Table S5 Evolutionary parameters obtained in Bayesian MCMC inference with constant size and lognormal relaxed.
(PDF)

Acknowledgments

We are grateful to all the patients who participated in our surveillance study. We appreciate the support of and helpful discussions with members of the Japanese Drug Resistance HIV-1 Surveillance Network: Atsushi Ajisawa, Hitoshi Chiba, Teruhisa Fujii, Yuko Fujikawa, Akira Fujita, Katsuyuki Fukutake, Hiroyuki Gatanaga, Tetsushi Goto, Shuji Hatakeyama, Igen Hongo, Masahide Horiba, Makiko Kondo, Makiko Kondo, Mitsuru Konishi, Shuzo Matsushita, Motoo Matsuura, Rumi Minami, Naoko Miyazaki, Haruyo Mori, Masaaki Noda, Chiho Otani, Takeyuki Sato, Satoshi Shirahata, Masashi Taki, Sadahiro Tamashima, Masap Tateyma, Masanori Tei, Kazue Uchida, Dai Watanabe, Yasuyuki Yamamoto, Kunio Yano, Mihoko Yotsumoto, Shigeru Yoshida.

We thank Dr. Frank Maldarelli for reading the manuscript critically. We also thank Claire Baldwin for her help in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: TS JH WS. Performed the experiments: JH TS YI. Analyzed the data: TS JH. Contributed reagents/materials/analysis tools: YY YI WS. Wrote the paper: TS JH WS.

References

1. Infectious Diseases Surveillance Center (2011) HIV/AIDS in Japan, 2010. IASR 32: 282–283.
2. Yamanaka T, Fujimura Y, Ishimoto S, Yoshioka A, Konishi M, et al. (1997) Correlation of titer of antibody to principal neutralizing domain of HIVMN strain with disease progression in Japanese hemophiliacs seropositive for HIV type 1. AIDS Res Hum Retroviruses 13: 317–326.
3. Kato S, Saito R, Hiraishi Y, Kitamura N, Matsumoto T, et al. (2003) Differential prevalence of HIV type 1 subtype B and CRF01_AE among different sexual transmission groups in Tokyo, Japan, as revealed by subtype-specific PCR. AIDS Res Hum Retroviruses 19: 1057–1063.
4. Weniger BG, Takebe Y, Ou CY, Yamazaki S (1994) The molecular epidemiology of HIV in Asia. AIDS 8 Suppl 2: S13–S28.
5. Ou CY, Takebe Y, Luo CC, Kalish M, Auwanit W, et al. (1992) Wide distribution of two subtypes of HIV-1 in Thailand. AIDS Res Hum Retroviruses 8: 1471–1472.
6. Ou CY, Takebe Y, Weniger BG, Luo CC, Kalish ML, et al. (1993) Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet 341: 1171–1174.
7. Lau KA, Wang B, Saksena NK (2007) Emerging trends of HIV epidemiology in Asia. AIDS Rev 9: 218–229.
8. Beyrer C, Vancott TC, Peng NK, Artenstein A, Duriasamy G, et al. (1998) HIV type 1 subtypes in Malaysia, determined with serologic assays: 1992–1996. AIDS Res Hum Retroviruses 14: 1687–1691.
9. Kusagawa S, Sato H, Kato K, Nohtomi K, Shiino T, et al. (1999) HIV type 1 env subtype E in Cambodia. AIDS Res Hum Retroviruses 15: 91–94.
10. Menu E, Truong TX, Lafon ME, Nguyen TH, Müller-Trutwin MC, et al. (1996) HIV type 1 Thai subtype E is predominant in South Vietnam. AIDS Res Hum Retroviruses 12: 629–633.
11. Nerurkar VR, Nguyen HT, Woodward CL, Hoffmann PR, Dashwood WM, et al. (1997) Sequence and phylogenetic analyses of HIV-1 infection in Vietnam: subtype E in commercial sex workers (CSW) and injection drug users (IDU). Cell Mol Biol (Noisy-le-grand) 43: 959–968.
12. Porter KR, Mascola JR, Hupudio H, Ewing D, VanCott TC, et al. (1997) Genetic, antigenic and serologic characterization of human immunodeficiency virus type 1 from Indonesia. J Acquir Immune Defic Syndr Hum Retrovirol 14: 1–6.
13. Wang W, Jiang S, Li S, Yang K, Ma L, et al. (2008) Identification of subtype B, multiple circulating recombinant forms and unique recombinants of HIV type 1 in an MSM cohort in China. AIDS Res Hum Retroviruses 24: 1245–1254.
14. Wang W, Xu J, Jiang S, Yang K, Meng Z, et al. (2011) The dynamic face of HIV-1 subtypes among men who have sex with men in Beijing, China. Curr HIV Res 9: 136–139.
15. Zhang X, Li S, Li X, Li X, Xu J, et al. (2007) Characterization of HIV-1 subtypes and viral antiretroviral drug resistance in men who have sex with men in Beijing, China. AIDS 21 Suppl 8: S59–S65.
16. Hattori J, Shiino T, Gatanaga H, Yoshida S, Watanabe D, et al. (2010) Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: nationwide surveillance from 2003 to 2008 in Japan. Antiviral Res 88: 72–79.
17. Kihara M, Ichikawa S, Kihara M, Yamasaki S (1997) Descriptive epidemiology of HIV/AIDS in Japan, 1985–1994. J Acquir Immune Defic Syndr Hum Retrovirol 14 Suppl 2:S3–12.
18. Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, et al. (2004) Unifying the epidemiological and evolutionary dynamics of pathogens. Science 303: 327–332.
19. Nelson MI, Holmes EC (2007) The evolution of epidemic influenza. Nat Rev Genet 8: 196–205.
20. Nelson MI, Simonsen L, Viboud C, Miller MA, Holmes EC (2007) Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. PLoS Pathog 3: 1220–1228.
21. Shiino T, Okabe N, Yasui Y, Sunagawa T, Ujike M, et al. (2010) Molecular evolutionary analysis of the influenza A(H1N1)pdm, May-September, 2009: temporal and spatial spreading profile of the viruses in Japan. PLoS One 5: e11057.
22. Magiorkinis G, Magiorkinis E, Paraskevis D, Ho SY, Shapiro B, et al. (2009) The global spread of hepatitis C virus 1a and 1b: a phylodynamic and phylogeographic analysis. PLoS Med 6: e1000198.
23. Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A (2003) The epidemiology and iatrogenic transmission of hepatitis C virus in Egypt: a Bayesian coalescent approach. Mol Biol Evol 20: 381–387.
24. Hughes GJ, Fearnhill E, Dunn D, Lycett SJ, Rambaut A, et al. (2009) Molecular phylodynamics of the heterosexual HIV epidemic in the United Kingdom. PLoS Pathog 5: e1000590.
25. Leigh Brown AJ, Lycett SJ, Weinert L, Hughes GJ, Fearnhill E, et al. (2011) Transmission Network Parameters Estimated From HIV Sequences for a Nationwide Epidemic. J Infect Dis 204: 1463–1469.
26. Lewis F, Hughes GJ, Rambaut A, Pozniak A, Leigh Brown AJ (2008) Episodic sexual transmission of HIV revealed by molecular phylodynamics. PLoS Med 5: e50.
27. Gatanaga H, Ibe S, Matsuda M, Yoshida S, Asagi T, et al. (2007) Drug-resistant HIV-1 prevalence in patients newly diagnosed with HIV/AIDS in Japan. Antiviral Res 75: 75–82.
28. UNGASS (2012) National Commitments and Policies Instrument (NCPI). Available: http://www.unaids.org/en/dataanalysis/knowyourresponse/ncpi/2012countries/Japan NCPI 2012.pdf. Accessed 20 June 2012.
29. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.
30. Lloyd AL, May RM (2001) Epidemiology. How viruses spread among computers and people. Science 292: 1316–1317.
31. Romano CM, de Carvalho-Mello IM, Jamal LF, de Melo FL, Iamarino A, et al. (2010) Social networks shape the transmission dynamics of hepatitis C virus. PLoS One 5: e11170.
32. Barabasi AL, Albert R (1999) Emergence of scaling in random networks. Science 286: 509–512.
33. R Development Core Team (2010) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Available: http://www.r-project.org/.
34. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 101: 11030–11035.
35. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.
36. Swofford DL (2003) PAUP. Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.
37. Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Uppsala University: Evolutionary Biology Centre.
38. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214.
39. Suchard MA (2001) Bayesian selection of continuous-time Markov chain evolutionary models. Mol Biol Evol 18: 1001–1013.
40. Baele G, Li WL, Drummond AJ, Suchard MA, Lemey P (2013) Accurate model selection of relaxed molecular clocks in bayesian phylogenetics. Mol Biol Evol 30: 239–243.
41. Xie W, Lewis PO, Fan Y, Kuo L, Chen MH (2011) Improving marginal likelihood estimation for Bayesian phylogenetic model selection. Syst Biol 60: 150–160.
42. Prosperi MC, Ciccozzi M, Fanti I, Saladini F, Pecorari M, et al. (2011) A novel methodology for large-scale phylogeny partition. Nat Commun 2: 321.
43. Drummond A, Forsberg R, Rodrigo AG (2001) The inference of stepwise changes in substitution rates using serial sequence samples. Mol Biol Evol 18: 1365–1371.
44. Drummond A, Rodrigo AG (2000) Reconstructing genealogies of serial samples under the assumption of a molecular clock using serial-sample UPGMA. Mol Biol Evol 17: 1807–1815.
45. Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, et al. (1999) Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol 73: 10489–10502.

46. Liao H, Tee KK, Hase S, Uenishi R, Li XJ, et al. (2009) Phylodynamic analysis of the dissemination of HIV-1 CRF01_AE in Vietnam. Virology 391: 51–56.
47. Li L, Liang S, Chen L, Liu W, Li H, et al. (2010) Genetic characterization of 13 subtype CRF01_AE near full-length genomes in Guangxi, China. AIDS Res Hum Retroviruses 26: 699–704.
48. An M, Han X, Xu J, Chu Z, Jia M, et al. (2012) Reconstituting the epidemic history of HIV strain CRF01_AE among men who have sex with men (MSM) in Liaoning, northeastern China: implications for the expanding epidemic among MSM in China. J Virol 86: 12402–12406.
49. Feng Y, He X, Hsi JH, Li F, Li X, et al. (2013) The rapidly expanding CRF01_AE epidemic in China is driven by multiple lineages of HIV-1 viruses introduced in the 1990s. AIDS 27: 1793–1802.
50. Ye J, Xin R, Yu S, Bai L, Wang W, et al. (2013) Phylogenetic and temporal dynamics of human immunodeficiency virus type 1 CRF01_AE in China. PLoS One 8: e54238.
51. Kondo M, Lemey P, Sano T, Itoda I, Yoshimura Y, et al. (2013) Emergence in Japan of an HIV-1 variant associated with transmission among men who have sex with men (MSM) in China: first indication of the International Dissemination of the Chinese MSM lineage. J Virol 87: 5351–5361.

OPEN ACCESS Freely available online

PLOS | ONE

Lack of Association between Intact/Deletion Polymorphisms of the *APOBEC3B* Gene and HIV-1 Risk

Mayumi Imahashi[1,2], Taisuke Izumi[3,4], Dai Watanabe[5], Junji Imamura[1], Kazuhiro Matsuoka[1], Hirotaka Ode[1], Takashi Masaoka[1], Kei Sato[6], Noriyo Kaneko[7], Seiichi Ichikawa[7], Yoshio Koyanagi[6], Akifumi Takaori-Kondo[3], Makoto Utsumi[1], Yoshiyuki Yokomaku[1], Takuma Shirasaka[5], Wataru Sugiura[1,8], Yasumasa Iwatani[1,8]*, Tomoki Naoe[1,2]

1 Department of Infectious Diseases and Immunology, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan, **2** Department of Hematology and Oncology, Graduate School of Medicine, Nagoya University, Nagoya, Japan, **3** Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **4** Japanese Foundation for AIDS Prevention, Chiyoda-ku, Tokyo, Japan, **5** Clinical Research Center, National Hospital Organization Osaka Medical Center, Osaka Japan, **6** Center for Human Retrovirus Research, Institute for Virus Research, Kyoto University, Kyoto, Japan, **7** Department of International Health Nursing, Graduate School of Nursing, Nagoya City University, Nagoya, Japan, **8** Department of AIDS Research, Graduate School of Medicine, Nagoya University, Nagoya, Japan

Abstract

Objective: The human APOBEC3 family of proteins potently restricts HIV-1 replication *APOBEC3B*, one of the family genes, is frequently deleted in human populations. Two previous studies reached inconsistent conclusions regarding the effects of *APOBEC3B* loss on HIV-1 acquisition and pathogenesis. Therefore, it was necessary to verify the effects of APOBEC3B on HIV-1 infection *in vivo*.

Methods: Intact (I) and deletion (D) polymorphisms of *APOBEC3B* were analyzed using PCR. The syphilis, HBV and HCV infection rates, as well as CD4$^+$ T cell counts and viral loads were compared among three *APOBEC3B* genotype groups (I/I, D/I, and D/D). HIV-1 replication kinetics was assayed *in vitro* using primary cells derived from PBMCs.

Results: A total of 248 HIV-1-infected Japanese men who have sex with men (MSM) patients and 207 uninfected Japanese MSM were enrolled in this study. The genotype analysis revealed no significant differences between the *APOBEC3B* genotype ratios of the infected and the uninfected cohorts ($p = 0.66$). In addition, HIV-1 disease progression parameters were not associated with the *APOBEC3B* genotype. Furthermore, the PBMCs from D/D and I/I subjects exhibited comparable HIV-1 susceptibility.

Conclusion: Our analysis of a population-based matched cohort suggests that the antiviral mechanism of APOBEC3B plays only a negligible role in eliminating HIV-1 *in vivo*.

