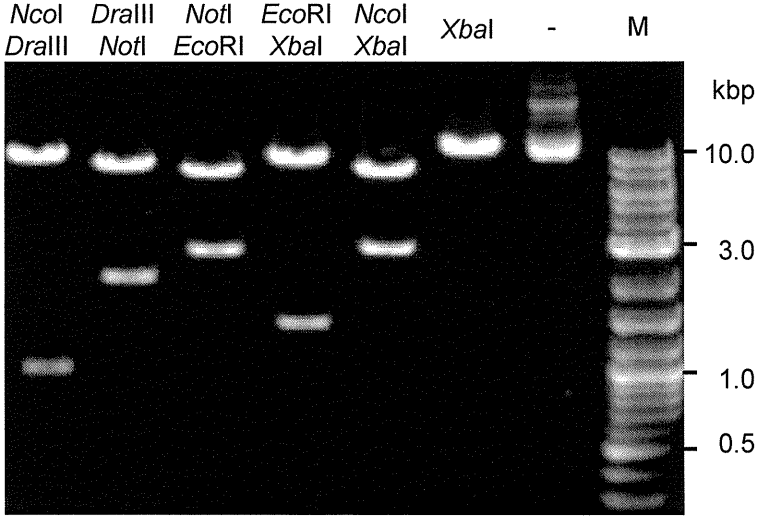


Fig.2

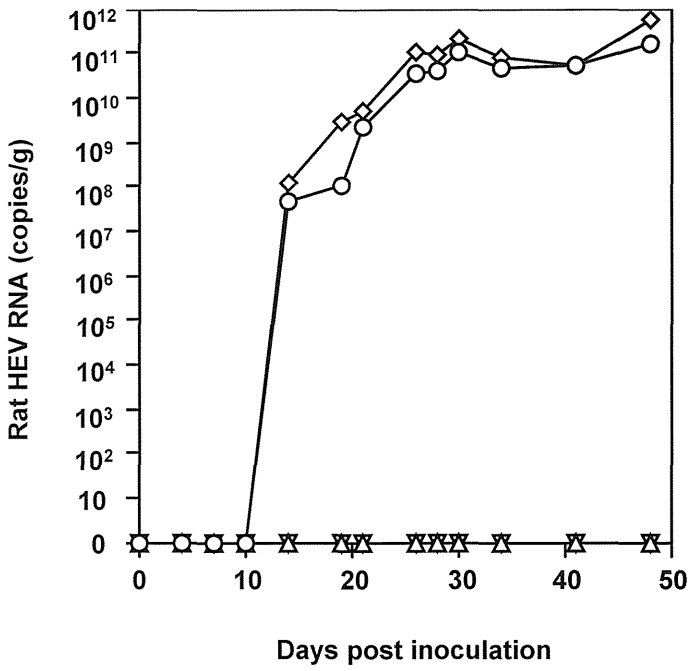

Rat HEV RNA (copies/g)
10^12
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0
10
20
30
40
50
Days post inoculation

Fig.3

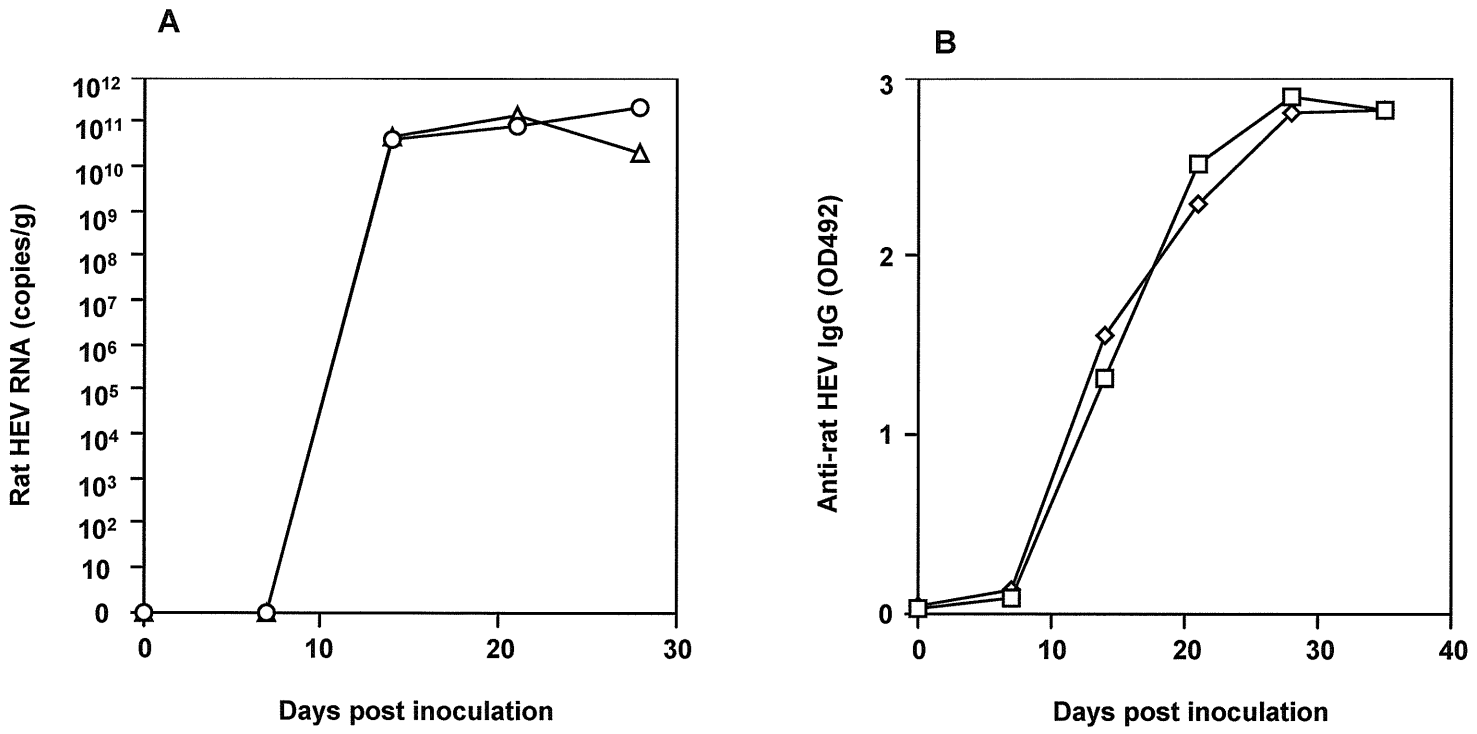

A
Rat HEV RNA (copies/g)
10^12
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0
10
20
30
Days post inoculation
B
Anti-rat HEV IgG (OD492)
3
2
1
0
0
10
20
30
40
Days post inoculation

Fig.4

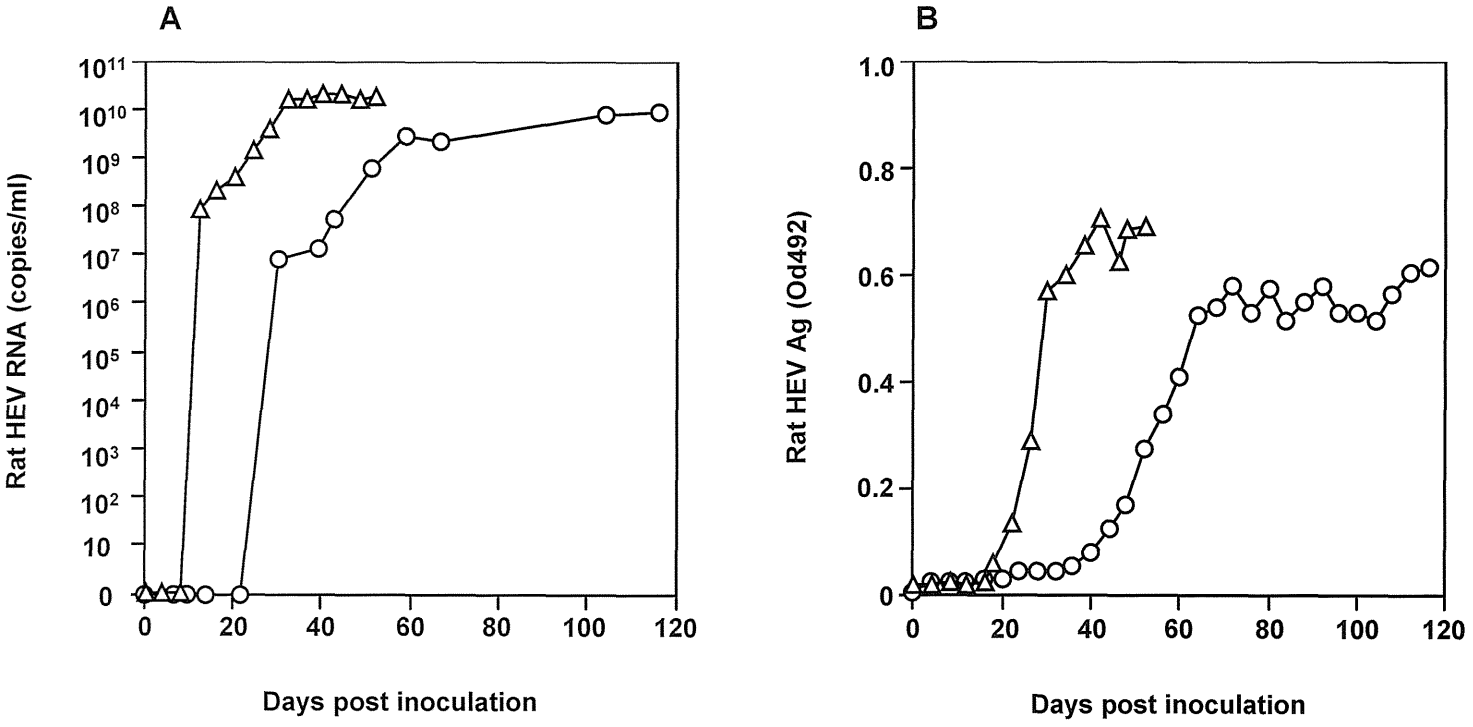
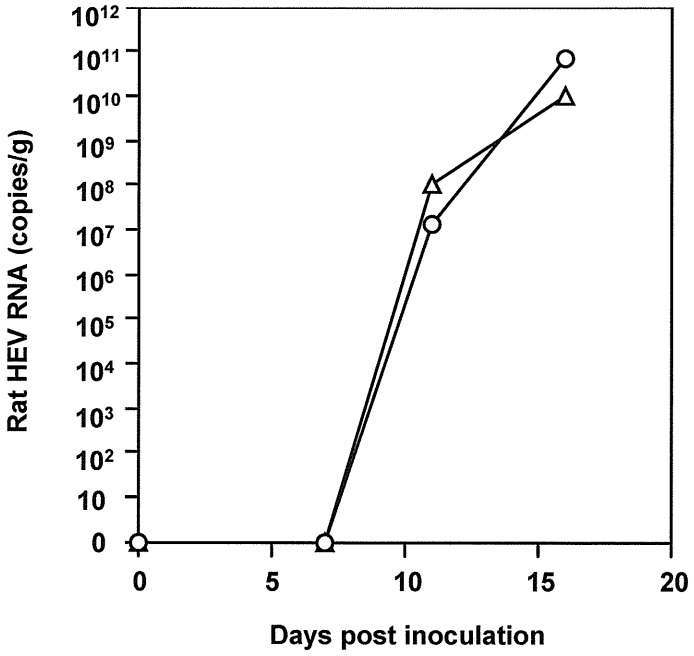