Citation: Imahashi M, Izumi T, Watanabe D, Imamura J, Matsuoka K, et al. (2014) Lack of Association between Intact/Deletion Polymorphisms of the *APOBEC3B* Gene and HIV-1 Risk. PLoS ONE 9(3): e92861. doi:10.1371/journal.pone.0092861

Editor: Roberto F. Speck, University Hospital Zurich, Switzerland

Received October 21, 2013; **Accepted** February 27, 2014; **Published** March 25, 2014

Copyright: © 2014 Imahashi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was partly supported by a grant from the National Hospital Organization Network (grant NHO H23-AIDS) and by grants for HIV/AIDS research from the Ministry of Health, Labor, and Welfare of Japan (grants H22-AIDS-004 and H24-AIDS-005). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscripts.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: iwataniy@nnh.hosp.go.jp

Introduction

Human APOBEC3 proteins are cellular cytidine deaminases that play crucial roles in the inhibition of retroviral replication, including that of HIV-1 [1–3]. The molecular mechanisms underlying APOBEC3-mediated HIV-1 restriction are primarily dependent on the editing [1,2] and/or non-editing activities [4,5] of these enzymes. The family of genes encoding the seven APOBEC3 proteins (APOBEC3A, B, C, DE, F, G, and H) is positioned in a tandem array on human chromosome 22 [6]. HIV-1 produces an accessory protein, Vif, that invalidates the antiviral functions of the APOBEC3 proteins by mediating the ubiquitination-proteasomal degradation of APOBEC3 in virus-producing cells [7]. APOBEC3C, DE, F, G, and H (haplotype II) are vulnerable to HIV-1 Vif-mediated degradation, whereas APOBEC3A and B are resistant [8–12].

Among the members of the APOBEC3 family, APOBEC3G has been consistently shown to possess powerful anti-HIV-1 activity in cell-based systems [1,2], and this protein may affect the pathogenesis of HIV-1 infection *in vivo* [13–19]. However, there is little consensus regarding the degree to which the other APOBEC3 family members, especially APOBEC3B, are able to restrict HIV-1 replication *in vitro* and *in vivo*. The anti-HIV-1 activity of APOBEC3B is undetectable when this gene is stably expressed in a human T cell line [20] and is detected only weakly after the transient transfection of HEK 293T or HeLa cells [20–22]. Because these findings have varied according to the experimental conditions employed, there is a fundamental

question whether the expression of human APOBEC3B, DE, and F plays a critical role in HIV-1 restriction *in vivo*. The potential role of APOBEC3B in modulating HIV-1 replication *in vivo* is of particular interest because this protein is resistant to HIV-1 Vif-mediated degradation [20,22–24].

A polymorphic deletion of a 29.5-kb segment between *APOBEC3A* exon 5 and *APOBEC3B* exon 8 has been identified in human populations; this polymorphism causes the loss of the entire APOBEC3B coding region [25]. A particularly high frequency of *APOBEC3B* deletion has been found among Asians [25]. According to Kidd et al., the deletion allele is rare in Africans (1%) and Europeans (6%), more common in East Asians (36%) and Amerindians (58%), and almost fixed in Oceanians (93%) [25].

Two independent groups have reported contrasting findings concerning the effects of the *APOBEC3B* gene deletion on HIV-1 acquisition and disease progression [26,27]. An et al. determined that the deletion allele genotype correlated with a higher risk of HIV-1 infection, whereas a study conducted by Itaya et al. concluded that the deletion polymorphism had no effect on HIV-1 acquisition and the rate of disease progression to AIDS. An et al. included 4 patients with homozygous deletions of *APOBEC3B* in their HIV-1-seropositive cohorts of 656 European and 296 African-American individuals but no homozygotes for the deletion in their seronegative groups, which prevented a proper evaluation of the impact of the deletion polymorphism on HIV-1 acquisition and pathogenesis [26]. In contrast, the study conducted by Itaya et al. in Japan utilized inappropriate enrollment [27], because the enrolled patients were all hemophiliacs who had survived HIV-1 infection for at least 10 years prior to the study and the information for individuals who had progressed to AIDS and death before the enrollment date was excluded.

To examine the impact of the *APOBEC3B* deletion polymorphism on HIV-1 infection risk *in vivo*, this study enrolled a matched cohort in Japan and investigated the impact of *APOBEC3B* gene intact/deletion polymorphisms on HIV-1 susceptibility and pathogenesis. In addition, we analyzed the effects of different *APOBEC3B* genotypes on HIV-1 replication kinetics *in vitro*.

Materials and Methods

Sample Collection

A total of 248 Japanese HIV-1-positive men who have sex with men (MSM) who were patients at Nagoya Medical Center (n = 203) and Osaka Medical Center (n = 45) were enrolled in this study from November 2011 to February 2013. The control group comprised 207 Japanese HIV-1-negative MSM who were recruited at the Nagoya Lesbian & Gay Revolution Plus (NLGR+) festival in June 2012. The study protocol was approved by the ethics committees of Nagoya Medical Center (registration number 2011-430) and Osaka Medical Center. Written informed consent was obtained from all the participants. The control subjects recruited at the NLGR+ festival provided anonymous consent. To collect information regarding their sex, nationality, age, and sexuality, anonymous questionnaires collated with linked numbers were obtained.

Genotyping

The *APOBEC3B* intact (I) and deletion (D) alleles were genotyped using a previously reported polymerase chain reaction (PCR) method [26] with slight modifications. Of note, the "intact (I)" in this study is used for the "insertion" that originally reported by Kidd et al. [25]. Briefly, the primer sets for amplifying the Deletion and Insertion 2 fragments were the same as those previously described [26], although one additional set of primers for the Insertion 1 fragement was replaced by the two following oligonucleotide primers: Insertion3_F: 5′-GAGTGGAAGCGCCTCCTC-3′ and Insertion3_R: 5′-CTCCTGGCCAGCCTAGC-3′. The QIAamp DNA Blood Mini Kit (Qiagen, Valencia, USA) was used according to the manufacturer's protocol to extract genomic DNA from whole blood (patients) or from buccal mucosa (controls).

Analysis of Viral Replication Capacity and Infectivity

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples from different HIV-1-negative donors with the I/I and D/D *APOBEC3B* genotypes (n = 5 for each) using Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). The PBMCs were then subjected to negative selection with the MACS CD4 T Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany) to purify primary $CD4^+$ T cells. The cells were activated with 1 µg/ml of phytohemagglutinin (PHA) (Pharmacia) for 72 hours, infected with HIV-1 NL4-3 for 24 hours with a multiplicity of infection (MOI) of 0.01, washed twice, and maintained in RPMI-1640 medium with 20% fetal bovine serum (FBS), penicillin (50 U/ml)/streptomycin (50 µg/ml) (Invitrogen, Carlsbad, USA), and 20 U/ml interleukin-2 (IL-2) (Roche Applied Science, Mannheim, Germany). The culture supernatants were assayed for the p24 antigen using the HIV-1 p24 Antigen Assay Kit (Coulter Corporation, Fullerton, USA) on the day of infection and on days 2, 4, 6, 8, 10, and 13 after infection. To analyze the viral infectivity of the infected PBMCs, culture supernatants were harvested six days post-infection and inoculated into TZM-bl cells [29] in black 96-well plates. The viral infectivity was assessed 48 hours post-infection by detecting β-galactosidase activity using the Galacto-Star System (Applied Biosystems, Foster City, USA).

Quantification of APOBEC3 mRNA

To analyze the mRNA expression levels of members of the APOBEC3 family, unstimulated $CD4^+$ cells from three different genotyped subjects were prepared for RNA isolation. The induction rates of mRNA transcription for APOBEC3A or APOBEC3G were analyzed using monocyte-derived macrophages (MDMs). Briefly, monocytes were isolated from PBMCs from each genotyped healthy donor using CD14 MicroBeads (Miltenyi Biotec). The enriched $CD14^+$ cells were plated at a cell density of 1×10^6/ml in 12-well plates in RPMI-1640 medium (Sigma, St. Louis, USA) with penicillin (50 U/ml)/streptomycin (50 µg/ml) for three hours, followed by the addition of 10% FBS and 10 ng/ml macrophage colony stimulating factor (M-CSF) (Peprotech, Rocky Hill, USA). Adherent cells were cultured for eight days to facilitate their differentiation into MDMs. Differentiated MDMs either received no stimulation or were stimulated with 100 U/ml of recombinant human interferon (IFN)-α (Sigma) for six hours and were then lysed for RNA isolation. As previously described [14,30], total RNA isolated using the QIAamp RNA Blood Mini Kit (Qiagen) was used to synthesize cDNA with the SuperScript III First-Strand Synthesis System (Invitrogen) using random hexamers. The cDNA levels were quantified using real-time PCR in a Thermal Cycler Dice Real Time System (TP800) (Takara Bio, Shiga, Japan). The real-time PCR was employed to analyze the levels of APOBEC3, β-actin, and GAPDH mRNA, and the assays were performed according to the manufacturer's protocol using SYBR Premix DimerEraser (Takara Bio). The primer sets for the real-time PCR were purchased from FASMAC Co., Ltd. (Atsugi, Japan) and the oligonucleotide sequences are

shown in Table S1. The gene expression levels were calculated using the ΔΔCt (Ct; cycle threshold) and are presented as the ratio of APOBEC3 mRNA to β-actin or GAPDH mRNA.

Statistical Analysis

The relationships between *APOBEC3B* genotype and baseline characteristics were assessed using the Fisher exact test for categorical variables. The Mann-Whitney *U*-test was used for continuous variables. All the statistical analyses were performed with the statistical software EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More specifically, this software is a version of R commander (version 1.6–3) modified to add statistical functions that are frequently used in biostatistics [31]. All the p values were two-tailed. The effects of *APOBEC3B* gene deletion on the disease progression of HIV-1 were evaluated based on the CD4+ T cell counts and log10 HIV-1 viral load (RNA copy number/ml) at more than two time points before the start of antiretroviral therapy (ART). Patients whose $CD4^+$ T cell counts and HIV-1 viral loads were measured at fewer than two time points were excluded from the statistical analyses of these factors. Other related infectious diseases were identified in the patients using the following definitions. If the rapid plasma reagin test and/or the *Treponema pallidum* latex agglutination (TPHA) test were positive, the patient was considered positive for syphilis. Patients were considered hepatitis B virus (HBV)-positive if either hepatitis B surface antigen (HBsAg) or hepatitis B core antibody (HBcAb) was present. In addition, patients were considered hepatitis C virus (HCV) carriers if they tested positive for HCV antibodies.

Results

APOBEC3B Genotype Frequencies in the Cohorts

The demographics of the HIV-1-positive and HIV-1-negative cohorts are shown in Table 1. A total of 248 HIV-1-infected Japanese MSM patients and 207 uninfected Japanese MSM were enrolled and analyzed in this study. To conduct a matched cohort study, all the participants were recruited from Nagoya and Osaka in the central area of Japan. First, a comparative analysis of the *APOBEC3B* genotype among the participants indicated that there were no significant differences in *APOBEC3B* genotype frequency between the HIV-1-positive (D/D 7.7%, I/D 44.0%, and I/I 48.4%) and HIV-1-negative (D/D 8.7%, I/D 39.6%, and I/I 51.7%) cohorts (p = 0.66) (Table 1). A comparison of the distributions of the *APOBEC3B* deletion allele in the HIV-1-positive and HIV-1-negative cohorts revealed that the D allele occurred in the HIV-1-positive (29.6%) and HIV-1-negative subjects (28.5%) at comparable rates (p = 0.71). We also analyzed the cDNA sequences of *APOBEC3B* I allele isolated from the Japanese healthy donors with the I/I or I/D genotypes (Table S1). There was one variant (rs#2076109): K62 (allele frequency, or AF = 0.4) (E62 as the reference) although we could not detect any other variants changing the amino acid sequences within the 15 alleles. According to the 1000 Genome database, the variant (AF = 0.373) appears globally distributed but not limited in Japan or Asia. In addition, we tested the antiviral effect of APOBEC3B E62 and the variant with an overexpression system using 293T cells (Figure S1). The results demonstrated that the E62 variant had equivalent antiviral activity to APOBEC3B K62 *in vitro*. These data suggest that the I alleles in our Japanese cohorts are not strongly biased in terms of genetic and functional features.

Next, we analyzed the HIV-1-positive individuals for the prevalence of HBV, HCV, and syphilis, as well as for HIV-1 disease progression at a minimum of two time points before the commencement of ART. The prevalence of each infectious disease is presented in Table 2. The frequencies of the three *APOBEC3B* genotypes (D/D 7.6%, I/D 45.4%, and I/I 47.0%) among the 132 HBV-positive patients were not significantly different from those of the 94 HBV-negative individuals (D/D 9.6%, I/D 41.5%, and I/I 48.9%) (p = 0.69). In addition, the *APOBEC3B* genotype distributions did not differ significantly between the HCV-positive and HCV-negative patients (p = 1.00) or between the syphilis-positive and syphilis-negative patients (p = 0.62) (Table 2).

We also assessed the rates of both $CD4^+$ T cell decline and plasma viral load increase at different time points after the first patient visit to the hospital prior to ART treatment. As shown in Figure 1, the changes in the $CD4^+$ T cell counts (cells/μl/day) and viral loads ($\log_{10}$ copies/ml/day) did not differ significantly according to *APOBEC3B* genotype (CD4: p = 0.054; viral load: p = 0.96). The data from the 46 patients (D/D 6.5%, I/D 41.3%, and I/I 52.2%) who began ART before the second measurement of the $CD4^+$ T cells and viral loads were excluded from the analysis. Of these 46 patients, 32 (D/D 3.1%, I/D 50.0%, and I/I 46.9%) began ART shortly after their first hospital visit due to AIDS onset; this decision was based on the domestic clinical guidelines of the Ministry of Health, Labor, and Welfare of Japan. There were no significant differences in the proportions of the *APOBEC3B* genotypes between the patients with $CD4^+$ T cell count and viral load data from at least two time points and the 46 patients without complete data (p = 0.91). Detailed demographic information on the HIV-1 (+) patients is shown in Table 3. Moreover, we analyzed the non-ART periods from the first diagnosis through the ART introduction and set two groups: longer and shorter than median days from diagnosis to ART. As the genotype frequencies were compared (Table 4), the results showed no significant difference in the *APOBEC3B* genotype between the two groups (p = 0.96).

Moreover, we performed deep sequencing of the HIV-1 proviral DNAs that were isolated from the I/I, I/D or D/D patients' PBMCs, and then analyzed the hypermutation rates on APOBEC3-prefered dinucleotide sequences: GG>AG and GA>AA mutations. The results showed that the hypermutation frequencies vary among different individuals although the levels of GA>AA hypermutation relative to the GG>AG are comparable among the three *APOBEC3B* genotypes (Figure S2). The data suggest that the APOBEC3B is not likely a major contributor to introduce hypermutations on the proviral DNAs in HIV-1(+) patients' PBMCs.

The Effects of *APOBEC3B* Genotype on Other APOBEC3 Expression Profiles

To assess whether the *APOBEC3B* gene deletion altered the expression of the other proximal *APOBEC3* genes, we compared mRNA expression profiles in fresh, unstimulated primary $CD4^+$ cells of each *APOBEC3B* genotype: D/D, I/D, and I/I. As shown in Figure 2A, the mRNA expression levels of APOBEC3A, which is the *APOBEC3* family member located closest to the *APOBEC3B* gene, were not significantly different between the I/I and D/D genotype groups (p = 0.63), although the levels would likely vary considerably among individuals. As expected, APOBEC3B mRNA expression levels were not detected in the D/D subjects (Figure 2A). The APOBEC3B mRNA levels in the I/D subjects were somewhat lower than in the I/I subjects, although this difference was not statistically significant (Figure 2A, p = 0.12). Moreover, the relative levels of APOBEC3C, DE, F, G, and H mRNA were comparable among the I/I, I/D, and D/D subjects (Figure 2A). We also analyzed APOBEC3B mRNA levels in

Table 1. *APOBEC3B* genotype frequency in HIV-1-positive patients and HIV-1-negative controls.

	HIV-1		
	Negative (%)	**Positive (%)**	p[a]
Genotype			
D/D	18/207 (8.7)	19/248 (7.7)	0.66
I/D	82/207 (39.6)	109/248 (44.0)	
I/I	107/207 (51.7)	120/248 (48.4)	
Allele			
D	118/414 (28.5)	147/496 (29.6)	0.71
I	296/414 (71.5)	349/496 (70.4)	

[a]Determined using the Fischer exact test.
doi:10.1371/journal.pone.0092861.t001

PBMCs isolated from healthy donors and HIV-1 seropositive patients with or without the ART. Similar to the pattern of APOBEC3B mRNA levels in the CD4+ T cells of three genotyped subjects (Figure 2A), the mRNA expression is slightly lower in the I/D genotyped PBMCs than in the I/I whereas no detectable level of APOBEC3B mRNA in the D/D PBMCs (Figure S3). The different expression levels between the I/D and I/I PBMCs were not statistically significant (Figure S3). Moreover, comparative analysis showed that the APOBEC3B mRNA level of each I/I or I/D genotype appears relatively higher in the HIV-1 (+) patients, regardless the ART-treatment, than in the uninfected donors. However, the difference was not statistically significant (Figure S3).

In the *APOBEC3B* D allele, the APOBEC3A mRNA contains a 3′-untranslated region of APOBEC3B's and is subject to the upstream regulatory elements of the *APOBEC3A*. Thus, we further assessed whether the degrees to which APOBEC3A and APOBEC3G mRNA expression was stimulated by IFN-α in MDMs differed between the *APOBEC3B* I/I and D/D genotypes. The APOBEC3G mRNA expression was used as a control because the gene is distal to the *APOBEC3B* loci on the genome. As shown in Figure 2B, IFN-α stimulation resulted in APOBEC3A mRNA increases in the I/I and D/D MDMs of 1,999±1,190-fold and 1,251±264-fold, respectively. The APOBEC3G mRNA levels increased upon IFN-α stimulation by 28.6±41.8-fold (I/I) and 38.9±18.0-fold (D/D). A comparison of the mRNA expression magnitudes between the two homozygous *APOBEC3B* genotypes revealed no significant differences ($p = 0.4$ and $p = 0.4$ for APOBEC3A and APOBEC3G, respectively).

The Effects of *APOBEC3B* Genotype on HIV-1 Susceptibility *in Vitro*

We further analyzed the viral replication kinetics in primary PBMCs isolated from D/D or I/I donors. At an MOI of 0.01, the efficiency of HIV-1 replication was comparable between the D/D and I/I genotypes (Figure 3A). The p24 antigen levels in the culture supernatant from the I/I and D/D PBMCs were $8.7\pm3.0\times10^5$ pg/ml and $1.3\pm0.2\times10^6$ pg/ml, respectively, on day 8 ($p = 0.31$) and $8.4\pm0.2\times10^5$ pg/ml and $1.3\pm0.3\times10^6$ pg/ml, respectively, on day 6 ($p = 0.13$). At the peak of infection (day 6), the virus-containing supernatants derived from D/D and I/I PBMCs exhibited comparable levels of infectivity ($p = 0.86$) (Figure 3B). These data suggest that the different *APOBEC3B* deletion genotypes are not associated with significantly different levels of HIV-1 susceptibility *in vitro*.

Table 2. *APOBEC3B* genotype frequency and clinical parameters in HIV-1-positive patients.

	APOBEC3B genotype			
	D/D (%)	**I/D (%)**	**I/I (%)**	p[a]
HBV				
Positive	10/132 (7.6)	60/132 (45.4)	62/132 (47.0)	0.69
Negative	9/94 (9.6)	39/94 (41.5)	46/94 (48.9)	
Unknown	0/22 (0)	10/22 (45.5)	12/22 (54.5)	
HCV				
Positive	0/7 (0)	3/7 (42.9)	4/7 (57.1)	1.00
Negative	19/241(7.9)	106/241 (44.0)	116/241 (48.1)	
Syphilis				
Positive	9/116 (7.8)	47/116 (40.5)	60/116 (51.7)	0.62
Negative	10/131 (7.6)	61/131 (46.6)	60/131 (45.8)	
Unknown	0/1 (0)	1/1 (100)	0/1(0)	

[a]Determined using the Fischer exact test.
doi:10.1371/journal.pone.0092861.t002

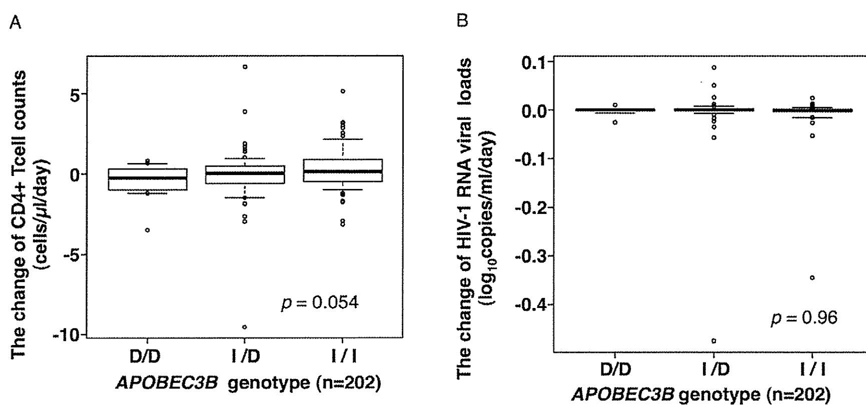


Figure 1. Analysis of effects of genotype on parameters of HIV disease progression in the HIV-1-infected cohort. (A) Changes in CD4^{+} T cell counts (cells/μl/day) (n = 202). (B) Changes in HIV-1 RNA levels (log_{10} copies/ml/day) in plasma (n = 202). The box plots show data between the 25th and 75th percentiles with central horizontal lines representing the median, and with whiskers showing the 10th and 90th percentiles. The open circles represent outliers with data >1.5-fold of the interquartile range. All the p values were determined using the Kruskal-Wallis test.
doi:10.1371/journal.pone.0092861.g001

Discussion

There is only limited information about the roles played by APOBEC3 family members *in vivo*, with the exception of APOBEC3G. Previously, two independent groups reported conflicting conclusions regarding the impact of the *APOBEC3B* gene deletion on human HIV-1 infection *in vivo*, and this issue remains unclear [26,27]. Therefore, to determine the effects of different *APOBEC3B* genotypes on HIV-1 infection *in vivo* and *in vitro*, we investigated the frequencies of intact and deletion polymorphisms of the *APOBEC3B* gene in a matched cohort in Japan.

The comparison of *APOBEC3B* genotypes in HIV-1-infected patients and HIV-1-negative controls revealed similar *APOBEC3B* genotype distributions in the two groups: D/D 7.7%, I/D 44.0%, and I/I 48.4% in the infected cohort versus D/D 8.7%, I/D 39.6%, and I/I 51.7% in the uninfected cohort (p = 0.66). In addition, no significant associations between the *APOBEC3B* genotype and the subclinical parameters of disease progression were observed among the HIV-1-positive patients. We also found no differences between the mRNA expression profiles of other APOBEC3 family members in PBMCs. Furthermore, the IFN-α-stimulated mRNA induction rates for APOBEC3A and APOBEC3G in MDMs did not differ between the D/D and I/I genotypes. Moreover, the HIV-1 susceptibility levels in PBMCs were comparable between the two genotypes. Considered together, our findings suggest that the loss of APOBEC3B is not significantly associated with HIV-1 acquisition and pathogenesis *in vivo* and with HIV-1 susceptibility *in vitro*, which fully supports the results of the cohort study conducted by Itaya et al [27].

There are two possible explanations for the lack of APOBEC3B involvement in HIV-1 restriction. First, the APOBEC3B protein cannot be incorporated into viral cores. Efficient HIV-1 restriction requires that APOBEC3 family proteins are packaged into virions through associations with viral and/or nonviral RNA [1,2,28–30] and that the proteins are localized to the plasma membrane in virus-producing cells [31]. APOBEC3G colocalizes with HIV-1 RNA and cellular RNA in P bodies [32] and are dispersed throughout the cytoplasm that facilitate interactions with HIV-1 Gag proteins and their incorporation into nascent virions [1,2]. In contrast, APOBEC3B predominantly localizes to the nucleus [20,21,33], which may prevent its incorporation into virions.

The second possible explanation is that the low expression level of APOBEC3B in PBMCs [22,34,35] is insufficient to block HIV-

Table 3. Demographics of the cohorts.

	HIV-1 Negative (n = 207)	HIV-1 Positive (n = 248)
Age (years), median [IQR][a]	33 [26–39]	40 [36–51]
Year of diagnosis, median [IQR] [a]	NA[b]	2008 [2005–2010]
ART naïve at entry, n (%)	NA[b]	20 (8%)
CD4^{+} cell count at entry (cells/mm^{3}), median [IQR] [a]	NA[b]	451 [294–534]
HIV-1 viral load at entry (copies/mL), median [IQR] [a]	NA[b]	61 [<40–410]
History of AIDS, n (%)	NA[b]	32 (13%)
Days from diagnosis to entry, median [IQR] [a]	NA[b]	1470 [539–2256]
Observation periods for disease progression (days), median [IQR] [a]	NA[b]	56 [28–88]
Days from diagnosis to ART, median [IQR] [a]	NA[b]	88 [38–599]

[a]IQR denotes interquartile range.
[b]NA, Not applicable.
doi:10.1371/journal.pone.0092861.t003

Table 4. *APOBEC3B* genotype frequency on the days from diagnosis to ART (n = 246).

	days from diagnosis to ART		
	88 days> (%)	**88 days< (%)**	**p[a]**
Genotype			
D/D	9 (3.7)	10 (4.1)	0.961
I/D	49 (19.9)	59 (24.0)	
I/I	57 (23.2)	62 (25.2)	

(Median days from diagnosis to ART = 88 days).
[a]Determined using the Fischer exact test.
The diagnosis date of 2 patients(each patient's genotype is I/I and I/D, respectively.) are unknown.
doi:10.1371/journal.pone.0092861.t004

1 replication, as shown in Figure 3. Similar to the HIV-1 results, overexpressed APOBEC3B potently suppresses HBV replication *in vitro* [36]. However, a study by Abe et al. on the frequency of the D/D genotype in HBV carriers demonstrated that the *APOBEC3B* gene deletion was not responsible for chronic HBV infection [37]. These data suggest that the high expression of APOBEC3B *in vitro* may produce exaggerated effects on both HIV-1 and HBV infection *in vitro*.

All the participants enrolled in this study were Japanese MSM, according to the information provided on anonymous questionnaires. Because approximately 80% of the HIV-1-positive patients in Japan are MSM [38], we investigated the effects of *APOBEC3B* deletion polymorphisms on this major mode of HIV-1 transmission rather than on the two other major modes (injection drug use and heterosexual intercourse). However, the effect of *APOBEC3B* genotype is less likely to be dependent on the mode of HIV-1 transmission because APOBEC3B mRNA expression in hematopoietic cells is lower and less tissue-specific than that of most of the other APOBEC3 family members [22,34,35].