A
Rat HEV RNA (copies/ml)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10
0
0
20
40
60
80
100
120
Days post inoculation
B
Rat HEV Ag (Od492)
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Days post inoculation

Fig.5


Rat HEV RNA (copies/g)
10^12
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0
5
10
15
20
Days post inoculation

Supplementary Table S1: Sequences of primers used for amplification and cloning of the ratHEV genome.

Designation	Sequence (5'-3')	Notes
63inf-1s	TTGCCATGG**TAATACGACTCACTATA**GCAACCCCCGATGGAGACCCATC	*Nco*I-site, **T7 promotor**
63inf-1as	TAGGCACAGAGTGGAACGTAGAC	*Dra*III-site
63inf-2s	GTTCCACTCTGTGCCTACAGATAT	*Dra*III-site
63inf-2as	ATCCGCGGCCGCAGTCATGTAGC	*Not*I-site
63inf-3s	GACTGCGGCCGCGGATGTAGTTC	*Not*I-site
63inf-3as	GATGGAATTCATATCCACCGACG	*Eco*RI-site
63inf-4s	ATATGAATTCCATCACTTCCACCG	*Eco*RI-site
63inf-4as	CTTTCTAGA**TTTTTTTTTTTTTTTTTT**GTCTTGCGGGGA	*Xba*I-site, **poly A**

Supplementary Table S2: Nucleotide and amino acid exchanges present in the ratHEV genomic clone as compared to the original sequence GU345042.

Nucleotide position	Nucleotide		Amino acid				Notes
	GU345042	Genomic clone	ORF	Position	GU345042	Genomic clone	
889	T	C	-*	-	-	-	-
1549	A	G	-	-	-	-	-
1588	C	T	-	-	-	-	-
2516	C	A	ORF1	836	Q	K	R in JN167537
2520	A	G	ORF1	837	D	G	G in JN167537
2528	C	T	ORF1	840	P	S	hypervariable region
2534	T	C	ORF1	842	S	P	hypervariable region
2540	C	T	ORF1	844	P	S	hypervariable region
2546	C	T	ORF1	846	P	S	hypervariable region
2903	C	T	-	-	-	-	-
3808	G	A	-	-	-	-	-
4078	C	T	-	-	-	-	-
4079	T	C	ORF1	1357	F	L	L in JN167537 and GU345043
4087	T	C	-	-	-	-	-
4088	C	T	-	-	-	-	-
4090	G	A	-	-	-	-	-
4150	T	C	-	-	-	-	-
4347	G	C	ORF1	1446	G	A	A in JN167537 and GU345043
5386	A	G	-	-	-	-	-
5493	A	G	ORF2	182	H	R	R in JN167537 and GU345043
5503	G	C	-	-	-	-	-
5656	T	G	-	-	-	-	-
5950	C	T	-	-	-	-	-

*- synonymous exchange

Am. J. Trop. Med. Hyg., 90(4), 2014, pp. 764–766
doi:10.4269/ajtmh.13-0562

Short Report: Molecular Detection of Hepatitis E Virus in Rivers in the Philippines

Tian-Cheng Li, Tingting Yang, Tomoyuki Shiota, Sayaka Yoshizaki, Hiromu Yoshida, Mariko Saito, Toshifumi Imagawa, Fidelino F. Malbas, Socorro P. Lupisan, Hitoshi Oshitani, Takaji Wakita, and Koji Ishii*

Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan; Department of Clinical Laboratory, Affiliated Hospital of Qingdao University Medical College, Qingdao 266003, China; Department of Virology, Tohoku University Graduate School of Medicine, Miyagi 980-8575, Japan; RITM-Tohoku Collaborating Research Center on Emerging and Re-Emerging Infectious Diseases, Filinvest Corporate City, Alabang, Muntinlupa City 1781, the Philippines; Research Institute for Tropical Medicine, Department of Health Compound, FILINVEST Corporate City, Alabang, Muntinlupa City 1781, the Philippines

Abstract. To understand the hepatitis E virus (HEV)-pollution status in the environment in the Philippines, a total of 12 water samples were collected from rivers in Manila City for detection of HEV RNA. Three of 12 samples were positive for HEV RNA indicating that HEV is circulating in the Philippines. Phylogenetic analysis classified all of the HEV sequences into genotype 3.

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae*.[1–3] The HEV is the causative agent of acute or fulminant hepatitis E, primarily transmitted by the fecal–oral route.[4] The relatively high mortality rate in HEV-infected pregnant women, up to 28%, is unique among hepatitis viruses.[1,5] Hepatitis E is a zoonotic disease, with swine, wild boars, and wild deer serving as the reservoir for human infections.[6–8] Four genotypes of HEV (G1–G4) have been detected in humans and G3 and G4 HEV are responsible for sporadic and autochthonous infections in both humans and other animal species worldwide.[9–12]

The HEV is a public health concern in many Asian and African countries where sanitation conditions are insufficient.[13,14] Large waterborne outbreaks with high attack rates among young adults have been described in regions characterized by poor sanitary conditions in countries such as China, India, Somalia, and Uganda.[15] However, there have been no reports of HEV infection in the Philippines. No information about hepatitis E patients or HEV infection in animals has been reported, and no sequence data have been deposited from this country. There is also no report of the HEV-pollution status of the environmental sewage water. With the hypothesis that environmental water samples may reflect the prevalence of HEV circulation, we examined river water samples to investigate HEV in the environment in one of the most densely populated cities in the world, Manila City, a metropolitan area in the Philippines with over 10 million residents.

A total of 12 water samples were collected from rivers that run through Manila City. Six sampling sites were selected (Figure 1). Sampling sites 1 to 3 were in the Pasig River, sites 4 and 5 were in the Paranaque River, and site 6 was in the Las Pinas River. These rivers receive the wastewater from the residents nearby. Water samples were drawn at all locations during both the dry season (December 23, 2012) and the wet season (July 23, 2013), and were named D1 to D6 and W1 to W6, respectively. The water samples were kept at 4°C during transport.

The concentration and purification of these water samples was carried out as described previously[16]; briefly, 500 mL of water was collected from each sampling site, and centrifuged at 3,000 rpm for 30 min at 4°C. Then, 2.5 mM $MgCl_2$ was added to the supernatant to a final concentration of 0.05 mM. The pH value was adjusted to 3.5. The solution was filtered through a 0.45-μm mixed cellulose ester membrane filter (Merck Millipore, Tokyo, Japan) by a positive-pressure pump. Absorbents on the filter were then eluted with 10 mL of 3% beef extract solution by ultrasonication, three times. The solution was centrifuged at 12,000 rpm for 30 min, and the supernatant was stored at −80°C until RNA extraction.

The RNA was extracted using the MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems, Carlsbad, CA) at 25°C for 10 min, 37°C for 120 min, and followed by 85°C for 5 min in a 20-μL reaction mixture containing 1 μL reverse transcriptase, 2 μL of the random primer, 1 μL RNase inhibitor, 2 μL RT buffer, 0.8 μL 10-mM deoxynucleoside triphosphates, 8 μL RNA, and 5.2 μL distilled water. A nested reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to amplify a portion of the ORF2 genome, based on the method described previously.[17]

By RT-PCR, three samples (W4, W5, and W6) of the 12 water samples were positive for HEV RNA. Excluding the primer sequences, the length of the nested RT-PCR products was 338 nucleotides corresponding to nt 5959-6296 in the ORF2 of the Myanmar strain (D10330). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). Each of 20 clones was sequenced. The clones with the same nucleotide sequence were counted as one strain. Finally, 21 HEV strains were obtained (GenBank accession nos. KF546257–KF546277), of which five strains were isolated from W4, 10 strains from W5, and six strains from W6. Phylogenetic analysis indicated that all 21 strains were G3 HEV. With the exception of strain W5-13, the other 20 strains' sequences belonged to subgenotype 3a,[18] separated into four clusters (cluster 1 to 4) with nucleotides sequence identities of 89.6–99.7% (Figure 2). In cluster 1, the sequences of three strains isolated from W6 were close to that of HEV strain EF530663 (isolated from a patient in Hungary) with nucleotide sequence identities of 92.3% to 92.6%. The nucleotide sequences of all nine of the strains in cluster 2 detected from W5 were close to that of

*Address correspondence to Koji Ishii, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. E-mail: litc@nih.go.jp