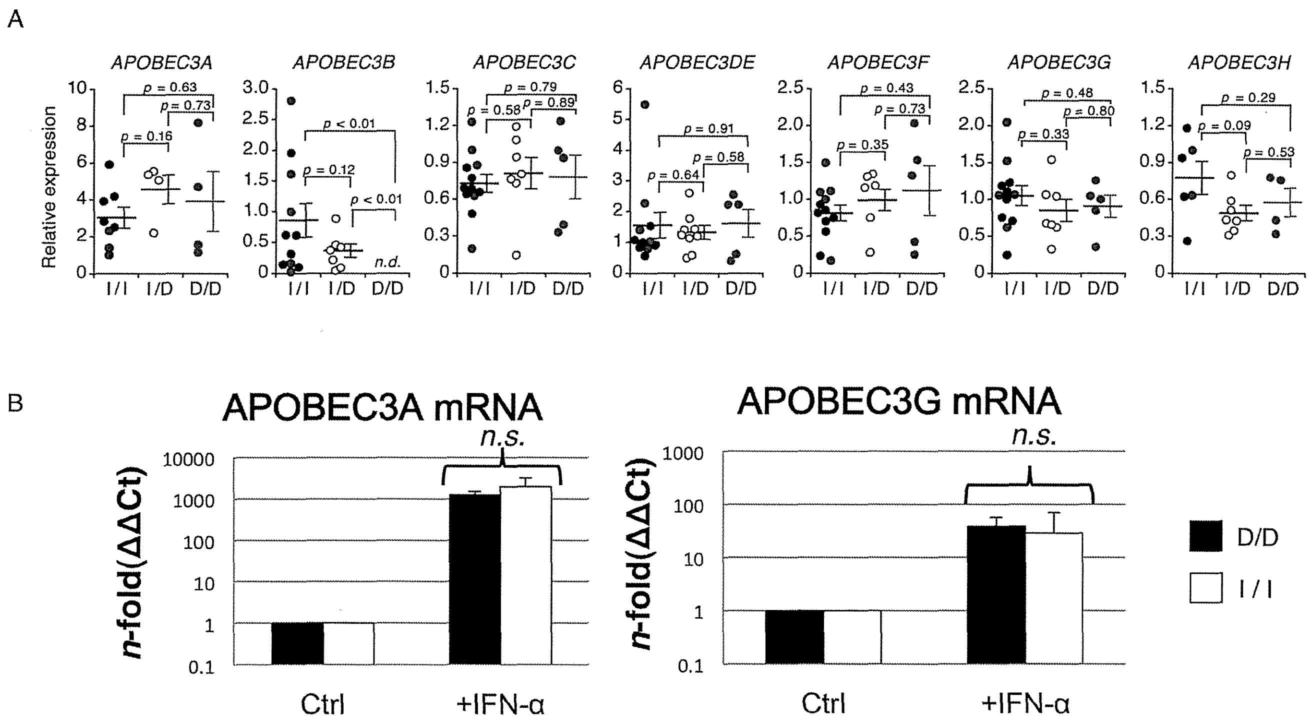


Figure 2. APOBEC3 mRNA expression levels depending on *APOBEC3B* genotype. (A) Comparison of mRNA expression levels of APOBEC3 in CD4$^+$ cells isolated from intact (I/I), hemizygous (I/D) and deletion (D/D) individuals of healthy donors. The relative mRNA expression levels of APOBEC3A (I/I, n = 8; I/D, n = 4; D/D, n = 4), APOBEC3B (I/I, n = 11; I/D, n = 7; D/D, n = 5), APOBEC3C (I/I, n = 12; I/D, n = 7; D/D, n = 5), APOBEC3DE (I/I, n = 11; I/D, n = 9; D/D, n = 5), APOBEC3F (I/I, n = 12; I/D, n = 7; D/D, n = 5), APOBEC3G (I/I, n = 12; I/D, n = 7; D/D, n = 5), and APOBEC3H (I/I, n = 6; I/D, n = 7; D/D, n = 4) were determined using quantitative RT-PCR and were normalized to GAPDH. The red (I/I) or gray (D/D) dots represent the expression levels of donors whose PBMCs were used for the *in vitro* kinetics of HIV-1 replication and infectivity in Figure. 3. The p values were calculated using Welch's *t*-test. The error bar represents the standard error of the mean (SEM). (B) APOBEC3A (A3A) and APOBEC3G (A3G) mRNA expression levels under basal conditions (Ctrl) and after stimulation with 100 U/ml (+IFN-α) of interferon (IFN)-α in CD14$^+$ MDMs isolated from healthy control subjects. The black and white bars indicate D/D (n = 3) and I/I (n = 4) individuals, respectively. The *p* values were calculated with the Mann-Whitney *U*-test. The error bars represent the standard deviation. *n.d.*, not detected. Ct, cycle threshold. *n.s.*, not significant ($p = 0.4$ for both cases).
doi:10.1371/journal.pone.0092861.g002

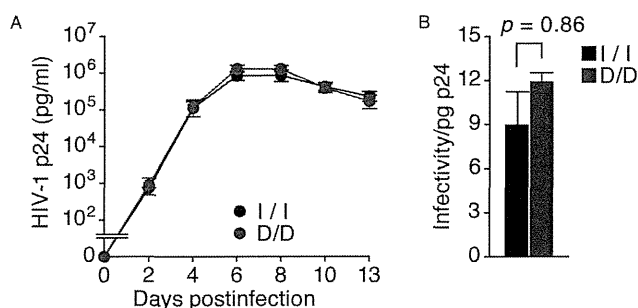


Figure 3. The kinetics and infectivity of HIV-1 depending on *APOBEC3B* genotype. (A) The kinetics of HIV-1 replication in PBMCs isolated from I/I (black dot) or D/D (gray dot) subjects (n = 5 each). (B) The infectivity values of virus-containing supernatants derived from I/I (black bar) and D/D (gray bar) PBMCs six days post-infection are provided relative to the values normalized with equal amounts of p24. The assay was performed using samples from three donors, and a representative result is shown. The p values were calculated using Welch's *t*-test. The error bars represent the SEM.
doi:10.1371/journal.pone.0092861.g003

In the D/D genotype, APOBEC3A mRNA expressed from the genome has a 3′-untranslated region corresponding to that of APOBEC3B. In addition, the genomic location of the APOBEC3G coding region is closer to the highly IFN-responsive transcriptional element of *APOBEC3A* in the D/D genome than to in the I/I. Therefore, we evaluated whether *APOBEC3B* gene deletion altered the IFN-stimulated gene induction of the other APOBEC3 family members. Our results suggest that the 29.5-kb genomic deletion of *APOBEC3B* does not significantly affect the expression profiles of the proximal *APOBEC3* family genes. Therefore, it is unlikely that the loss of the *APOBEC3B* gene in the D/D population leads to functional compensation via the mRNA expression modulation of the other APOBEC3 family members. Interestingly, Biasin et al. have demonstrated that increased levels of APOBEC3G mRNA in PBMCs, (primarily $CD14^+$ MDMs) following exposure to IFN-α correlated with HIV-1 susceptibility both *in vivo* and *in vitro* [13]. Our results showed that the induction magnitude of APOBEC3G mRNA upon the IFN-α stimulation was similar between the I/I and D/D genotypes (Figure 2B). This suggests that different HIV-1 susceptibility observed by Basin et al. is unlikely linked to the *APOBEC3B* intact/deletion genotypes.

Recent studies of tumors such as breast cancers [39–41] and lymphomas [42] have shown that increased expression of APOBEC3B *in vivo* was linked to the chronic induction of mutations and/or instability in genomic DNA. We did not observe any significant diagnosable HIV-associated cancers in our short-term cohort study. It may be necessary to continue our prospective studies for a longer period. In addition, because other studies have suggested that *APOBEC3B* gene deficiency is associated with higher susceptibility to two other ancient pathogens, human T-cell leukemia virus type 1 [43,44] and *Plasmodium falciparum* (the causative agent of malaria) [45], it would be beneficial to further investigate the correlations between *APOBEC3B* genotype and susceptibility to unknown pathogens.

Conclusions

Our analysis of a population-based matched cohort provided important evidence that the loss of the *APOBEC3B* gene is not associated with risk of HIV-1 infection and disease progression. In addition, the *in vitro* kinetics of HIV-1 replication and the infectivity of the virus in PBMCs were comparable between the D/D and I/I subjects. These results suggest that the APOBEC3B antiviral mechanism plays only a negligible role in eliminating HIV-1 *in vivo*. This finding may explain why HIV-1 has not evolved a Vif-based strategy to counteract APOBEC3B restriction. Further analyses to explore the role(s) of APOBEC3B in human are also required in other cohorts with diverse genetic backgrounds in Asia.

Supporting Information

Figure S1 Overexpression of two APOBEC3B variants and the antiviral effect of the variants *in vitro*. **(A)** A DNA fragment of the complete *APOBEC3B* open reading frame was amplified by RT-PCR from each RNA sample of healthy donors with APOBEC3B K62 (A3B K62) and E62 (A3B E62). Each of the fragment was replaced into the *APOBEC3G* gene position of the pcDNA A3G (Myc-His) WT (A3G WT) plasmid as previously described [8]. The primer sets for amplification of APOBEC3B cDNA were used as follows: the 1st PCR, 5′- gagcgggacagggacaagcg and 5′- aacccaggtctctgccttcc; the 2nd PCR, 5′-tcgagcggccgcatgaatccacagatcagaaatccg and 5′- cgatacaagcttgtttccctgattctggagaatggc. The resultant APOBEC3B expression plasmids, pcDNA APOBEC3B K62 and pcDNA APOBEC3B E62, contain a C-terminal MycHIS tag (consisting of Myc and hexa-histidine epitopes). The sequences of both the insert and the boundary regions for the APOBEC3B expression plasmids were verified by DNA sequencing. The expression or control (Vector) plasmids were transfected into human embryonic kidney cells (HEK 293T) by using FuGENE HD (Promega, Madison, USA). At 48 hr after transfection, cell lysates were prepared with Laemmli buffer containig 2.5% 2-Mercaptoethanol and analyzed by western blot. Protein bands were probed with anti-β-tubulin rabbit polyclonal antibody (1/2,500) (ab6046, Abcam, Cambridge, USA) or anti-His mAb (1/3,000) (D291-3, Medical & Biological Laboratories Co., Nagoya, Japan) as previously reported [8]. **(B)** The effect of two APOBEC3B variants on HIV-1 infectivity *in vitro* was analyzed. For virus production, 293T cells were cotransfected with 1 μg of pNL4-3 WT (HIV-1 WT) or pNL4-3*vif*(–) (HIV-1 *vif*(–)) plus 1 (black) or 0.1 (gray) μg of pcDNA APOBEC3B K62, pcDNA APOBEC3B E62, pcDNA 3.1 (–) (Vector), or pcDNA A3G (Myc-His) WT. Because it has been reported that the antiviral effect of APOBEC3B on HIV-1 *in vitro* can be observed when overexpressed in 293T cells but not T cell lines [20], we used 293T cells for the virus production. Virus infectivity was determined using TZM-bl cells [29]. Relative infectivity as relative light units (RLU) was calculated by normalizing for the amount of input CA, determined by p24 antigen ELISA (ZeptoMetrix, Buffalo, USA). Three independent experiments were performed. Results from one representative experiments are shown. A3G, APOBEC3G.
(TIF)

Figure S2 Quantitative hypermutation analysis of APOBEC3-prefered dinucleotide motifs in the proviral DNA isolated from PBMCs of HIV-1 (+) patients. **(A)** Genomic DNAs from patients' PBMC (n = 4, for each *APOBEC3B* genotype I/I, I/D, and D/D) were extracted using the QIAamp DNA Blood Mini Kit. The proviral DNA fragments were prepared by nested PCR using the PrimeSTAR GXL DNA Polymerase (Takara Bio). For the first PCR, a 2,877-bp DNA fragment of *pol* (RT-IN) region (nt 2,388–5,264 according to the numbering positions of HXB2 strain, K03455) and a 1,095-bp fragment of *vif* region (nt 4,899–5,993) were independently amplified with 300 nM of each primer set: *pol*, DRRT1L (5′-atgatagggggaattg-

gaggttt) and DRIN1R (5′-cctgtatgcagaccccaatatg); *vif*, DRVIF1F (5′-cgggtttattacagggacagcag) and DRVIF1R (5′-gctgtctccgcttcttcctgccat). For the nested PCR, a 2,735-bp (*pol*, nt 2,485–5,219) and an 859-bp (*vif*, nt 4,953–5,812) fragment were generated using primer sets, DRRT7L (5′-gacctacacctgtcaacataattgg)/DRRT7R (5′-cctagtgggatgtgtacttctgaactta) and DRVIF2F (5′-ctctggaaaggtgaaggggcagta)/DRVIF2R (5′-gaataatgcctattctgctatg), respectively. The resulting PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and quantified with the Quant-iT dsDNA BR kit (Life Technologies). Paired-end DNA libraries were prepared using the Nextera DNA sample prep kit (Illumina, San Diego, USA) according to the manufacture's protocol. The DNA libraries were sequenced on a MiSeq (Illumina) using the MiSeq reagent kit v2 to produce 250 bp ×2 paired-end reads. The reads generated by deep sequencing were mapped onto the reference sequence of HXB2 strain by BWA 0.7.3a program (http://bio-bwa.sourceforge.net). Then, sequences of a 150-base pairs-long region were extracted and the sequences containing bases with quality scores under 30 were omitted by our *in house* program. **(B)** Among the extracted sequences, the hypermutation types and the numbers of the dinucletitide sequences, GG>AG (red) and GA>AA (blue), were analyzed. In order to detect hypermutations, the unique sequences with >5-fold coverage depth were used. The frequency (%) of hypermutaion is shown as mutation rates per dinucleotide (GG or GA) sequence with two color-coded scales below. The positions of the hypermutations in each patient sample are represented based on the nucleotide position of HXB2 strain. Since no sequences at the 3′-end part of *vif* (5470–5619) in sample ID #15, 47 and 73 were mapped onto HXB2 (panel A), the hypermutation frequency in the portion (5,485–5,619) is not shown. **(C)** The cumulative histograms represent number of hypermuated positions (y-axis) for GG>AG (red) or GA>AA (blue) at the degree of hypermutation (%) (x-axis). Bars were denoted for every 10% of the frequency degree.
(PDF)

Figure S3 Relative expression levels of APOBEC3B mRNA in three different *APOBEC3B* genotyped subjects of healthy donors or HIV-1-infected patients. Total RNA samples were isolated from PBMCs of three different genotyped subjects, intact (I/I); hemizygous (I/D); and deletion (D/D) individuals, of healthy donors (Uninfected), or HIV-1 (+) patients before (Naïve) or after (Treated) cART. Each genotype includes 3 samples for each status. Relative APOBEC3B mRNA expression levels were determined by using RT-qPCR using the Thermal Cycler Dice Real Time System (TP800) (Takara Bio, Shiga, Japan). The qPCR cycle at which amplification was detectable above a background threshold (threshold cycle, or Ct) was calculated and normalized to β-Actin. The relative expression levels are presented as the $\Delta\Delta$Ct (*n*-fold) of APOBEC3B mRNA to β-actin mRNA. cDNA sytheisis and qPCR was performed in duplicate for each sample, and the mean values and standard deviations for each genotype group (n = 3) are shown. The p values were calculated using Kruskal-Wallis test. The error bar represents the standard deviation. *n.d.*, not detected.
(TIF)

Table S1 Oligonucleotide primers used for real-time PCR of *APOBEC3* and control. A real-time PCR assay was performed for *APOBEC3* and control genes (Gene symbol) using each of forward primer (S) and reverse primer (AS) sets.
(DOC)

Table S2 APOBEC3B variations in the I/D and I/I genotyped healthy donors. APOBEC3B cDNAs from I/D and I/I-genotyped healthy donors (n = 5 each) were amplified by the nested RT-PCR and cloned into pUC118 plasmids. The *APOBEC3B* cDNA sequences were determined by DNA sequencing. The individual APOBEC3B variants analyzed are shown. A3B, APOBEC3B.
(DOC)

Acknowledgments

The authors thank all of the patients and volunteers who agreed to participate in this study. We also thank the NLGR organization for cooperating in the recruitment of the MSM volunteers in Nagoya. The TZM-bl cells were provided by J. C. Kappes and X. Wu through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, NIH Reagent Program.