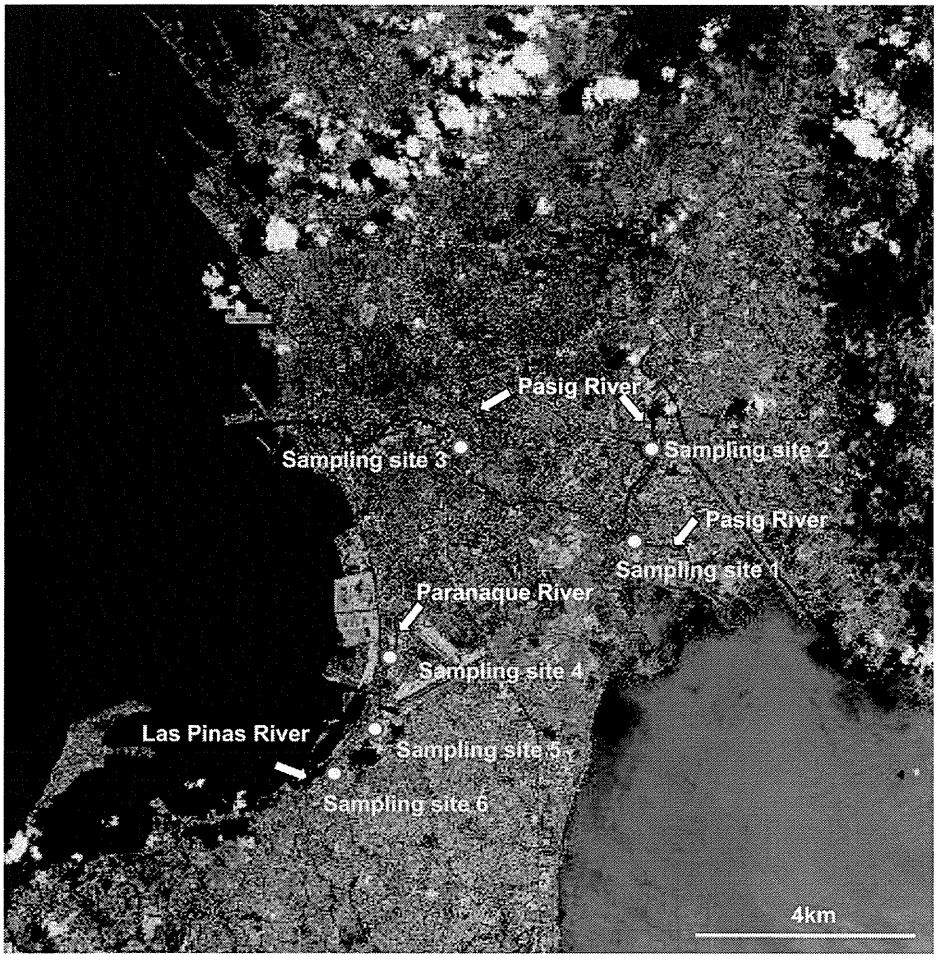


FIGURE 1. Map of Manila City and the sampling sites. Circles indicate the sampling sites. Each river is labeled with an arrow.

a Japan swine HEV strain (AB094215) with identities of 91.1–92.6%. Cluster 3 contained six strains three from W4 and three from W6. Their sequences were close to that of AB671098, isolated from a Japanese donor, with nucleotide sequence identities of 93.5–94.4%. Cluster 4 comprised two strains from W4, with sequences close to the Japan strain AB 807429 (identities of 91.7–92.0%). The strain W5-13 does not belong to any known sub-genotype and shares identities of 84.0–84.3%, 90.2–91.7%, 85.5–88.2%, and 83.7–84.0%, with the Philippines HEV strains in clusters 1 to 4, respectively. The strain W5-13 thus constitutes a new sub-genotype of G3 HEV.

A basic local alignment tool (BLAST) analysis showed that the nucleotide sequence identities between these HEV strains detected in the Philippines and other HEV strains that have been published in GenBank were lower than 94.4%, indicating that area-specific HEV strains are circulating in the Philippines. All 21 of the HEV strains we detected in the river water were collected during the wet season, suggesting that the wet season presents a higher risk of individuals in the area contracting HEV infections.

The results of this study beg the question, what is the source of HEV detected in the Manila City rivers? Because no epidemiological information about HEV in the Philippines is currently available, for human patients, animal outbreaks, or genetic sequences, it is difficult to speculate about the sources of HEV. However, because the HEV is primarily transmitted by the fecal–oral route, HEV might be present in rivers containing human or animal stool. In this study, all of the HEV strains were detected from sampling sits 4–6, located in the Paranaque River and the Las Pinas River. None of the water samples from the Pasig River (sampling sites 1–3) were found to be HEV RNA positive. The Paranaque River and the Las Pinas River are considerably smaller than the Pasig River, and flow through a residential area having high population density. The degree of wastewater pollution is higher for sampling sites 4–6 than for sampling sites 1–3. All of the HEV detected in the river water samples belonged to G3. Genotype 3 HEV can be isolated not only from infected humans but is known to be zoonotic and has also been isolated from domestic swine and wild boars, wild deer, mongoose, and rabbits.[6,7,9,11,19,20] The rivers were probably contaminated with HEV by human or animal excrement, or both.

In conclusion, we have detected and here reported HEV in the Philippines for the first time, and showed that G3 HEV in particular is circulating in the rivers of Manila City. To fully elucidate and address the HEV infection situation in the Philippines, it will be necessary to collect and analyze hepatitis patients' information and investigate the prevalence of HEV infection in swine and wild animals in these areas.

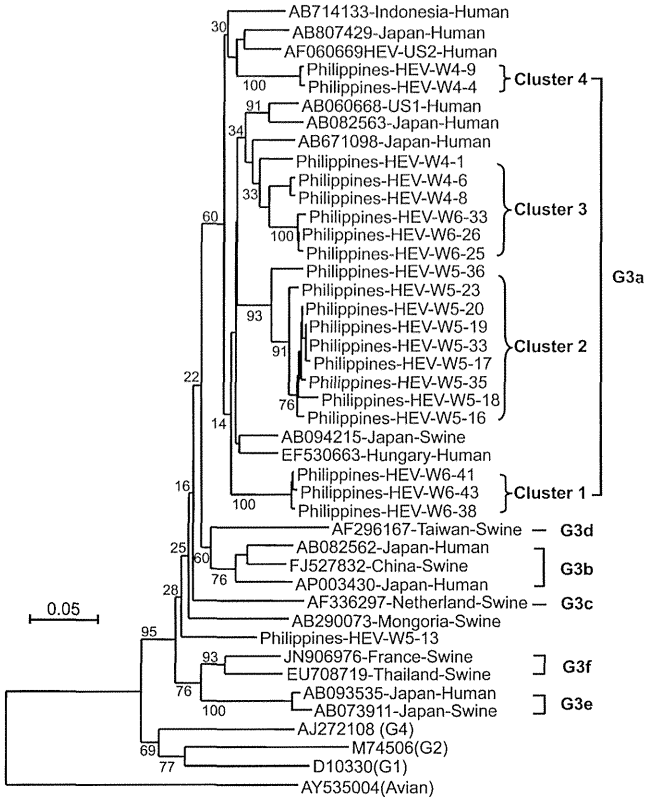


FIGURE 2. Phylogenetic analysis of HEV isolated from river water samples in Manila City, the Philippines. Nucleic acid sequence alignment was performed using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by Kimura's two-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method based on the partial genome (338 nt) of HEV ORF2 of the genotypes 1–4 and avian HEV isolates. The scale bar indicates nucleotide substitutions per site.

Received September 29, 2013. Accepted for publication January 14, 2014.

Published online March 3, 2014.

Acknowledgments: We thank Hiroyuki Katayama (University of Tokyo) for helpful discussions.

Financial support: This work was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan, and the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministries of Education, Culture, Sports, Science and Technology of Japan.

Authors' addresses: Tian-Cheng Li, Tomoyuki Shiota, Sayaka Yoshizaki, Takaji Wakita, and Koji Ishii, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, E-mails: litc@nih.go.jp, t-shiota@nih.go.jp, yoshizak@nih.go.jp, wakita@nih.go.jp, and kishii@nih.go.jp. Tingting Yang, Department of Clinical Laboratory, Affiliated Hospital of Qingdao University Medical College, Qingdao, China, E-mail: ttyang629@126.com. Hiromu Yoshida, Mariko Saito, Toshifumi Imagawa, and Hitoshi Oshitani, Department of Virology, Tohoku University Graduate School of Medicine, Miyagi, Japan, E-mails: hyoshida@nih.go.jp, saitom@med.tohoku.ac.jp, imagawat@med.tohoku.ac.jp, and oshitanih@med.tohoku.ac.jp. Fidelino F. Malbas and Socorro P. Lupisan, RITM-Tohoku Collaborating Research Center on Emerging and Re-Emerging Infectious Diseases, Filinvest Corporate City, Alabang, Muntinlupa City 1781, the Philippines, E-mails: fidelmalbas@yahoo.com and socorrolupisan@yahoo.com.

REFERENCES

1. Purcell RH, Emerson SU, 2008. Hepatitis E: an emerging awareness of an old disease. *J Hepatol 48:* 494–503.
2. Meng XJ, Anderson DA, Arankalle VA, Emerson SU, Harrison TJ, Jameel S, Okamoto H, 2012. Hepeviridae. King AM, Carstens MJ, Lefkowitz EJ, eds. *Virus Taxonomy*. Ninth Report of the ICTV. London: Elsevier/Academic Press, 1021–1028.
3. Emerson SU, Purcell RH, 2003. Hepatitis E virus. *Rev Med Virol 13:* 145–154.
4. Balayan MS, Andjaparidze AG, Savinskaya SS, Ketiladze ES, Braginsky DM, Savinov AP, Poleschuk VF, 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology 20:* 23–31.
5. Navaneethan U, Al Mohajer M, Shata MT, 2008. Hepatitis E and pregnancy: understanding the pathogenesis. *Liver Int 28:* 1190–1199.
6. Tei S, Kitajima N, Takahashi K, Mishiro S, 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet 362:* 371–373.
7. Li TC, Chijiwa K, Sera N, Ishibashi T, Etoh Y, Shinohara Y, Kurata Y, Ishida M, Sakamoto S, Takeda N, Miyamura T, 2005. Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis 11:* 1958–1960.
8. Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, Heyries L, Raoult D, Gerolami R, 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. *J Infect Dis 202:* 825–834.
9. Zhao C, Ma Z, Harrison TJ, Feng R, Zhang C, Qiao Z, Fan J, Ma H, Li M, Song A, Wang Y, 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. *J Med Virol 81:* 1371–1379.
10. Yamamoto H, Suzuki J, Matsuda A, Ishida T, Ami Y, Suzaki Y, Adachi I, Wakita T, Takeda N, Li TC, 2012. Hepatitis E virus outbreak in monkey facility, Japan. *Emerg Infect Dis 18:* 2032–2034.
11. Nakamura M, Takahashi K, Taira K, Taira M, Ohno A, Sakugawa H, Arai M, Mishiro S, 2006. Hepatitis E virus infection in wild mongooses of Okinawa, Japan: demonstration of anti-HEV antibodies and a full-genome nucleotide sequence. *Hepatol Res 34:* 137–140.
12. Meng XJ, 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol 140:* 256–265.
13. Arankalle VA, Tsarev SA, Chadha MS, Alling DW, Emerson SU, Banerjee K, Purcell RH, 1995. Age-specific prevalence of antibodies to hepatitis A and E viruses in Pune, India, 1982 and 1992. *J Infect Dis 171:* 447–450.
14. Chandra V, Taneja S, Kalia M, Jameel S, 2008. Molecular biology and pathogenesis of hepatitis E virus. *J Biosci 33:* 451–464.
15. Kamar N, Bendall R, Legrand-Abravanel F, Xia NS, Ijaz S, Izopet J, Dalton HR, 2012. Hepatitis E. *Lancet 379:* 2477–2488.
16. Tao Z, Song Y, Wang H, Zhang Y, Yoshida H, Ji S, Xu A, Song L, Liu Y, Cui N, Ji F, Li Y, Chen P, Xu W, 2012. Intercity spread of echovirus 6 in Shandong Province, China: application of environmental surveillance in tracing circulating enteroviruses. *Appl Environ Microbiol 78:* 6946–6953.
17. Li TC, Ochiai S, Ishiko H, Wakita T, Miyamura T, Takeda N, 2012. A retrospective study on imported hepatitis E in Japan. *Travel Med Infect Dis 10:* 80–85.
18. Lu L, Li C, Hagedorn CH, 2006. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol 16:* 5–36.
19. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU, 1997. A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA 94:* 9860–9865.
20. Li TC, Saito M, Ogura G, Ishibashi O, Miyamura T, Takeda N, 2006. Serologic evidence for hepatitis E virus infection in mongoose. *Am J Trop Med Hyg 74:* 932–936.

TCF1 and LEF1 act as T-cell intrinsic HTLV-1 antagonists by targeting Tax

Guangyong Ma[a], Jun-ichirou Yasunaga[a,1], Hirofumi Akari[b], and Masao Matsuoka[a,1]

[a]Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan; and [b]Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan

Edited by Patrick L. Green, The Ohio State University, Columbus, Ohio, and accepted by the Editorial Board January 12, 2015 (received for review October 5, 2014)

Human T-cell leukemia virus type 1 (HTLV-1) is a delta-type retrovirus that induces malignant and inflammatory diseases during its long persistence in vivo. HTLV-1 can infect various kinds of cells; however, HTLV-1 provirus is predominantly found in peripheral CD4 T cells in vivo. Here we find that TCF1 and LEF1, two Wnt transcription factors that are specifically expressed in T cells, inhibit viral replication through antagonizing Tax functions. TCF1 and LEF1 can each interact with Tax and inhibit Tax-dependent viral expression and activation of NF-κB and AP-1. As a result, HTLV-1 replication is suppressed in the presence of either TCF1 or LEF1. On the other hand, T-cell activation suppresses the expression of both TCF1 and LEF1, and this suppression enables Tax to function as an activator. We analyzed the thymus of a simian T-cell leukemia virus type 1 (STLV-1) infected Japanese macaque, and found a negative correlation between proviral load and TCF1/LEF1 expression in various T-cell subsets, supporting the idea that TCF1 and LEF1 negatively regulate HTLV-1 replication and the proliferation of infected cells. Thus, this study identified TCF1 and LEF1 as Tax antagonistic factors in vivo, a fact which may critically influence the peripheral T-cell tropism of this virus.

HTLV-1 | Tax | TCF1 | LEF1

Human T-cell leukemia virus type 1 (HTLV-1) causes a malignancy named adult T-cell leukemia (ATL) and several inflammatory diseases including HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1, 2). HTLV-1 encodes a critical transactivator, Tax, that induces the activation and subsequent clonal expansion of infected T cells in vivo (2, 3). Tax is transcribed from the viral promoter 5′ long terminal repeat (LTR), where it further enhances HTLV-1 viral transcription by recruiting cellular CREB protein to Tax-responsive elements (TRE). However, Tax expression is frequently silenced in ATL cells due to genetic and epigenetic changes in the viral 5′ LTR and the *tax* gene (4–7), a possible consequence of host immune surveillance (8). On the other hand, the viral 3′ LTR remains intact and is responsible for consistent expression of the HTLV-1 bZIP factor (HBZ), a negative strand encoded accessory gene, in all ATL cells (9).

T-cell factor 1 (TCF1) and lymphoid-enhancer binding factor 1 (LEF1) are transcription factors of the Wnt pathway that bind to β-catenin to coactivate the downstream cascade (10, 11). They are predominantly expressed in T-lineage cells, with immature thymocytes having the highest expression (12). Thymocyte development was impaired in TCF1 knockout mice (13). Although LEF1 knockout did not significantly affect T-cell development, deficiency in both TCF1 and LEF1 resulted in a complete block at the immature single positive stage, indicating a functional redundancy of TCF1/LEF1 and their indispensible role in driving T-cell development (14). In contrast, their functions in peripheral T cells remain poorly characterized although a quite different role has been suggested due to their reduced expression upon T-cell receptor (TCR) engagement in CD8 T cells (15).

HTLV-1 is peripheral mature T-cell tropic. However, the mechanism of this tropism remains to be elucidated. Here we find that TCF1 and LEF1 are T-cell intrinsic factors that suppress HTLV-1 replication via antagonizing Tax. They interact with Tax and suppress its transactivating abilities. As a result, viral transcription and replication are greatly suppressed by either TCF1 or LEF1, resulting in selective viral replication in TCF1/LEF1 low-expressing T cells. At the same time, Tax is able to down-regulate TCF1/LEF1 by inducing STAT5a expression. We further demonstrate that thymocytes from a simian T-cell leukemia virus type 1 (STLV-1) infected Japanese macaque have low viral abundance and low 5′ LTR activity, negatively correlating with their high expression of TCF1 and LEF1.

Results

TCF1/LEF1 Are Expressed at Low Levels in HTLV-1–Infected T Cells. Previously we reported that HBZ impaired the DNA-binding ability of TCF1/LEF1 and thereby suppressed the canonical Wnt pathway, shaping an HTLV-1 favorable host environment (16). Interestingly, upon further study, we found that TCF1 and LEF1 mRNA and protein levels were invariably low in HTLV-1–infected cell lines, in contrast to most HTLV-1–negative T-cell lines except Kit225 (Fig. 1 *A* and *B*). Fresh ATL cells exhibited reduced expression of TCF1 and LEF1 compared with CD4 T cells from a healthy donor (Fig. 1*C*). Moreover, by analyzing microarray data of HTLV-1–infected individuals including asymptomatic carriers (AC), HAM/TSP, and ATL patients (GSE19080 and GSE33615), we observed similar down-regulation of TCF1 and LEF1 (Fig. S1 *A* and *B*).

Significance

HTLV-1 is a peripheral T-cell tropic virus and induces proliferation of CD4+ T cells, resulting in T-cell malignancy and inflammatory diseases. Recent studies demonstrated that several restriction factors inhibiting HIV are also inhibitory to HTLV-1. We identified two T-cell–specific proteins, TCF1 and LEF1, as HTLV-1 restriction factors that determine the peripheral T-cell tropism of this virus by targeting Tax. They are highly expressed in immature thymocytes and thereby become a natural intrinsic barrier for HTLV-1 replication in the thymus. However, their expression can be down-regulated by Tax, as well as by activation and differentiation of T cells. These findings provide a mechanistic understanding of how HTLV-1 induces T-cell malignancies in the periphery but never in the thymus.

Author contributions: G.M., J.-i.Y., and M.M. designed research; G.M. and J.-i.Y. performed research; H.A. contributed new reagents/analytic tools; G.M., J.-i.Y., and M.M. analyzed data; G.M., J.-i.Y., and M.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. P.L.G. is a guest editor invited by the Editorial Board.

[1]To whom correspondence may be addressed. Email: mmatsuok@virus.kyoto-u.ac.jp or jyasunag@virus.kyoto-u.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1419198112/-/DCSupplemental.

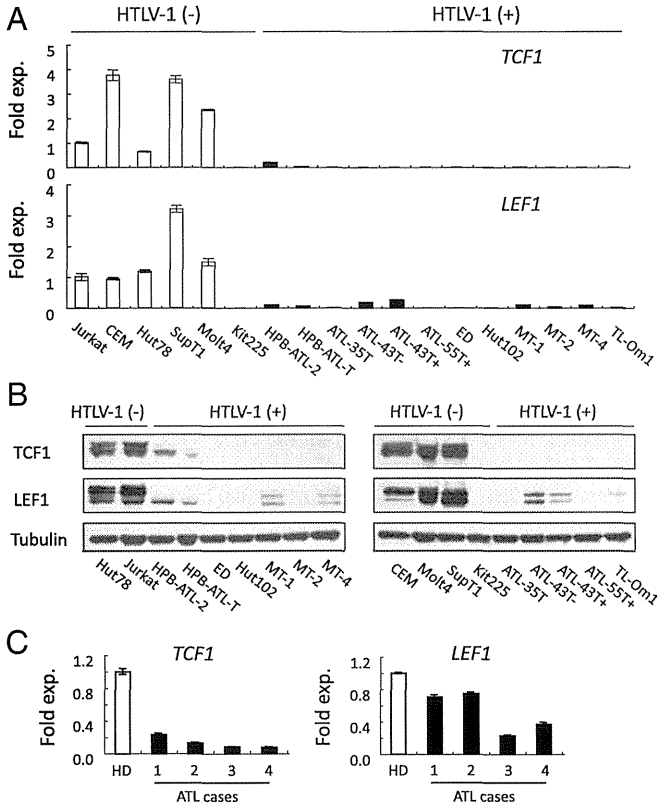


Fig. 1. TCF1 and LEF1 are expressed at low levels in HTLV-1–infected T cells. (*A*) TCF1 and LEF1 mRNA expression is invariably low in HTLV-1–infected cell lines. Total RNA was extracted for each cell line and subjected to quantitative real-time PCR (qPCR) analysis. Results are shown as relative mRNA expression of TCF1 or LEF1 normalized to that of 18S rRNA. (*B*) TCF1 and LEF1 protein expression of cell lines used in *A*. α-tubulin expression was used as a control. (*C*) TCF1 and LEF1 mRNA expression is lower in fresh ATL cases. Peripheral CD4 T cells from a healthy donor (HD) and four ATL patients were subjected to RNA extraction and following qPCR analysis. Results are shown as relative mRNA expression of TCF1 or LEF1 normalized to that of 18S rRNA. "Fold exp." indicates fold expression of normalized mRNA level of TCF1 or LEF1.