Author Contributions

Conceived and designed the experiments: MI TI NK SI YK ATK MU YY WS YI TN. Performed the experiments: MI TI KM TM KS YI. Analyzed the data: MI TI DW JI KS HO YK ATK YY WS YI. Contributed reagents/materials/analysis tools: MI DW JI SI MU YY TS. Wrote the paper: MI TI YK ATK WS YI TN.

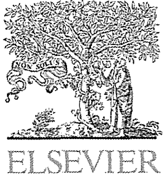
References

1. Albin JS, Harris RS (2010) Interactions of host APOBEC3 restriction factors with HIV-1 in vivo: implications for therapeutics. Expert Rev Mol Med 12: e4.
2. Goila-Gaur R, Strebel K (2008) HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology 5: 51.
3. Sheehy AM, Gaddis NC, Choi JD, Malim MH (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418: 646–650.
4. Bishop KN, Verma M, Kim EY, Wolinsky SM, Malim MH (2008) APOBEC3G inhibits elongation of HIV-1 reverse transcripts. PLoS Pathog 4: e1000231.
5. Iwatani Y, Chan DS, Wang F, Maynard KS, Sugiura W, et al. (2007) Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G. Nucleic Acids Res 35: 7096–7108.
6. LaRue RS, Andrésdóttir V, Blanchard Y, Conticello SG, Derse D, et al. (2009) Guidelines for naming nonprimate APOBEC3 genes and proteins. J Virol 83: 494–497.
7. Yu X, Yu Y, Liu B, Luo K, Kong W, et al. (2003) Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. Science 302: 1056–1060.
8. Kitamura S, Ode H, Nakashima M, Imahashi M, Naganawa Y, et al. (2012) The APOBEC3C crystal structure and the interface for HIV-1 Vif binding. Nat Struct Mol Biol 19: 1005–1010.
9. Li MM, Wu LI, Emerman M (2010) The range of human APOBEC3H sensitivity to lentiviral Vif proteins. J Virol 84: 88–95.
10. Russell RA, Smith J, Barr R, Bhattacharyya D, Pathak VK (2009) Distinct domains within APOBEC3G and APOBEC3F interact with separate regions of human immunodeficiency virus type 1 Vif. J Virol 83: 1992–2003.
11. Smith JL, Pathak VK (2010) Identification of specific determinants of human APOBEC3F, APOBEC3C, and APOBEC3DE and African green monkey APOBEC3F that interact with HIV-1 Vif. J Virol 84: 12599–12608.
12. Zhen A, Wang T, Zhao K, Xiong Y, Yu XF (2010) A single amino acid difference in human APOBEC3H variants determines HIV-1 Vif sensitivity. J Virol 84: 1902–1911.
13. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, et al. (2007) Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. J Infect Dis 195: 960–964.
14. Chaipan C, Smith JL, Hu WS, Pathak VK (2013) APOBEC3G restricts HIV-1 to a greater extent than APOBEC3F and APOBEC3DE in human primary CD4+ T cells and macrophages. J Virol 87: 444–453.
15. Do H, Vasilescu A, Diop G, Hirtzig T, Heath SC, et al. (2005) Exhaustive genotyping of the CEM15 (APOBEC3G) gene and absence of association with AIDS progression in a French cohort. J Infect Dis 191: 159–163.
16. Kourteva Y, De Pasquale M, Allos T, McMunn C, D'Aquila RT (2012) APOBEC3G expression and hypermutation are inversely associated with human immunodeficiency virus type 1 (HIV-1) burden in vivo. Virology 430: 1–9.

17. Mulder LC, Ooms M, Majdak S, Smedresman J, Linscheid C, et al. (2010) Moderate influence of human APOBEC3F on HIV-1 replication in primary lymphocytes. J Virol 84: 9613–9617.
18. Ulenga NK, Sarr AD, Thakore-Meloni S, Sankale JL, Eisen G, et al. (2008) Relationship between human immunodeficiency type 1 infection and expression of human APOBEC3G and APOBEC3F. J Infect Dis 198: 486–492.
19. Vetter ML, Johnson ME, Antons AK, Unutmaz D, D'Aquila RT (2009) Differences in APOBEC3G expression in CD4+ T helper lymphocyte subtypes modulate HIV-1 infectivity. PLoS Pathog 5: e1000292.
20. Hultquist JF, Lengyel JA, Refsland EW, LaRue RS, Lackey L, et al. (2011) Human and rhesus APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H demonstrate a conserved capacity to restrict Vif-deficient HIV-1. J Virol 85: 11220–11234.
21. Pak V, Heidecker G, Pathak VK, Derse D (2011) The role of amino-terminal sequences in cellular localization and antiviral activity of APOBEC3B. J Virol 85: 8538–8547.
22. Yu Q, Chen D, Konig R, Mariani R, Unutmaz D, et al. (2004) APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. J Biol Chem 279: 53379–53386.
23. Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ, et al. (2004) Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr Biol 14: 1392–1396.
24. Doehle BP, Schafer A, Cullen BR (2005) Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. Virology 339: 281–288.
25. Kidd JM, Newman TL, Tuzun E, Kaul R, Eichler EE (2007) Population stratification of a common APOBEC gene deletion polymorphism. PLoS Genet 3: e63.
26. An P, Johnson R, Phair J, Kirk GD, Yu XF, et al. (2009) APOBEC3B deletion and risk of HIV-1 acquisition. J Infect Dis 200: 1054–1058.
27. Itaya S, Nakajima T, Kaur G, Terunuma H, Ohtani H, et al. (2010) No evidence of an association between the APOBEC3B deletion polymorphism and susceptibility to HIV infection and AIDS in Japanese and Indian populations. J Infect Dis 202: 815–816; author reply 816–817.
28. Bogerd HP, Cullen BR (2008) Single-stranded RNA facilitates nucleocapsid: APOBEC3G complex formation. RNA 14: 1228–1236.
29. Iwatani Y, Takeuchi H, Strebel K, Levin JG (2006) Biochemical activities of highly purified, catalytically active human APOBEC3G: correlation with antiviral effect. J Virol 80: 5992–6002.
30. Svarovskaia ES, Xu H, Mbisa JL, Barr R, Gorelick RJ, et al. (2004) Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. J Biol Chem 279: 35822–35828.
31. Burnett A, Spearman P (2007) APOBEC3G multimers are recruited to the plasma membrane for packaging into human immunodeficiency virus type 1 virus-like particles in an RNA-dependent process requiring the NC basic linker. J Virol 81: 5000–5013.
32. Wichroski MJ, Robb GB, Rana TM (2006) Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. PLoS Pathog 2: e41.
33. Stenglein MD, Harris RS (2006) APOBEC3B and APOBEC3F inhibit L1 retrotransposition by a DNA deamination-independent mechanism. J Biol Chem 281: 16837–16841.
34. Koning FA, Newman EN, Kim EY, Kunstman KJ, Wolinsky SM, et al. (2009) Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets. J Virol 83: 9474–9485.
35. Refsland EW, Stenglein MD, Shindo K, Albin JS, Brown WL, et al. (2010) Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. Nucleic Acids Res 38: 4274–4284.
36. Bonvin M, Greeve J (2007) Effects of point mutations in the cytidine deaminase domains of APOBEC3B on replication and hypermutation of hepatitis B virus in vitro. J Gen Virol 88: 3270–3274.
37. Abe H, Ochi H, Maekawa T, Hatakeyama T, Tsuge M, et al. (2009) Effects of structural variations of APOBEC3A and APOBEC3B genes in chronic hepatitis B virus infection. Hepatol Res 39: 1159–1168.
38. Hattori J, Shiino T, Gatanaga H, Yoshida S, Watanabe D, et al. (2010) Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: nationwide surveillance from 2003 to 2008 in Japan. Antiviral Res 88: 72–79.
39. Burns MB, Lackey L, Carpenter MA, Rathore A, Land AM, et al. (2013) APOBEC3B is an enzymatic source of mutation in breast cancer. Nature 494: 366–370.
40. Burns MB, Temiz NA, Harris RS (2013) Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat Genet 45: 977–983.
41. Xuan D, Li G, Cai Q, Deming-Halverson S, Shrubsole MJ, et al. (2013) APOBEC3 deletion polymorphism is associated with breast cancer risk among women of European ancestry. Carcinogenesis.
42. Shinohara M, Io K, Shindo K, Matsui M, Sakamoto T, et al. (2012) APOBEC3B can impair genomic stability by inducing base substitutions in genomic DNA in human cells. Sci Rep 2: 806.
43. Mahieux R, Suspene R, Delebecque F, Henry M, Schwartz O, et al. (2005) Extensive editing of a small fraction of human T-cell leukemia virus type 1 genomes by four APOBEC3 cytidine deaminases. J Gen Virol 86: 2489–2494.
44. Ooms M, Krikoni A, Kress AK, Simon V, Munk C (2012) APOBEC3A, APOBEC3B, and APOBEC3H haplotype 2 restrict human T-lymphotropic virus type 1. J Virol 86: 6097–6108.
45. Jha P, Sinha S, Kanchan K, Qidwai T, Narang A, et al. (2012) Deletion of the APOBEC3B gene strongly impacts susceptibility to falciparum malaria. Infect Genet Evol 12: 142–148.

G Model
DESC 2688 No. of Pages 8
ARTICLE IN PRESS

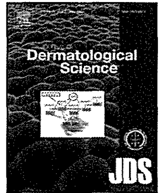
Journal of Dermatological Science xxx (2014) xxx–xxx



Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com



Review article

Innate and intrinsic antiviral immunity in skin

Tatsuyoshi Kawamura *, Youichi Ogawa, Rui Aoki, Shinji Shimada

Department of Dermatology, Faculty of Medicine, University of Yamanashi, Japan

ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 2 May 2014
Accepted 13 May 2014

Keywords:
Innate immunity
Intrinsic immunity
C-type lectins (CLRs)
Toll-like receptors (TLRs)
NOD-like receptors (NLRs)
RIG-I like receptors (RLRs)

ABSTRACT

As the body's most exposed interface with the environment, the skin is constantly challenged by potentially pathogenic microbes, including viruses. To sense the invading viruses, various types of cells resident in the skin express many different pattern-recognition receptors (PRRs) such as C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytosolic DNA sensors, that can detect the pathogen-associated molecular patterns (PAMPs) of the viruses. The detection of viral PAMPs initiates two major innate immune signaling cascades: the first involves the activation of the downstream transcription factors, such as interferon regulatory factors (IRFs), nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), which cooperate to induce the transcription of type I interferons and pro-inflammatory cytokines. The second signaling pathway involves the caspase-1-mediated processing of IL-1β and IL-18 through the formation of an inflammasome complex. Cutaneous innate immunity including the production of the innate cytokines constitutes the first line of host defence that limits the virus dissemination from the skin, and also plays an important role in the activation of adaptive immune response, which represents the second line of defence. More recently, the third immunity "intrinsic immunity" has emerged, that provides an immediate and direct antiviral defense mediated by host intrinsic restriction factors. This review focuses on the recent advances regarding the antiviral immune systems, highlighting the innate and intrinsic immunity against the viral infections in the skin, and describes how viral components are recognized by cutaneous immune systems.

© 2014 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

Contents

1. Introduction 00
2. Antiviral innate immunity in skin 00

Abbreviations: AIDS, Acquired immune deficiency syndrome; AIM2, Absent in melanoma 2; AMPs, Antimicrobial peptides; APCs, Antigen presenting cells; AP-1, Activator protein 1; APOBEC3, Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3; ASC, Apoptosis-associated speck-like protein containing a caspase recruitment domain; BMDC, Bone marrow-derived DC; CARD, Caspase activation and recruitment domain; CCR5, CC chemokine receptor 5; cGAS, Cyclic GMP-AMP synthase; CMV, Cytomegalovirus; CLEC, C-type lectin-like receptor; CLR, C-type lectin receptor; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, Danger-associated molecular pattern; DC, Dendritic cell; DCIR, DC immunoreceptor; DC-SIGN, DC-specific intercellular adhesion molecule-3-grabbing non-integrin; DNA-PKcs, DNA-dependentprotein kinase; ds, Double-stranded; EBV, Epstein Barr virus; EBER, snonpolyadenylated, noncoding RNA that forms stem-loop structure by intermolecular base-pairing; ER, Endoplasmic reticulum; GM-CSF, Granulocyte macrophage colony-stimulating factor; HBD, Human β defensin; HIV, Human immunodeficiency virus; HMGB1, High mobility group box-1; HNP, Human neutrophil peptides; HPV, Human papilloma virus; HSV, Herpes simplex virus; IFN, Interferon; IL, Interleukin; IRF, Interferon regulatory factor; KSHV, Kaposi's sarcoma-associated herpesvirus; LARG, Leukaemia-associated Rho guanine nucleotide exchange factor; LCs, Langerhans cells; LTR, Long terminal repeat; MAP, Mitogen-activated protein; MAVS, Mitochondrial antiviral signaling protein; MDA5, Melanoma differentiation-associated gene 5; MDP, Muramyl dipeptide; MyD88, Myeloid differentiation protein 88; NF-κB, Nuclear factor-κB; NLR, NOD-like receptor; NLRP3, NACHT, LRR and PYD domain-containing protein 3; NO, Nitric oxide; NOD, Nucleotide-binding oligomerization domain; PAMP, Pathogen-associated molecular pattern; PBMC, Peripheral blood mononuclear cells; pDC, Plasmacytoid dendritic cells; PKR, Protein kinase R; PRR, Pathogen recognition receptor; RLR, RIG-like receptor; RIG-I, Retinoic acid inducible gene I; ROS, Reactive oxygen species; RT, Reverse transcriptase; SAMHD1, SAM domain and HD domain-containing protein 1; si, Short hairpin; ss, Single-stranded; STDs, Sexually transmitted diseases; STAT, Signal transducer and activator of transcription; STING, Stimulator of IFN genes; SYK, Spleen tyrosin kinase; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor α; TRAF, TNF receptor-associated factor; TREX1, Three primerepair exonuclease 1; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN; TRIM5α, Tripartite motif 5α; VSV, Vesicular stomatitis virus; VV, Vaccinia virus; VZV, Varicella zoster virus; WNV, West Nile virus.