TCF1 and LEF1 Interact with Tax and Impair its Transactivating Ability. TCF family members have been recently reported to inhibit HIV type 1 (HIV-1) basal transcription (17). Therefore, we analyzed effects of TCF1 and LEF1 on transcription from the HTLV-1 LTR. As observed in HIV-1, we found that Tax-mediated activation of WT-Luc, which contains five tandem repeats of the TRE from HTLV-1 5′ LTR, was inhibited by TCF1 or LEF1 (Fig. 2*A*). Moreover, activation of the NFκB and AP1 pathways by Tax was also suppressed by TCF1 or LEF1 (Fig. 2*A*). Neither the activator of the Wnt pathway β-catenin nor the inhibitor Axin2 had such effects (Fig. S2*A*), indicating that the effects of TCF1 and LEF1 were mediated in a Wnt-independent manner. Furthermore, neither TCF1 nor LEF1 could inhibit the activation of these reporters by other transcription factors (Fig. S2*B*), suggesting that TCF1 and LEF1 specifically impair Tax function. We performed coimmunoprecipitation (co-IP) and found that TCF1 and LEF1 could each associate physically with Tax in vivo (Fig. 2*B*). Using a series of deletion mutants of Tax, we found that TCF1 and LEF1 predominantly bound to the C-terminal region of Tax (Fig. S2*C*). The PDZ-binding motif (PBM) is known to be localized in the C-terminal end of Tax (3). We found that removal of the PBM greatly impaired Tax binding to TCF1 or LEF1 (Fig. 2*C*), indicating that the PBM of Tax is critical for its binding with TCF1/LEF1. However, Tax bound to distinct regions of TCF1 and LEF1. The central regulation domain of TCF1 was indispensable for binding to Tax whereas all three domains were required for LEF1 to bind to Tax properly (Fig. S3*A*). Reporter assays with WT-Luc also functionally verified this result (Fig. S3*B*).

Nevertheless, due to their broad-spectrum antagonism of Tax, we suspected TCF1 and LEF1 might competitively bind to Tax over other host factors that are hijacked by Tax for transactivation of the viral LTR. CREB is recruited by Tax for its activation of the HTLV-1 5′ LTR (3). We found that TCF1 or LEF1 dose-dependently displaced CREB from Tax (Fig. 2*D*), which suggests that TCF1 and LEF1 each hinder the interaction between Tax and CREB. Thus, these data demonstrate that TCF1 and LEF1 are Tax antagonists that likely execute their inhibition via direct interaction with Tax.

TCF1 and LEF1 Inhibit HTLV-1 Replication by Antagonizing Tax. Next we examined the biological effects of this antagonism on Tax. HTLV-1 replication depends on Tax-driven transcription from the 5′ LTR. To address whether TCF1 and LEF1 are detrimental to HTLV-1 replication, we used an infectious clone of HTLV-1, pX1MT-M (18). HTLV-1 virus production measured by p19 ELISA was inhibited by TCF1 or LEF1 in a dose-dependent manner (Fig. 3*A*). Furthermore, expression of viral proteins that rely on Tax, such as gp46, p19, p24, and even Tax itself, was suppressed by TCF1 or LEF1 (Fig. 3*A*). We also found that endogenous TCF1 or LEF1 is also able to suppress HTLV-1 replication (Fig. S4).

On the other hand, HBZ transcription, which is initiated from viral 3′ LTR and slightly enhanced by Tax (19), was not suppressed but rather enhanced by TCF1 or LEF1 (Fig. 3*B*), in sharp contrast to Tax (Fig. 3*B*). To see whether this was associated with differential regulation of the HTLV-1 5′ and 3′ LTRs

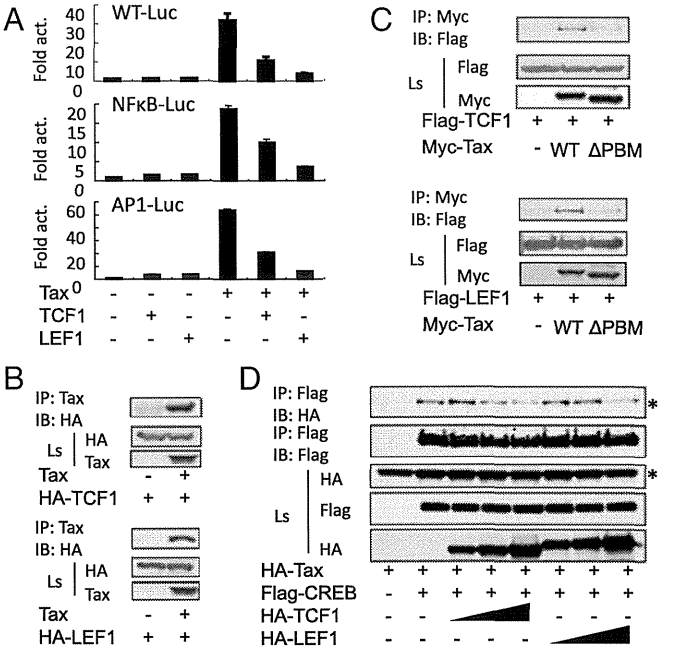


Fig. 2. TCF1 and LEF1 each interact with Tax and impair its transactivating ability. (*A*) TCF1 and LEF1 each repress Tax-mediated activation of WT-Luc (*Top*), NFκB-Luc (*Middle*), and AP1-Luc (*Bottom*). Reporter assays were performed in Jurkat cells. (*B*) Physical interactions between TCF1 and Tax (*Upper*), and LEF1 and Tax (*Lower*). (*C*) A ΔPBM mutant of Tax has impaired binding to TCF1 (*Upper*) and LEF1 (*Lower*) compared with WT Tax. (*D*) Physical interactions between Tax and CREB are inhibited by TCF1 or LEF1 in a dose-dependent manner. Tax-specific bands are denoted with an asterisk. All immunoprecipitations were performed in 293FT cells. "Ls" indicates the whole cell lysate.

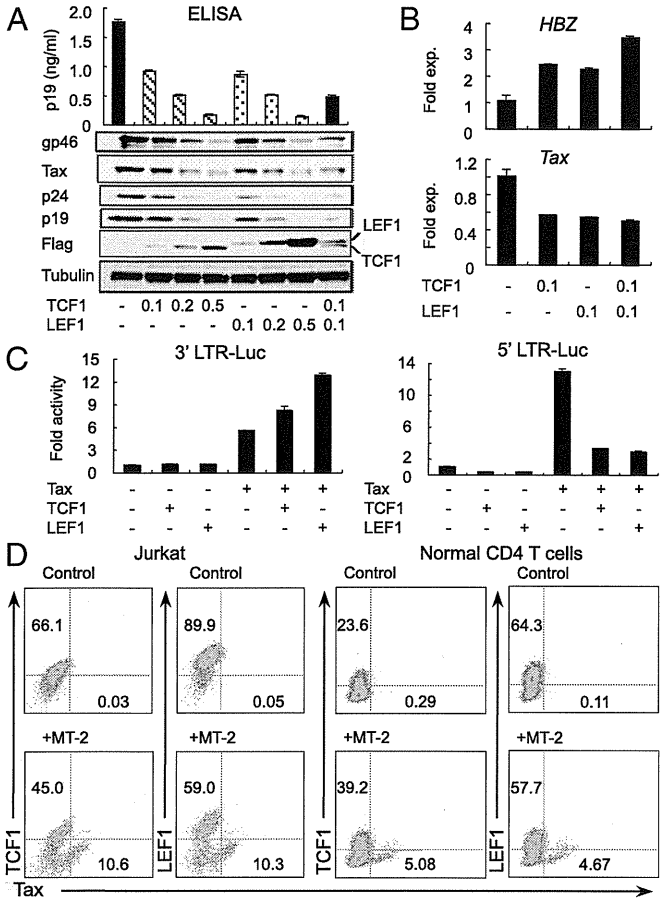


Fig. 3. TCF1 and LEF1 each inhibit HTLV-1 replication by antagonizing Tax. (*A*) TCF1 and LEF1 each inhibits HTLV-1 production (*Upper*) and protein expression (*Lower*). pX1MT-M (0.5 μg) was transfected with or without TCF1 or LEF1 into 293FT cells. 48 h later, supernatants were collected for p19 ELISA and cells were lysed for Western blot. (*B*) TCF1 and LEF1 each inhibit Tax transcription (*Lower*) but not HBZ transcription (*Upper*). pX1MT-M (0.5 μg) was transfected with or without TCF1 or LEF1 into 293FT cells. 44 h later, RNA was extracted for qPCR analysis. (*C*) TCF1 and LEF1 each slightly enhance Tax-mediated 3′ LTR-Luc (*Left*) activation, whereas they significantly suppress 5′ LTR activation (*Right*). Reporter assays were performed in Jurkat cells. (*D*) Jurkat or normal human CD4 T cells were either cultured alone (*Upper*) or cocultivated with lethally irradiated (150 Gy) MT-2 cells (*Lower*) at a 2:1 ratio. 48 h later (when MT-2 cells were all dead), cells were stained for intracellular Tax and TCF1 or LEF1. Numerals indicate percentages of gated populations. Fold exp. indicates fold expression.

by TCF1/LEF1, we performed reporter assays with the complete 5′ and 3′ LTR sequences. Tax mildly activated the 3′ LTR, and this activation was enhanced by TCF1 or LEF1 (Fig. 3*C*). This observation explains why HBZ transcription increased in the presence of TCF1/LEF1 (Fig. 3*B*). Consistent with the WT-Luc result (Fig. 2*A*), TCF1 or LEF1 significantly suppressed Tax-induced 5′ LTR activation (Fig. 3*C*). To evaluate the effect of TCF1/LEF1 upon HTLV-1 de novo infection, we cocultivated Jurkat or normal CD4 T cells with lethally irradiated MT-2 cells. Tax expression was detected predominantly in the TCF1/LEF1 low-expressing fraction (Fig. 3*D*), suggesting that TCF1/LEF1 restricts HTLV-1 de novo viral expression and its replication.

Tax Down-Regulates TCF1 and LEF1 via STAT5a. Antigen encounter or T-cell activation were reported to trigger TCF1/LEF1 down-regulation (15). We confirmed that phorbol myristate acetate (PMA)/ionomycin (P/I) stimulation down-regulate TCF1 and LEF1 in Jurkat and primary CD4 T cells (Fig. 4*A* and Fig. S5*A*). Therefore, we suspected that reduced expression of TCF1 and LEF1 in HTLV-1–infected cells is also caused by Tax, which is known to activate T cells (3). As expected, Tax induced the expression of the same activation markers as P/I stimulation (Fig. S5*B*), and suppressed the expression of TCF1 and LEF1 in Jurkat cells (Fig. 4*A*). Furthermore, cadmium-induced Tax expression in JPX-9, a modified Jurkat line that expresses Tax under a metallothionein promoter (20), also down-regulated TCF1 and LEF1 (Fig. 4*B*). However, Tax (Fig. S5*C*) did not inhibit transcription from the TCF1 and LEF1 promoters. To see whether the NFκB, NFAT, or AP1 pathways, the three major TCR downstream pathways, are involved in TCF1/LEF1 down-regulation (21), we activated them by electroporation of the corresponding transcription factors into Jurkat (Fig. S5*D*). However, neither single nor combined activation of these pathways clearly suppressed TCF1 or LEF1 expression (Fig. S5*E*). JAK/STAT signaling, a major cytokine pathway of T cells that becomes active following T-cell activation (22), has been found to be constitutively active in HTLV-1–infected T cells (23). Because STAT proteins are transcription factors that activate this pathway (24), we examined the effect of STAT5a, which is reported to be a target of Tax (25). First, we confirmed that STAT5a expression was induced upon P/I stimulation and Tax expression (Fig. 4*C*). Then we overexpressed either the wild type or the constitutively active form of STAT5a in Jurkat cells, and found significantly decreased expression of TCF1 and LEF1 (Fig. 4*D*).

Higher Expression of TCF1 and LEF1 Is Associated with Low STLV-1 Proviral Load in Vivo. The above results suggest that Tax function and HTLV-1 replication are impaired in TCF1/LEF1 high expressing cells, most likely in thymocytes that express higher levels of TCF1/LEF1. To analyze the relationship between TCF1/LEF1 expression and proviral load (PVL) in vivo, a model of HTLV-1 infection was required. We have reported that

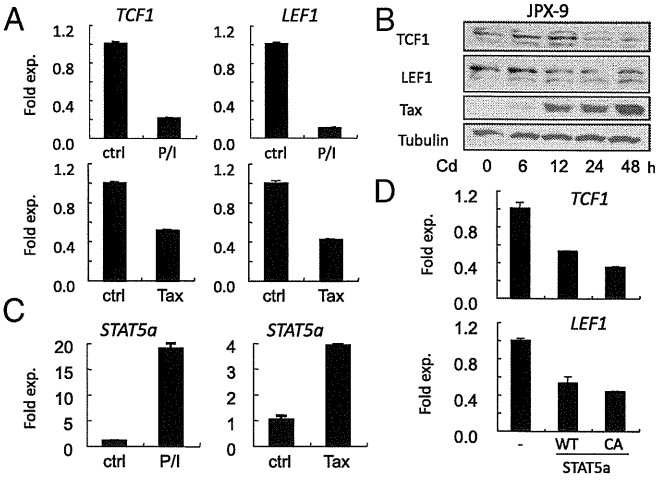


Fig. 4. Tax down-regulates the expression of TCF1 and LEF1 via STAT5a. (*A*) P/I stimulation (*Upper*) or Tax overexpression (*Lower*) inhibits TCF1/LEF1 transcription in Jurkat. For P/I stimulation, cells were treated with 50 ng/mL of PMA and 500 ng/mL of ionomycin (P/I) for 5 h and then subjected to RNA extraction and qPCR analysis. Overexpression of Tax was achieved by electroporation and 24 h later, RNA was extracted for qPCR. (*B*) Tax induction in JPX-9 down-regulates the expression of TCF1 and LEF1. JPX-9 was cultured in RPMI supplemented with 20 μM of cadmium (Cd) to induce Tax expression. At indicated time points, cells were lysed for Western blot analysis. (*C*) P/I stimulation or Tax overexpression induces STAT5a expression in Jurkat. P/I stimulation and Tax overexpression were performed as in *A*. (*D*) Overexpression of STAT5a down-regulates TCF1 and LEF1. Jurkat was transfected with wild type (WT) or constitutively active (CA) STAT5a by electroporation. 24 h later, RNA was extracted for qPCR. Fold exp. indicates fold expression.

STLV-1 encoded Tax and STLV-1 bZIP factor (SBZ) possess functions similar to those of HTLV-1 Tax and HBZ, and an STLV-1–infected Japanese macaque developed T-cell lymphoma (26), indicating that STLV-1–infected Japanese macaques can serve as a suitable model of HTLV-1 infection. STLV-1 Tax is highly homologous to HTLV-1 Tax (26). Similar to HTLV-1 Tax, it also has a typical PDZ-binding motif (ETDV) in its C-terminal end. We sorted various T-cell subsets from an STLV-1–infected Japanese macaque (Fig. S6) and found that CD4+CD8+ thymocytes (T-DP) showed the highest expression levels of TCF1 and LEF1 (Fig. 5*A*) whereas their PVL was the lowest (Fig. 5*B*). This result is consistent with our hypothesis that TCF1 and LEF1 inhibit viral expansion through impairing both the function and expression of Tax (Fig. 2 and 3). CD4+ thymocytes (T-CD4) were about twofold higher in TCF1/LEF1 expression (Fig. 5*A*) than their counterparts in the periphery (P-CD4). However, the PVL of P-CD4 T cells was 10-fold higher than that of T-CD4 T cells (Fig. 5*B*). Similar measurements were made in thymic (T-CD8) and peripheral CD8 T cells (P-CD8) (Fig. 5*A* and *B*). Interestingly, only a 1.3-fold increase of PVL in P-CD8 over T-CD8 was observed, in contrast to a 10-fold increase in P-CD4 over T-CD4 (Fig. 5*B*). Along with the fact that thymic CD8 and CD4 T cells had similar PVLs, this observation implies a much smaller expansion of infected CD8 T cells in the periphery than of CD4 T cells, an observation in agreement with a previous report showing that HTLV-1's in vivo tropism is a consequence of predominant expansion of peripheral CD4 over CD8 T cells (27).

Next we compared the levels of transcriptional activity from the 5′ and 3′ LTRs of the provirus in STLV-1–infected cells. We did this by normalizing either Tax or SBZ transcription to PVL. Recall that TCF1/LEF1 regulate transcription of these genes in opposing manners (Fig. 3*B*). The 5′ LTR was clearly more active in peripheral CD4 or CD8 T cells than their thymic counterparts (Fig. 5*C*). In contrast, transcription from the 3′ LTR was more active in thymocytes, although the differences were not so big as with the 5′LTR (Fig. 5*D*). Memory (CD45RA-) CD4 T cells from the spleen of the STLV-1–infected Japanese macaque showed lower TCF1 and LEF1 expression but much higher PVL than naïve (CD45RA+) CD4 T cells (Fig. 5 *E* and *F*), which is in agreement with the fact that HTLV-1–infected cells have mostly a memory phenotype (28).

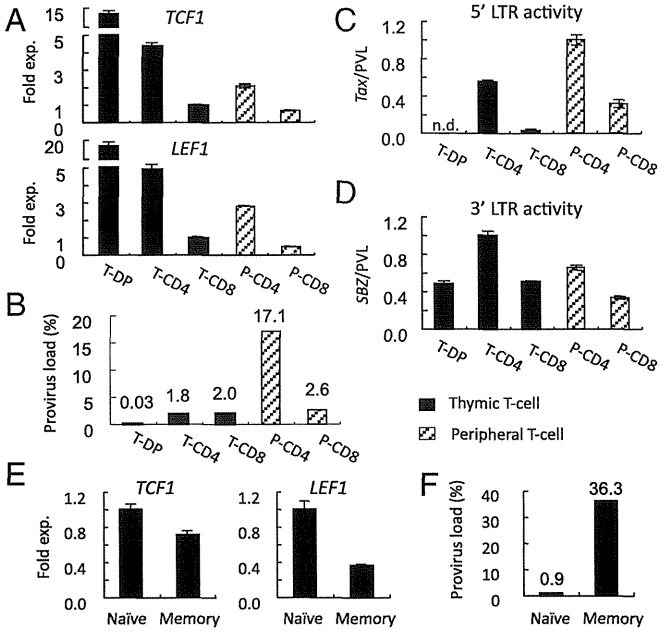


Fig. 5. TCF1 and LEF1 expression correlate negatively with STLV-1 proviral load in vivo. (*A*) TCF1 (*Upper*) and LEF1 (*Lower*) transcription in sorted CD4/CD8 double positive (T-DP), CD4 single positive (SP) thymocytes (T-CD4), CD8 SP thymocytes (T-CD8) and peripheral CD4 (P-CD4) and CD8 (P-CD8) SP T-cells from an STLV-1–infected Japanese macaque, determined by qPCR. (*B*) Genomic DNAs of sorted T cells were analyzed for STLV-1 proviral load. Numerals indicate number of virus copies in 100 cells. (*C*) Relative 5′ LTR activity of infected T cells. Tax mRNA expression was normalized to PVL of the same subset to represent relative transcription efficiency from 5′ LTR. (*D*) Relative 3′ LTR activity of infected T cells determined by normalizing SBZ mRNA expression to PVL. (*E*) TCF1 (*Left*) and LEF1 (*Right*) mRNA expression in sorted CD3+CD4+CD45RA+ (naive) and CD45RA- (memory) T cells from the STLV-1–infected Japanese macaque, determined by qPCR. Fold exp. indicates fold expression. (*F*) Genomic DNAs of naïve and memory T cells were analyzed for STLV-1 proviral load. Numerals indicate number of virus copies in 100 cells.