* Corresponding author at: University of Yamanashi, Department of Dermatology, 1110 Shimokato, Chuo, Yamanash 409-3898, Japan. Tel.: +81 55 273 6766 ; fax: +81 55 273 6766.
E-mail address: tkawa@yamanashi.ac.jp (R. Aoki).

http://dx.doi.org/10.1016/j.jdermsci.2014.05.004
0923-1811/© 2014 Japanese Society for Investigative Dermatology. Published by Elsevier Ireland Ltd. All rights reserved.

2.1. Toll-like receptors (TLRs) 00
2.2. NOD-like receptors (NLRs) 00
2.3. RIG-I-like receptors (RLRs) and intracellular DNA sensors 00
2.4. C-type lectin receptors (CLRs) 00
3. Intrinsic antiviral immunity in skin 00
4. Conclusion 00
Acknowledgments 00
References 00

1. Introduction

The skin plays a central role in host defence against a broad array of potentially pathogenic microbes, including viruses. Cutaneous innate immunity constitutes the first line of host defence that limits the virus dissemination from the local sites of infection, and also plays an important role in the activation of adaptive immune response, which represents the second line of defence. Over the past decade, remarkable progress has been made towards understanding the innate immune responses, especially to viral detection. More recently, the host's third immunity "intrinsic immunity" has emerged. Unlike the innate and adaptive immune systems, intrinsic immunity provides an immediate and direct antiviral defense mediated by intrinsic restriction factors, which are mostly preexistent in certain cell types.

There are many viral skin infections, which range from the common to the rare, from the intractable to the self-healing and from those causing just local infection in the skin to those with associated systemic diseases (Table 1). To counter viral invasion, the cells resident in the skin, including keratinocytes, Langerhans cells (LCs), dermal dendritic cells (DCs), macrophages, mast cells, and fibroblasts, express many different pattern-recognition receptors (PRRs) that can detect the pathogen-associated molecular patterns (PAMPs) of the invading viruses, which in turn activate antiviral innate immune responses.

This review focuses on the recent advances regarding the host antiviral immune systems, highlighting the innate and intrinsic immunity against the viruses which are transmitted via the skin or associated with cutaneous symptoms, and describes how viral components are recognized by cutaneous immune systems.

2. Antiviral innate immunity in skin

The conserved microbial components known as PAMPs are recognized by host PRRs. Viral recognition by the innate immune system is more challenging than recognition of other pathogen classes, because any given viral protein is unlikely to be shared among diverse viruses [1,2]. However, remarkable progress has been made over the past few years towards understanding the contribution of PRRs, such as C-type lectins (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I like receptors (RLRs) and other cytosolic PRRs, to viral detection.

In various viral infections in the skin, the detection of viral PAMPs via PRRs initiates two distinct innate immune signaling cascades: the first involves the activation of the transcription factors interferon regulatory factor 3 (IRF3) and/or IRF7, nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), which cooperate to induce the transcription of type I interferons (IFNs, e.g. IFN-α and IFN-β), chemokines and pro-inflammatory cytokines (Fig. 1 and Fig. 3). Through the secretion of type I IFNs, the response can be amplified and spread to surrounding uninfected skin cells and thereby activate hundreds of IFN-stimulated genes (ISGs), most of which encode products with profound antiviral effects, such as the degradation of viral nucleic acids or inhibition of viral gene expression [3,4]. The second signaling pathway results in the formation of an inflammasome complex, which activates caspase-1, a protease which processes pro-interleukin (IL) 1-β and pro-IL-18 to generate active cytokines ready for secretion (Fig. 2). In general, TLRs and RLRs are involved in the expression of type I IFNs or proinflammatory cytokines and chemokines, whereas viral detection by NLRs leads to caspase-1-mediated processing of IL-1β. The importance of viral recognition via these PRRs and subsequent cytokines production in the skin is illustrated by the

Table 1
Representative viruses transmitted via skin or associated with cutaneous symptoms.

dsDNA viruses	
Poxviridae	Molluscum contagiosum virus
Herpesviridae	α: human Herpesvirus 1, 2, 3 (HSV-1, HSV-2, VZV)
	β: human Herpesvirus 5, 6, 7 (HCMV, HHV-6, HHV-7)
	γ: human Herpesvirus 4, 8 (EBV, KSHV)
Papillomaviridae	Human papillomavirus (HPV) 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 57, 60
Smallpox	Vaccinia virus (VV)
ssDNA viruses	
Parvoviridae	Human parvovirus B19
DNA and RNA reverse transcribing virus	
Retroviridae	Human T-lymphotropic virus 1 (HTLV-1)
Lentivirus	Human immunodeficiency virus 1 (HIV-1)
Negative stranded ssRNA viruses	
Rhabdoviridae	Rabies virus
Paramyxoviridae	Measles virus
Positive stranded ssRNA viruses	
Picornaviridae	Human enterovirus A, Coxsackie virus A
Flaviviridae	Dengue virus (DV), Japanese encephalitis virus (JEV), West Nile virus (WNV)
Togaviridae	Rubella virus

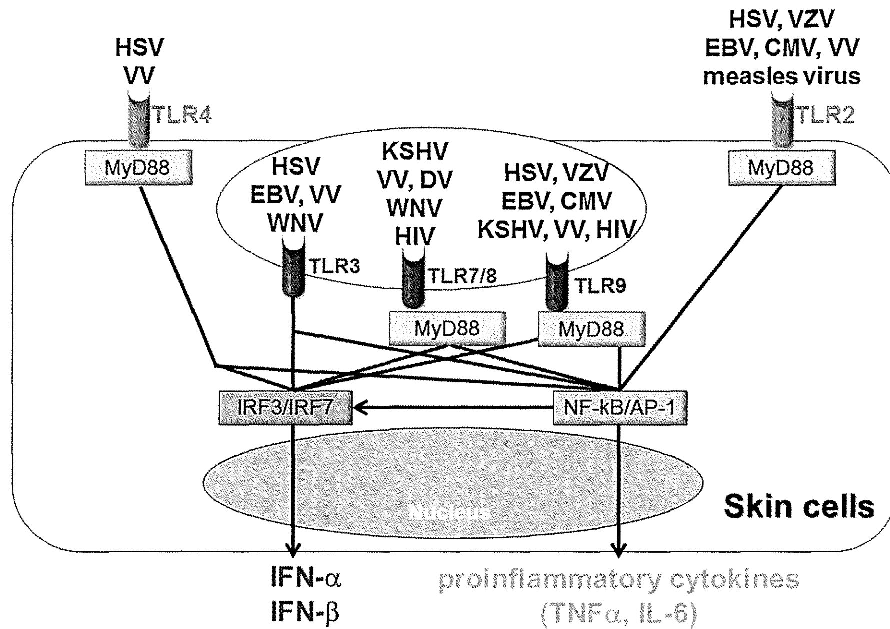


Fig. 1. TLRs-mediated viral recognition and signaling pathways in skin cells. Upon ligand stimulation by the indicated skin-associated viruses, TLRs, except the TLR3, recruit the adaptor MyD88. This allows NF-κB to translocate into the nucleus, simultaneously, activates the MAP kinase pathway triggering the activation of AP-1. Together, NF-κB and AP-1 induce the expression of pro-inflammatory cytokines. Upon viral ligand stimulation, TLR7 and TLR9 associate with MyD88 leading to nuclear translocation of IRF7. Stimulation of TLR3 and TLR4 permits nuclear translocation of IRF3. IRF3 or IRF7, along with NF-kB and AP-1, cooperate to induce the expression of type I IFNs, IFN-β and IFN-α, respectively.

extreme susceptibility to percutaneous viral infection of mice lacking the PRRs and the cytokines.

2.1. Toll-like receptors (TLRs)

Nucleic acids of both DNA and RNA viruses (Table 1), such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG rich motif (CpGDNA), are considered as PAMPs and can be recognized by several endosomal members of the TLRs family such as TLRs-3, -7, -8 and -9 [1]. TLR3 recognizes double-stranded (ds) RNA; TLR7 and TLR8 recognize single-stranded (ss) RNA; and TLR9 recognizes CpG motifs in DNA, and all of these nucleic acid-sensing TLRs induce the nuclear translocation of IRF3 or IRF7 and the expression of type I IFNs, leading to effective antiviral immunity (Fig. 1). Since viral particles are generally endocytosed and degraded in late endosomes or lysosomes, viral DNA and RNA are released into these intracellular acidic compartments, allowing viral nucleic acids to be in close contact with endosomal TLRs. Alternatively, nucleic acids present in the cytoplasm may be engulfed by an autophagosome, which subsequently fuse with the endosome, and recognized by the TLRs [5].

Recent studies have revealed that TLR2can be stimulated by several skin-associated viruses, including herpes simplex viruses (HSV), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), human and mouse cytomegaloviruses (HCMV, MCMV), vaccinia virus (VV) and measles virus (Fig. 1) [2,6–9]. In most of these cases, viral ligand stimulation of TLR2, in the combination with TLR1 or TLR6, recruits the adaptor MyD88, resulting in translocation of NF-κB into the nucleus and triggering the activation of AP-1, which cooperate to induce the expression of proinflammatory cytokines. For example, VZV and measles virus have been demonstrated to activate monocytes to produce proinflammatory cytokines in a TLR2-dependent manner [10,11]. Surprisingly, although it is well known bacterial ligands for TLR2 were unable to drive type I IFNs production, VV and MCMV have recently been demonstrated to be able to induce type I IFNs via TLR2 [12], thus acting as beneficial to the host. On the contrary, viral interaction with TLR2can be detrimental to its host. HSV-1 recognition by TLR2 induces the expression of inflammatory cytokines in the brain that causes lethal encephalitis [6]. TLR2 activation by measles virus not only induces proinflammatory cytokines but also upregulates surface expression of CD150, the receptor for measles virus, indicating that activation of TLR2-dependent signals might contribute viral spread and pathogenicity.

TLR3 is hypothesized to enable recognition of DNA viruses since virtually all viruses produce dsRNA at some point during replication [13]. Indeed, it has been demonstrated that HSV-1 activates keratinocytes to produce type I IFN in a TLR3-dependent manner (Fig. 1) [14]. By contrast, only a few RNA viruses have been shown to induce a TLR3-dependent innate immune response, although RNA viruses are important producers of dsRNA [9]. In Epstein-Barr virus (EBV)-associated hemophagocytic lymphohistiocytosis and infectious mononucleosis, TLR3contributes to the sudden release of inflammatory cytokines, as the release of EBV-encoded small RNA (EBAR) from EBV-infected cell, which are giving rise to dsRNA-like molecules, activate immune cells via TLR3 signaling to produce type I IFNs and TNF-α [15]. New insights have recently emerged regarding the TLR3-mediated recognition of viral dsRNA; Class A scavenger receptors bind to extracellular viral dsRNA, which is released into the extracellular space after lysis of infected cells, and mediate uptake and presentation of dsRNA to TLR3 in the endosome [16]. As well as TLR3, recent study revealed that TLR4 stimulation also promotes activation of IRF3 and signal to NFκB [17]. Indeed, TLR4has been shown to be important for the induction of anti-viral cytokines in the response to HSV and VV (Fig. 1) [2].

TLR7/8 and TLR9 detect viral RNA and DNA, respectively, in the endosomal lumen of virus-infected cells and their signaling pathways require MyD88 and IRF7 to induce type I IFNs, especially IFN-α. TLR7/8can be stimulated by several skin-associated viruses, including Kaposi's sarcoma-associated herpesvirus (KSHV), VV,

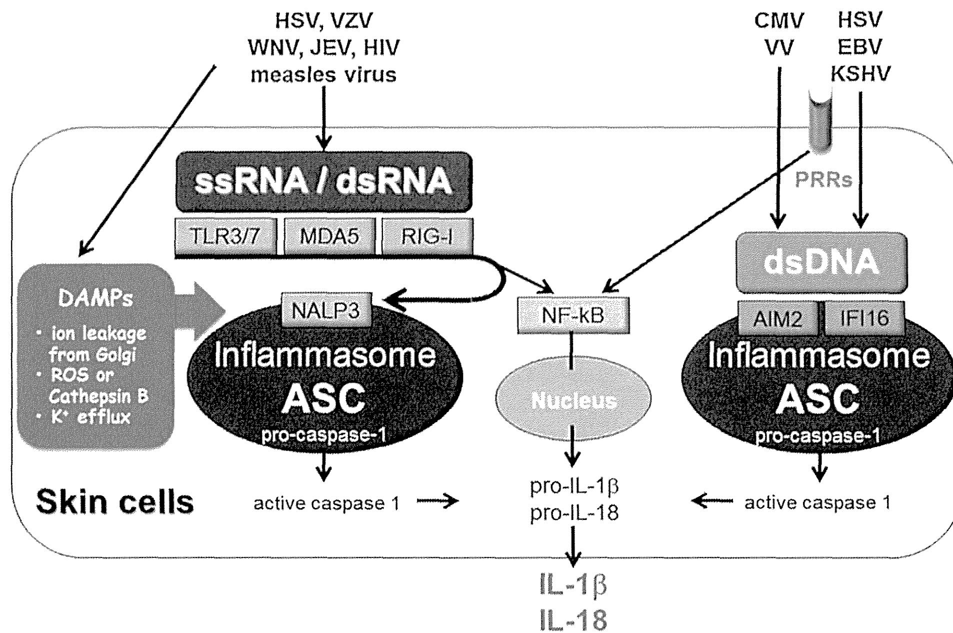


Fig. 2. Viral recognition and IL-1β/IL-18 production by skin cells. During the indicated skin-associated virus infections, viral ssRNA or dsRNA is recognized by endosomal TLRs (TLR3/7) or cytoplasmic RLRs (RIG-I or MDA5), leading to the priming of NLRP3 inflammasome. Following the priming, the NLRP3 inflammasome is assembled and activated in response to virus-induced DAMPs. AIM2 or IFI16 in the cytosol bind viral dsDNA and engage the adaptor protein ASC to form a caspase-1-activating inflammasome. Inflammasome requires another signal for IL-1β/IL-18 production, which comes from the virus that triggers TLRs-, RLRs- or other PRR-dependent signaling pathway and allows pro-IL-1β production through NF-κB-mediated signaling.

dengue virus (DV), West Nile virus (WNV) and HIV-1, and TLR9can sense all herpes viruses, VV and HIV-1 (Fig. 1) [8]. However, it is of note that for these endosomal TLRs, induction of type I IFNs generally occurs in plasmacytoid DCs (pDCs), "professional IFN producers" [8,18], which are essentially absent in normal skin but have been described in lesions of inflammatory diseases of the skin (e.g. psoriasis vulgaris, contact dermatitis, and lupus erythematosus) [19]. Intriguingly, in mice models, after i.v. inoculation of HSV, TLR9-dependent recognition of CpG motifs in HSV DNA mediates IFN-α secretion by pDCs, whereas pDC have a negligible impact on local type I IFN production after vaginal or cutaneous HSV infection [20]. Nevertheless, it is still possible that pDCs may play some roles in human anti-HSV immune responses, because pDCs have been shown to abundantly infiltrate the reactivated lesions of genital herpes and persist for months after healing [21].

2.2. NOD-like receptors (NLRs)

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are cytoplasmic PRRs and has been recently implicated to recognize viral PAMPs. NLRs that contain a caspase activation and recruitmentdomain (CARD) are part of the NLRC subfamily including NOD2, whereas, NLRs that possess a PYD form the NLRP (also known as NALP) subfamily including NALP3. NLRP3 is essentially involved in the activation of the inflammasome, whereas NOD2 plays a role in the activation of NF-κB and mitogen-activated protein (MAP) kinase pathways. Using a variety of cell types including DCs, macrophages, fibroblasts and melanoma cells, a critical role for the NLRP3 inflammasome has been demonstrated during the infection of various skin-associated viruses, including HSV, VZV, WNV, Japanese encephalitis virus (JEV), measles virus and HIV (Fig. 2) [8,22–26]. Because NALP3 is unlikely to bind directly to viral nucleic acids, NLRP3 inflammasome is considered to be activated through an indirect mechanism. One possibility is that viral RNA is sensed by endosomal TLRs or cytoplasmic RLRs, such as retinoic acid-Inducible Gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), leading to the priming of NLRP3 inflammasome for activation and the upregulation of pro-IL-1β through NF-κB-mediated signaling pathways (Fig. 2) [27].

Virus-mediated NLRP3 activation requires another signal to induce the production of mature IL-1β by a wide variety of danger-associated molecular pattern (DAMPs) including (i) ion leakage from intracellular organelles (e.g. the Golgi) into the cytosol, (ii) the generation of reactive oxygen species resulting from PRR signaling, endoplasmic reticulum (ER) and mitochondrial stress, or virus-induced damage to endosomes, (iii) the P2 × 7 ion channel is opened in response to extracellular ATP from damaged or necrotic cells allowing for potassium efflux, (iv) during entry, virus infection damages endosomes and releases proteases such as cathepsin B into the cytosol, (v) pore-forming toxins, (vi) uric acid crystals (Fig. 2) [9,27]. Indeed, several viral infections have been shown to cause these variety of DAMPs, which are then recognized by NLRP3, which in turn, recruits the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and then ASC interacts with the pro-caspsase-1 which becomes activated and allows the mature IL-1β production [27].

A recently discovered receptor for cytosolic dsDNA, AIM2 (absent in melanoma 2), has been shown to bind DNA and engage ASC to form a caspase-1-activating inflammasome. Knockdown of AIM2 in macrophages abrogates caspase-1 activation in response to dsDNA of VV and MCMV [28], indicating that AIM2 plays a role in sensing DNA viruses (Fig. 2). In addition, another DNA receptor, IFN-γ-inducible protein 16 (IFI16) has been reported to induce ASC-dependent inflammasome activation in a variety of cell types, including DCs, macrophages and fibroblasts, during infection with DNA viruses such as HSV-1, EBV and KSHV [29].

2.3. RIG-I-like receptors (RLRs) and intracellular DNA sensors

An additional family of cytosolic receptors, RLRs, including RIG-I, MDA5 and LGP2 (laboratory of genetics and physiology 2), are expressed in most skin cell types and are greatly increased with type I IFNs exposure after virus infection [30,31]. RIG-I and

G Model
DESC 2688 No. of Pages 8
ARTICLE IN PRESS

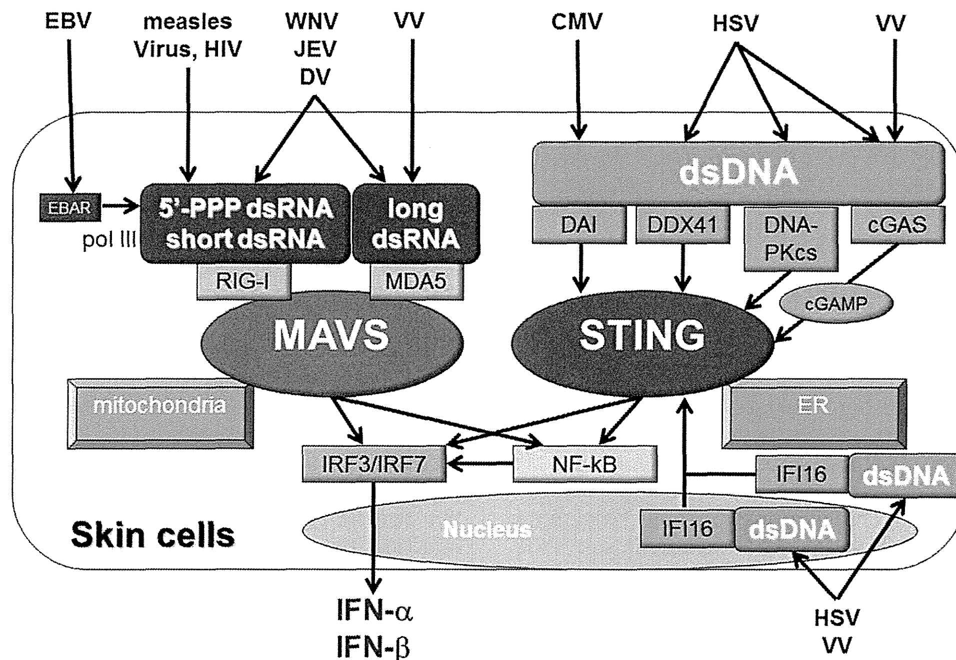


Fig. 3. RLRs- and cytosolic DNA sensors-mediated viral recognition and IFN production by skin cells. During the indicated skin-associated virus infections, viral dsRNA is recognized by cytoplasmic RLRs (e.g. RIG-I or MDA), leading to the production of type I IFNs via the adaptor protein MAVS and the transcription factors, NFκB, IRF3 and IRF7. DNA introduced by invading DNA viruses and/or damaged host cells are released and can meet sensors in the cytosol of skin cells. Multiple DNA sensors, such as DAI, DNA-PKcs, DDX41, cGAS and IFI16, are considered to activate STING located at the endoplasmic reticulum (ER), culminating in the activation the transcription factors NFκB, IRF3 and IRF7, which then lead to induction of type I IFNs.

MDA5contain two CARDs in tandem at their amino terminus that are important for their signaling functions. In general, infection by RNA viruses leads to the generation of long dsRNA in the cytosol that is structurally different from host cellular RNA, which is single stranded with short and often imperfectly matched stem loops [32]. RIG-I is preferentially involved in the detection of short dsRNA (up to 1 kb) or stem-loop RNA species bearing a 5′-triphosphate (5′ppp) ends, which serve in part to define a non-self RNA PAMP, whereas MDA-5 is more sensitive to long dsRNA (more than 2 kb) containing branched structures [9,31,32]. Viral RNA recognition by RIG-I and/or MDA5 leads to the production of type I IFNs via the adaptor protein: mitochondrial antiviral signaling protein (MAVS) and the transcription factors, namely NFκB, IRF3 and IRF7 (Fig. 3) [30,32]. RIG-I and MDA-5 recognize different viruses; RIG-I is essential for immune defense against some skin-associated viruses such as EBV, measles virus and HIV, whereas MDA-5 is required for IFNs induction by HSV and VV [8,31]. Some members of the Flaviviridae such as DV, JEV and WNV have been shown to activate both RIG-I and MDA5 for type I IFNs production in a variety of skin cell types (Fig. 3) [31,33]. In addition to the RLRs, the helicases DDX1, DDX21, DHX9, and DHX36have been linked to recognition of cytoplasmic RNA in DCs [8].

RNA polymerase III (pol III) has the fundamental role in transcribing transfer RNAs and other small non-coding RNA molecules. Intriguingly, pol III is also able to generate dsRNA bearing a 5'ppp moiety with AT-rich DNA as templates and the dsRNA is then recognized by RIG-I [29,34]. In EBV infection, pol III plays a role is in the generation of the EBERs (nonpolyadenylated, noncoding RNA that forms stem-loop structure by intermolecular base-pairing), giving rise to dsRNA-like molecules, that are subsequently recognized by RIG-I (Fig. 3) [34]. However, in this case, pol III acts in its conventional role, i.e. in the transcription of functional RNAs, rather than as a sensor per se [32].

During viral infection, DNA introduced by invading DNA viruses and/or released from damaged host cells can meet intracellular sensors and triggers the activation of innate immune responses, especially when DNA is not effectively cleared by DNases, including DNase I in the extracellular space, DNase II in lysosomes and three prime repair exonuclease 1 (TREX1) in the cytoplasm. In addition to the endosomal TLRs-dependent IFN response elicited by intracellular DNA, it has recently been demonstrated that adaptor protein STING (stimulator of IFN genes) expressed in the endoplasmic reticulum is also essential for IFN-β induction by intracellular dsDNA and/or DNA viruses via activation of the transcription factors IRF3, TANK-binding kinase 1 (TBK1) and NF-κB [29,30]. Although the identity of the upstream DNA receptors that cause the activation of STING has remained controversial, it has been proposed that STING-dependent signaling pathway may be activated by multiple DNA sensors; these include DAI (DNA-dependent activator of IFN-regulatory factors), IFI16, the helicase DDX41, DNA-PKcs (DNA-dependentprotein kinase) and cGAS (cyclic GMP-AMP synthase) (Fig. 3) [29,30].

DAI has been shown to play a role in human fibroblasts during HCMV infection, and another DNA receptors, IFI16, DDX41 and DNA-PKcs, induce IFN-β in response to intracellular DNA in a variety of skin cell types during HSV-1 infection (Fig. 3) [8,29]. It is of note that IFI16 shuttles between the nucleus and the cytoplasm and sense DNA in both compartments [29]. A newly identified enzyme, cGAS, binds intracellular DNA and mediate the production of cyclic GMP-AMP (cGAMP), which functions as a second messenger that binds and activates STING [30]. Thus, cGAS acts as a DNA receptor, and indeed, knockdown of cGAS inhibits IFN-β induction in THP-1 macrophages or L929 fibroblastic cell line by DNA viruses such as HSV-1 and VV [29,30].

2.4. C-type lectin receptors (CLRs)

CLRs are also an important family of PRRs and involved in the recognition of certain viruses. CLRs, such as DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), Langerin, mannose receptor

Table 2
C-type lectin receptors and related skin-associated viruses.

CLRs	Viral ligands	Expression
DC-SIGN (CD209)	HIV-1, CMV, DV, measles virus	Myeloid DCs, macrophages
Langerin (CD207)	HIV-1, measles virus	Langerhans cells, (dermal DCs)
Mannose receptor (CD206)	HIV-1, DV	Myeloid DCs, macrophages
DEC-205(CD205)	HIV-1	Myeloid DCs
CLEC4A (DCIR)	HIV-1	Myeloid DCs, macrophages, plasmacytoid DCs
CLEC5A	DV, JEV	Monocytes, macrophages
CLEC9A (DNGR-1)	HIV-1	Myeloid DCs

(MR), C-type lectin-like receptor (CLEC) 4A (also known as DC immunoreceptor; DCIR), CLEC 5A and CLEC9A (also known as DNGR-1), have been shown to bind several skin-associated viruses (Table 2) [35–37]. These CLRs are mostly expressed in cutaneous antigen presenting cells (APCs) such as LCs, DCs and macrophages (Table 2), and recognize mannose, fucose and glycosylated structures of the viruses via conserved carbohydrate recognition domains. Although most CLRs do not act as "self-sufficient" PRRs for the viruses, they mediate internalization of the viral ligand or the virus itself into intracellular compartments, leading to its degradation and subsequent antigen presentation by APCs [35,36]. Alternatively, the few CLRs trigger distinct signaling pathways via a number of kinases, including spleen tyrosin kinase (SYK) and Src kinase, that modulate the induction of specific cytokines [35,36]. In addition, recent studies have suggested that several CLRs, such as DC-SIGN and CLEC4A, induce signaling pathways that modulate TLR-induced gene expression at the transcriptional or post-transcriptional level [35].