Discussion

During coevolution between virus and the host, host cells acquire many restriction factors that suppress viral replication (29, 30). HTLV-1 is derived from STLV-1 in monkeys, just like HIV-1 is derived from SIV. Many restriction factors have been reported for HIV-1 (31). However, restriction factors for HTLV-1 have not been studied extensively. It has been reported that APOBEC3G suppresses replication of HTLV-1 whereas Gag protein inhibits incorporation of APOBEC3G into the virion (32). Recently, SAMHD1 has been reported to suppress replication of HTLV-1 in monocytes (33). Tax is indispensable for HTLV-1 replication because expression of most viral genes, including all HTLV-1 structural genes, depends on transcription from the 5′ LTR that is activated by Tax. Moreover, Tax also plays a key role in dysregulating the cellular environment toward one which favors viral propagation, such by activation and transformation of an infected T cell (2). It is presumed that the T-cell tropism of HTLV-1 is more likely determined by postinfection events triggered by the virus because viral receptors are expressed in a wide variety of host cells (34). This study suggests that TCF1 and LEF1 are factors that restrict the tropism of this virus to peripheral T cells. In thymocytes expressing high levels of TCF1 and LEF1, these factors impair the functions of Tax, likely hindering not only viral replication but also the proliferation of the infected cells.

Restriction of tropism to peripheral T cells is likely a useful adaptation for HTLV-1. If HTLV-1 could replicate efficiently in the thymus, it might cause serious damage to the host immune system and thus the host. Furthermore, this virus is transmitted via breast-feeding or sexual transmission through infected T cells, so infected T cells must enter breast milk or semen. Most T cells in breast milk are peripheral T cells with an effector/memory phenotype (35). Restriction by TCF1/LEF1 would explain viral tropism to peripheral T cells and facilitate transmission of the virus.

Neoplasm of immature T cells has not been reported in HTLV-1–infected individuals. However, transgenic expression of Tax in the thymus induced immature T-cell lymphomas (36). These findings suggest that overexpression of Tax is oncogenic even for thymocytes, but that Tax expression or functions are normally impaired in the thymus of infected individuals. This study presents a mechanism for how thymocytes are relatively resistant to HTLV-1 infection and leukemogenesis in vivo, by identifying TCF1 and LEF1 as antagonists for Tax. We discovered an unexpected Wnt-independent role of TCF1 and LEF1 as Tax antagonists and demonstrated that this antagonism renders thymocytes less permissive for HTLV-1 replication compared with peripheral T cells.

The roles of TCF1/LEF1 have been well established in the thymus; they are indispensable in driving T-cell development (37). Nevertheless, their functions in the periphery remain

unknown. Recent studies showed that down-regulation of TCF1/LEF1 always occurs in activated or differentiated peripheral T cells (38). HTLV-1 may exploit this down-regulation to achieve its expansion, because down-regulation of TCF1/LEF1 allows Tax to execute its functions. A previous report also indicated that preactivated primary T cells are easier to transform by HTLV-1 (39). Down-regulation of TCF1/LEF1 upon T-cell activation/differentiation would allow Tax expression and subsequent HTLV-1 expansion.

Down-regulation of TCF1/LEF1 also occurs as T cells develop or differentiate, from DP to SP in the thymus (Fig. 5*A*), or from naïve to memory in the periphery (Fig. 5*E*). Therefore, our results also imply an interesting possibility that HTLV-1 might achieve its expansion as infected T cells differentiate or even by driving differentiation of infected T cells to reduce TCF1/LEF1 expression. Indeed, a recent report using humanized mice showed altered T-cell development upon HTLV-1 infection in that the mature SP population, instead of immature DN or DP, becomes dominant in the thymus (40). This finding suggests that thymocytes are propelled to develop by HTLV-1 or the virus selectively expands in the more differentiated subsets. Similarly, in a previous study of peripheral T cells, we demonstrated that HTLV-1–infected T cells were mostly memory cells and the number of naïve cells was significantly decreased (28). Our current results also reveal the preferential infection of CD4 effector/memory T cells by STLV-1. However, to clarify the roles of T-cell development/differentiation in contributions of HTLV-1 expansion, further studies are needed.

STLV-1–infected Japanese macaque has been demonstrated to be a suitable model for HTLV-1 infection (26). It also served as an ideal model to analyze the impact of the antagonism of TCF1/LEF1 against Tax in vivo. However, due to the complexity of viral infections in vivo, other factors such as the susceptibility to viral infections, postinfection mitotic potential and cytotoxic T-cell killing efficiency might affect the consequence of an infection in a specific T-cell subset. Indeed, the tropism of the virus for peripheral CD4 T cells over peripheral CD8 T cells does not appear to be explained by TCF1/LEF1 levels. More detailed investigations in STLV-1–infected Japanese macaques are expected to clarify these points in the future.

TCF1/LEF1 regulate the HTLV-1 5′ and 3′ LTR activities in opposing manners via their interplay with Tax (Fig. 3*C*). This may result in distinct expression levels of Tax and HBZ in vivo in different T-cell subsets or during various stages of infection. Interestingly, valproate, a histone deacetylase inhibitor, was reported to induce Tax expression while suppressing that of HBZ (41). These intriguing observations that the HTLV-1 5′ and 3′ LTR are regulated in opposite ways by multiple mechanisms, in addition to frequently observed contradictory functions of Tax and HBZ, may suggest a complex but fine-tuned viral pathogenesis. For instance, although activation of NF-κB pathway has been considered a critical function of Tax for cellular transformation (2, 3), the recent studies have reported that hyperactivation of NF-κB pathway induces cellular senescence whereas HBZ suppresses this action of Tax, thereby enabling clonal expansion (42). This study shows that TCF1/LEF1 inhibit Tax-mediated NF-κB activation by direct binding to Tax. Furthermore, TCF1/LEF1 inhibit various functions of Tax, whereas HBZ selectively modulates signaling pathways (43, 44). Thus, Tax and HBZ collaboratively function for clonal expansion and viral replication, whereas TCF1/LEF1 inhibit functions of Tax by direct interaction, which leads to suppression of viral replication and proliferation of infected cells.

In summary, we here identify TCF1 and LEF1 as previously unidentified Tax antagonists that likely restrict viral expansion in the thymus. The critical interplay of TCF1 and LEF1 with Tax during HTLV-1 infection may shed light on how HTLV-1 achieves its tropism and persistence in peripheral T cells in vivo.

Materials and Methods

Primary Samples Ethics Statement. The experiments using primary samples in this study were conducted according to the principles expressed in the Declaration of Helsinki. This study was approved by the Institutional Review Board of Kyoto University (approval numbers G310 and E2005). All ATL patients and healthy individuals provided written informed consent for the collection of samples and subsequent analysis. A Japanese macaque used in this study was 3 y old and naturally infected with STLV-1. The monkey was reared in the Primate Research Institute, Kyoto University. All animal studies were conducted in accordance with the protocols of experimental procedures approved by the Animal Welfare and Animal Care Committee of the Primate Research Institute (approval number 2011–095).

Cell Lines. ATL-derived T-cell lines (HPB-ATL-2, HPB-ATL-T, ATL-43T-, ATL-43T+, ATL-55T+, ED, MT-1, and TL-Om1), HTLV-1–transformed T-cell lines (ATL-35T, Hut102, MT-2, and MT-4) were used in this study. Jurkat, CEM, Hut78, SupT1, Molt4, and Kit225 are HTLV-1–negative T-cell lines. All T-cell lines were maintained in RPMI supplemented with 10% (vol/vol) FBS, whereas Kit225, ATL-43T+, and ATL-55T+ were maintained in the media supplemented with 100 U/mL of recombinant IL-2. 293FT (Life Technologies) is a subline of HEK293, which originated from a human embryonic kidney cell.

Plasmids. Expression vectors for TCF1, LEF1, and Tax were described (16, 45). Flag-CREB was made by subcloning the CREB coding sequence into pCAG-Flag. WT-Luc and 5′ LTR-Luc were kind gifts from J. Fujisawa, Kansai Medical University, Osaka. pX1MT-M was a generous gift from D. Derse, National Cancer Institute, Frederick, MD. NFκB-Luc and AP1-Luc were purchased from Stratagene. 3′ LTR-Luc was described (19).

Antibodies. Rabbit monoclonal antibodies for TCF1 (C63D9) and LEF1 (C12A5) were purchased from Cell Signaling Technology. HRP conjugated mouse anti-HA (12A5) antibody was purchased from Sigma. Mouse monoclonal antibodies against HTLV-1 gp46, p24, and p19 were purchased from Zeptometrix. Mouse monoclonal antibodies for FLAG (M2), Myc (9E10), α-tubulin (DM1A), and Tax (MI73) were described (16). For flow cytometric analysis of cell surface markers, APC-Cy7 anti-CD3 (SP34-2), PerCP-Cy5.5 anti-CD4 (OKT4), V500 anti-CD8 (RPA-T8), and PE anti-CD45RA (5H9) were used. PerCP-Cy5.5 anti-CD4 (OKT4) was purchased from Biolegend, whereas the others were from BD.

Detection of Tax and TCF1/LEF1 by Flow Cytometry. Intracellular staining for Tax and TCF1/LEF1 was performed using the kit from eBioscience. DyLight 649 conjugated donkey anti-rabbit IgG and FITC conjugated goat anti-mouse IgG were purchased from Biolegend. Normal mouse IgG was purchased from Santa Cruz and used for blocking nonspecific binding.

ELISA. Supernatants from cultured cells were centrifuged at 1,710 × *g* for 5 min to remove debris and then diluted and quantified for p19 by ELISA (Zeptometrix) according to manufacturer's instructions.

Sorting by FACS Aria II. See Fig. S6 for details.

Electroporation, real-time PCR, knockdown, Western blot, coimmunoprecipitation, and reporter assays were performed as described (16).

ACKNOWLEDGMENTS. We thank Drs. J. Fujisawa and D. Derse for providing reagents and Dr. L. Kingsbury for proofreading. We appreciate the help from Dr. Tani-ichi for cell sorting. This study was supported by a Grant-in-aid for Scientific Research on Innovative Area from the Ministry of Education, Science, Sports, and Culture of Japan (to M.M.) (22114003), and a grant from the Japan Leukemia Research Fund (to M.M.). This study was conducted by the Cooperation Research Program of the Primate Research Institute, Kyoto University.

1. Gallo RC (2005) History of the discoveries of the first human retroviruses: HTLV-1 and HTLV-2. *Oncogene* 24(39):5926–5930.
2. Matsuoka M, Jeang KT (2007) Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* 7(4):270–280.
3. Grassmann R, Aboud M, Jeang KT (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* 24(39):5976–5985.
4. Tamiya S, et al. (1996) Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. *Blood* 88(8):3065–3073.

5. Takeda S, et al. (2004) Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer* 109(4):559–567.
6. Furukawa Y, Kubota R, Tara M, Izumo S, Osame M (2001) Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* 97(4):987–993.
7. Koiwa T, et al. (2002) 5′-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. *J Virol* 76(18): 9389–9397.
8. Bangham CR (2009) CTL quality and the control of human retroviral infections. *Eur J Immunol* 39(7):1700–1712.
9. Satou Y, Yasunaga J, Yoshida M, Matsuoka M (2006) HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci USA* 103(3):720–725.
10. Staal FJ, Clevers HC (2005) WNT signalling and haematopoiesis: A WNT-WNT situation. *Nat Rev Immunol* 5(1):21–30.
11. Staal FJ, Luis TC, Tiemessen MM (2008) WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol* 8(8):581–593.
12. Oosterwegel M, et al. (1993) Differential expression of the HMG box factors TCF-1 and LEF-1 during murine embryogenesis. *Development* 118(2):439–448.
13. Verbeek S, et al. (1995) An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374(6517):70–74.
14. Okamura RM, et al. (1998) Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity* 8(1):11–20.
15. Willinger T, et al. (2006) Human naive CD8 T cells down-regulate expression of the WNT pathway transcription factors lymphoid enhancer binding factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo. *J Immunol* 176(3):1439–1446.
16. Ma G, Yasunaga J, Fan J, Yanagawa S, Matsuoka M (2013) HTLV-1 bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells. *Oncogene* 32(36):4222–4230.
17. Narasipura SD, et al. (2012) Role of β-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes. *J Virol* 86(4):1911–1921.
18. Mitchell MS, et al. (2007) Phenotypic and genotypic comparisons of human T-cell leukemia virus type 1 reverse transcriptases from infected T-cell lines and patient samples. *J Virol* 81(9):4422–4428.
19. Yoshida M, Satou Y, Yasunaga J, Fujisawa J, Matsuoka M (2008) Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene. *J Virol* 82(19):9359–9368.
20. Azimi N, et al. (1998) Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF-kappaB site. *Proc Natl Acad Sci USA* 95(5):2452–2457.
21. Macian F (2005) NFAT proteins: Key regulators of T-cell development and function. *Nat Rev Immunol* 5(6):472–484.
22. Welte T, et al. (1999) STAT5 interaction with the T cell receptor complex and stimulation of T cell proliferation. *Science* 283(5399):222–225.
23. Migone TS, et al. (1995) Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. *Science* 269(5220):79–81.
24. Lin JX, Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene* 19(21):2566–2576.
25. Nakamura N, et al. (1999) Human T-cell leukemia virus type 1 Tax protein induces the expression of STAT1 and STAT5 genes in T-cells. *Oncogene* 18(17):2667–2675.
26. Miura M, et al. (2013) Characterization of simian T-cell leukemia virus type 1 in naturally infected Japanese macaques as a model of HTLV-1 infection. *Retrovirology* 10:118.
27. Kannian P, et al. (2012) Distinct transformation tropism exhibited by human T lymphotropic virus type 1 (HTLV-1) and HTLV-2 is the result of postinfection T cell clonal expansion. *J Virol* 86(7):3757–3766.
28. Yasunaga Ji, et al. (2001) Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: Its implications in the immunodeficient state. *Blood* 97(10):3177–3183.
29. Wolf D, Goff SP (2008) Host restriction factors blocking retroviral replication. *Annu Rev Genet* 42:143–163.
30. Bieniasz PD (2004) Intrinsic immunity: A front-line defense against viral attack. *Nat Immunol* 5(11):1109–1115.
31. Blanco-Melo D, Venkatesh S, Bieniasz PD (2012) Intrinsic cellular defenses against human immunodeficiency viruses. *Immunity* 37(3):399–411.
32. Derse D, Hill SA, Princler G, Lloyd P, Heidecker G (2007) Resistance of human T cell leukemia virus type 1 to APOBEC3G restriction is mediated by elements in nucleocapsid. *Proc Natl Acad Sci USA* 104(8):2915–2920.
33. Sze A, et al. (2013) Host restriction factor SAMHD1 limits human T cell leukemia virus type 1 infection of monocytes via STING-mediated apoptosis. *Cell Host Microbe* 14(4): 422–434.
34. Manel N, Battini JL, Taylor N, Sitbon M (2005) HTLV-1 tropism and envelope receptor. *Oncogene* 24(39):6016–6025.
35. Bertotto A, et al. (1990) Human breast milk T lymphocytes display the phenotype and functional characteristics of memory T cells. *Eur J Immunol* 20(8):1877–1880.
36. Hasegawa H, et al. (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. *Nat Med* 12(4):466–472.
37. Weber BN, et al. (2011) A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* 476(7358):63–68.
38. Xue HH, Zhao DM (2012) Regulation of mature T cell responses by the Wnt signaling pathway. *Ann N Y Acad Sci* 1247:16–33.
39. Merl S, et al. (1984) Efficient transformation of previously activated and dividing T lymphocytes by human T cell leukemia-lymphoma virus. *Blood* 64(5):967–974.
40. Villaudy J, et al. (2011) HTLV-1 propels thymic human T cell development in "human immune system" Rag2$^{-/-}$ gamma c$^{-/-}$ mice. *PLoS Pathog* 7(9):e1002231.
41. Belrose G, et al. (2011) Effects of valproate on Tax and HBZ expression in HTLV-1 and HAM/TSP T lymphocytes. *Blood* 118(9):2483–2491.
42. Zhi H, et al. (2011) NF-κB hyper-activation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog* 7(4):e1002025.
43. Zhao T, et al. (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113(12):2755–2764.
44. Matsuoka M, Yasunaga J (2013) Human T-cell leukemia virus type 1: Replication, proliferation and propagation by Tax and HTLV-1 bZIP factor. *Curr Opin Virol* 3(6): 684–691.
45. Peloponese JM, Jr, Yasunaga J, Kinjo T, Watashi K, Jeang KT (2009) Peptidylproline cis-trans-isomerase Pin1 interacts with human T-cell leukemia virus type 1 tax and modulates its activation of NF-kappaB. *J Virol* 83(7):3238–3248.

Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pam2 lipopeptides systemically increase myeloid-derived suppressor cells through TLR2 signaling

Akira Maruyama [1], Hiroaki Shime[*, 1], Yohei Takeda, Masahiro Azuma, Misako Matsumoto, Tsukasa Seya[*]

Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan

ARTICLE INFO

Article history:
Received 24 December 2014
Available online xxx

Keywords:
Myeloid-derive suppressor cells (MDSCs)
Pam2 lipopeptides
Toll-like receptor 2
Immunosuppression
Antitumor immunotherapy

ABSTRACT

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that exhibit potent immunosuppressive activity. They are increased in tumor-bearing hosts and contribute to tumor development. Toll-like receptors (TLRs) on MDSCs may modulate the tumor-supporting properties of MDSCs through pattern-recognition. Pam2 lipopeptides represented by Pam2CSK4 serve as a TLR2 agonist to exert antitumor function by dendritic cell (DC)-priming that leads to NK cell activation and cytotoxic T cell proliferation. On the other hand, TLR2 enhances tumor cell progression/invasion by activating tumor-infiltrating macrophages. How MDSCs respond to TLR2 agonists has not yet been determined. In this study, we found intravenous administration of Pam2CSK4 systemically up-regulated the frequency of MDSCs in EG7 tumor-bearing mice. The frequency of tumor-infiltrating MDSCs was accordingly increased in response to Pam2CSK4. MDSCs were not increased by Pam2CSK4 stimuli in TLR2 knockout (KO) mice. Adoptive transfer experiments using CFSE-labeled MDSCs revealed that the TLR2-positive MDSCs survived long in tumor-bearing mice in response to Pam2CSK4 treatment. Since the increased MDSC population sustained immune-suppressive properties, our study suggests that Pam2CSK4-triggered TLR2 activation enhances the MDSC potential and suppress antitumor immune response in tumor microenvironment.

1. Introduction

TLR2 signaling pathway plays a critical role in induction of protective immunity against infection [1,2]. TLR2 enhances dendritic cell/macrophage functions that cause host defense, but exerts a controversial effect on cancer development [2]. Recent reports demonstrated that treatment with purified TLR2 ligands such as Pam2CSK4, Pam3CSK4, MALP2 or related synthetic compounds inhibited tumor growth in mice tumor implant models [3,4]. Pam2 lipopeptides trigger activation of TLR2 in combination with TLR6 or TLR1 in conventional DCs, which leads to maturation of the DCs through the MyD88-dependent signaling pathway, resulting in NK cell activation and CTL proliferation [5–7].

In tumor-bearing mice with systemic exposing to TLR2 agonists, however, an opposite effect was reported: TLR2 signal-induced inflammation may contribute to tumor progression. TLR2 is also expressed on immune cells with regulatory properties that include tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) as well as tumor cells [8–10]. Host cell-derived endogenous TLR2 ligand, such as versican, a chondroitin sulfate proteoglycan derived from cancer cells, stimulates macrophages to produce TNF-α, which enhances lung metastasis of cancer cells [11]. Furthermore, Pam2CSK4 primes DC activation to induce expansion of $Foxp3^{+}CD25^{+}CD4^{+}$ regulatory T cells (Treg) and cause immune tolerance against cancer [12,13]. These reports suggest that TLR2 signaling may modulate the myeloid cell function, which promotes growth, invasion, or metastasis of tumor cells. There might be cell type-to-cell type difference in TLR2 response to its ligands, which critically determines their mode for regulation against tumor progression or survival.

MDSCs are heterogenous populations of immature myeloid cells that have immunosuppressive activity. MDSCs are expanded in

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; CTL, cytotoxic T lymphocyte; DAMP, damage-associated molecular pattern; DC, dendritic cell; MALP-2, macrophage activating lipopeptide-2; NK, natural killer; Pam2, 16 S-[2,3-bis(palmitoyl)propyl]cysteine; TLR, toll-like receptor.

* Corresponding authors. Fax: +81 11 706 7866.
E-mail addresses: shime@med.hokudai.ac.jp (H. Shime), seya-tu@pop.med.hokudai.ac.jp (T. Seya).

[1] The first two authors were equally contributed.

http://dx.doi.org/10.1016/j.bbrc.2015.01.011
0006-291X/