Most studies for CLRs in cutaneous viral infection have long been focused on HIV-1. HIV-1can bind various CLRs expressed by cutaneous innate immune cells (Table 2), which also express CD4 and coreceptors (CCR5 and CXCR4), required for HIV-1infection (Fig. 4) [35–38]. There are two major mechanisms of HIV-1 transmission between cells; one is *cis*-infection, where target cells are infected with progeny virions, which are released by productively infected cells, via CD4 and coreceptors, and the other is trans-infection, where target cells are infected with virions, which was captured by the neighboring donor cells without productive infection, via the CLRs-mediated virological synapse or the exosome-like pathway [35]. DC-SIGN, MR, and CLEC4A promote *cis*-infection of innate immune cells through increased interactions between HIV-1 glycoprotein envelope gp120 and CD4, but also facilitate viral capture and CD4+ T-lymphocyte trans-infection by mediating viral endocytosis into nondegradative endosomes permitting the intracellular storage of intact virions [35,36]. In contrast, Langerin impairs infection of LCs by HIV-1 via subsequent internalization within birbeck granules, where the virus is degraded [39]. Nevertheless, there is a saturation of langerin at higher virus concentrations that overwhelms the protective mechanism of action [39] and CD4/CCR5-mediated *cis*-infection of LCs is considered to be a major pathway involved in sexual transmission of HIV-1 [40,41].

3. Intrinsic antiviral immunity in skin

The intrinsic antiviral immunity provides an immediate and direct antiviral defense mediated by intrinsic restriction factors that are mostly preexistent in certain cell types. Unlike TLRs and RLRs, which inhibit viral infection indirectly by activating signaling cascades that result in the transcription of genes encoding antiviral factors such as type I IFNs (Fig. 1 and Fig. 3), intrinsic restriction factors bind viral components and directly inhibit all steps of viral

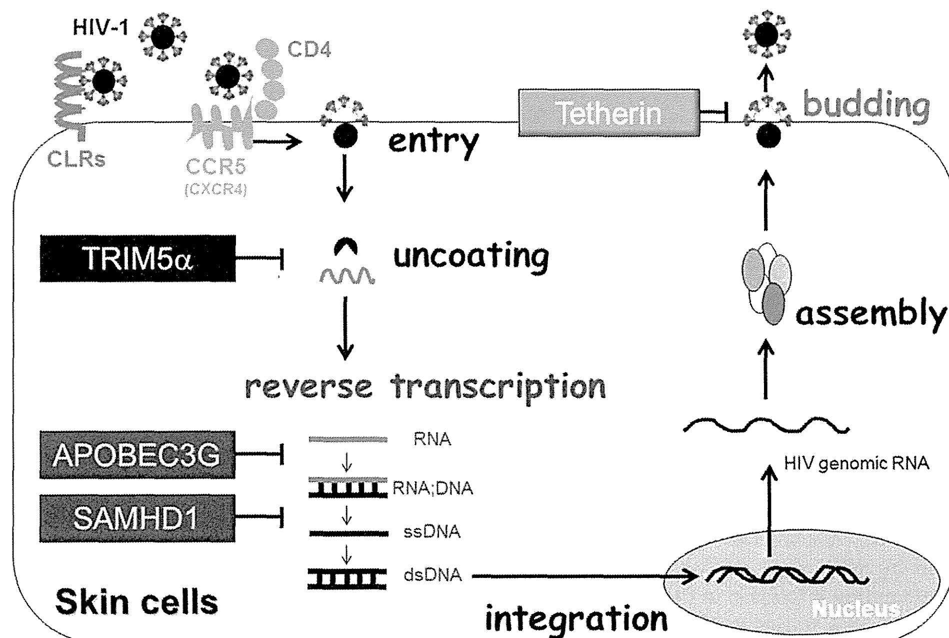


Fig. 4. Potential impact of intrinsic antiviral restriction factors on HIV-1cycle. Certain skin cell types, especially LCs and DCs, express intrinsic antiviral restriction factors, such as TRIM5α, APOBEC3G, SAMHD1 and tetherin, capable of binding viral components and directly blocking different stages of HIV replication cycle.

replication (Fig. 4) [42]. Importantly, as most of these factors are encoded by ISGs, they can be further induced by type I IFNs to amplify their antiviral activity [32].

HIV-1can infect various skin cells, which express CD4 and CCR5, including LCs, DCs, macrophages and CD4+ T cells. However, the magnitude of infection with HIV-1 is cell type specific, and CD4+ T cells are more susceptible to infection with HIV-1 than LCs/DCs and macrophages. Recent studies have revealed that the distinct susceptibility to HIV-1 among those cell types largely attribute to the host intrinsic restriction factors, such as APOBEC3G (Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G) and SAMHD1 (SAM domain- and HD domain-containing protein 1) (Fig. 4) [32,42]. APOBEC3G was one of the first intrinsic antiviral factors identified as acting against HIV-1. In cells productively infected with HIV-1, host APOBEC3G is packaged into HIV-1 virions, and in the infection to other target cells, APOBEC3G edits C–U in HIV DNA, which results in G-to-A mutation in the HIV genome, leading to the diminished replication. The deoxynucleoside triphosphate: SAMHD1 degrades deoxynucleoside triphosphates (dNTPs), and inhibit HIV-1 reverse transcription by decreasing the level of cellular dNTPs to below the level required for the synthesis of viral DNA. Intriguingly, in DCs, SAMHD1have an additional role in limiting the induction of type I IFNs in response to HIV-1 replication [43]. The capsid-binding protein TRIM5α (Tripartite motif 5α) also acts as intrinsic restriction factor and binds viral capsid targeting the virus for proteasomal degradation prior to reverse transcription. Human TRIM5α mediates mild restriction of HIV-2, but does not cause a significant inhibition to HIV-1, while rhesus TRIM5α restricts HIV-1. Tetherin has also been identified as the restriction factor responsible for blocking the release of nascent HIV-1 particles from infected cells [32,42]. At the HIV-1 budding site, tetherin incorporates into the cell and virus membranes and prevents efficient viral release by tethering HIV-1 particles to the cell, and consequently, tethered particles are internalized by endocytosis and are subsequently degraded in the endosomes (Fig. 4). Tetherin has an additional immunological role: the binding of tetherin to ILT7, a membrane receptor selectively expressed in pDCs, leads to the inhibition of TLR-mediated IFN responses [44].

In the initial phase of sexual transmission of HIV-1 via mucosa or skin, although DCs, macrophages and T cells in sub-mucosa or dermis can be potential targets for HIV-1, an emerging body of evidence now indicates that LCs located within epithelium/epidermis are initial cellular targets and play crucial roles in spreading HIV-1 because keratinocytes are not susceptible to HIV-1 and intercellular desmosomes and tight junctions provide a potent protective barrier to viral invasion [40,45–48]. During the first week after sexual exposure to HIV-1, HIV-1 infection does not appear to trigger a strong innate immune response in mucosa or skin, probably because HIV-1 may avoid triggering antiviral innate immune responses by not replicating efficiently in LCs. In addition to langerin-mediated degradation of HIV-1 in LCs as mentioned above, the host restriction factors may be associated with the poor replication in LCs. Indeed, we have recently found that LCs do functionally express the intrinsic restriction factors, APOBEC3G and SAMHD1 [41,49].

Intrinsic immunity mediated by host intracellular restriction factors play important roles in restricting not only HIV-1 but also other viral replication. For instance, tetherin targets many other enveloped viruses, such as Ebola virus and KSHV. Moreover, IFITM (IFN-inducible transmembrane) proteins has been recently identified as a host restriction factor for influenza A virus and they also restrict cytoplasmic entry of some flaviviruses, such as DV and WNV [32,42]. By contrast, the primate lentiviruses such as HIV-1have had to evolve multiple mechanisms to antagonize the restriction factors. For example, the Vpx and Vif proteins targets SAMHD1 and APOBEC3G respectively for ubiquitination and subsequent degradation in virus producing cells [42]. Furthermore, the lentiviral Vpu protein antagonizes tetherin by altering its normal subcellular localization.

4. Conclusion

Innate immune receptors are essential to initiate antiviral innate immune responses including production of innate cytokines, such as type I IFNs. In addition to the innate cytokines, upon percutaneous viral invasion, the skin cells also provide an early warning system by releasing stored and inducible cytokines as DAMPs or "alarmin". For example, recent study has highlighted the important roles of IL-33 as an alarmin in cutaneous HSV infection [50]. Although numerous previous studies have been dedicated to address which innate immune receptors are most critical for antiviral innate immunity, few have done so using human skin resident cells. Further studies focusing specifically on primary human skin cells are required. Understanding the mechanisms of cutaneous antiviral innate and intrinsic immunity may have important implications for the design of vaccines and the antiviral therapy.

Acknowledgments

We would like to thank Kazutoshi Harada, Naotaka Shibagaki, and Hiroaki Mitsuya for their helpful discussions.

References

[1] Kawai T, Akira S. Innate immune recognition of viral infection. Nat Immunol 2006;7:131–7.
[2] O'Neill LA, Bowie AG. Sensing and signaling in antiviral innate immunity. Curr Biol 2010;20:R328–33.
[3] Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. Nat Rev Immunol 2008;8:911–22.
[4] Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity 2006;25:373–81.
[5] Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007;315:1398–401.
[6] Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, et al. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci U S A 2004;101:1315–20.
[7] Sato A, Linehan MM, Iwasaki A. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. Proc Natl Acad Sci U S A 2006;103:17343–8.
[8] Melchjorsen J. Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity – clues for treatments and vaccines. Viruses 2013;5:470–527.
[9] Vandevenne P, Sadzot-Delvaux C, Piette J. Innate immune response and viral interference strategies developed by human herpesviruses. Biochem Pharmacol 2010;80:1955–72.
[10] Wang JP, Kurt-Jones EA, Shin OS, Manchak MD, Levin MJ, Finberg RW. Varicella-zoster virus activates inflammatory cytokines in human monocytes and macrophages via Toll-like receptor 2. J Virol 2005;79:12658–66.
[11] Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, et al. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J Virol 2002;76:8729–36.
[12] Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol 2009;10:1200–7.
[13] Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology 1996;219:339–49.
[14] Zhang SQ, Zhang Z, Luo X, Yang S, Chai Y, Huang HL, et al. Interleukin 29 enhances expression of Toll receptor 3 and mediates antiviral signals in human keratinocytes. Inflamm Res 2011;60:1031–7.
[15] Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, et al. Epstein–Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med 2009;206:2091–9.
[16] DeWitte-Orr SJ, Collins SE, Bauer CM, Bowdish DM, Mossman KL. An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA,

mediating entry and leading to subsequent type I IFN responses. PLoS Pathog 2010;6:e1000829.
[17] Tseng PH, Matsuzawa A, Zhang W, Mino T, Vignali DA, Karin M. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat Immunol 2010;11:70–5.
[18] Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8:594–606.
[19] Bangert C, Friedl J, Stary G, Stingl G, Kopp T. Immunopathologic features of allergic contact dermatitis in humans: participation of plasmacytoid dendritic cells in the pathogenesis of the disease? J Invest Dermatol 2003;121:1409–18.
[20] Swiecki M, Wang Y, Gilfillan S, Colonna M. Plasmacytoid dendritic cells contribute to systemic but not local antiviral responses to HSV infections. PLoS Pathog 2013;9:e1003728.
[21] Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, et al. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. Nat Med 2009;15:886–92.
[22] Johnson KE, Chikoti L, B Chandran. Herpes simplex virus 1 infection induces activation and subsequent inhibition of the IFI16 and NLRP3 inflammasomes. J Virol 2013;87:5005–18.
[23] Nour AM, Reichelt M, Ku CC, Ho MY, Heineman TC, Arvin AM. Varicella-zoster virus infection triggers formation of an interleukin-1beta (IL-1beta)-processing inflammasome complex. J Biol Chem 2011;286:17921–33.
[24] Ramos HJ, Lanteri MC, Blahnik G, Negash A, Suthar MS, Brassil MM, et al. IL-1beta signaling promotes CNS-intrinsic immune control of West Nile virus infection. PLoS Pathog 2012;8:e1003039.
[25] Kaushik DK, Gupta M, Kumawat KL, Basu A. NLRP3 inflammasome: key mediator of neuroinflammation in murine Japanese encephalitis. PLoS One 2012;7:e32270.
[26] Komune N, Ichinohe T, Ito M, Yanagi Y. Measles virus V protein inhibits NLRP3 inflammasome-mediated interleukin-1beta secretion. J Virol 2011;85:13019–26.
[27] Lupfer C, Kanneganti TD. The expanding role of NLRs in antiviral immunity. Immunol Rev 2013;255:13–24.
[28] Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 2010;11:395–402.
[29] Unterholzner L. The interferon response to intracellular DNA: why so many receptors? Immunobiology 2013;218:1312–21.
[30] Goubau D, Deddouche S, Reis ESC. Cytosolic sensing of viruses. Immunity 2013;38:855–69.
[31] Loo YM, Gale Jr. M. Immune signaling by RIG-I-like receptors. Immunity 2011;34:680–92.
[32] Yan N, Chen ZJ. Intrinsic antiviral immunity. Nat Immunol 2012;13:214–22.
[33] Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, et al. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell 2010;140:397–408.
[34] Chiu YH, Macmillan JB, Chen ZJ. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell 2009;138:576–91.
[35] Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol 2009;9:465–79.
[36] Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. Immunity 2011;34:651–64.
[37] Redelinghuys P, Brown GD. Inhibitory C-type lectin receptors in myeloid cells. Immunol Lett 2011;136:1–12.
[38] Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature 2009;458:899–903.
[39] de Witte L, Nabatov A, Pion M, Fluitsma D, de Jong MA, de Gruijl T, et al. Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. Nat Med 2007;13:367–71.
[40] Kawamura T, Kurtz SE, Blauvelt A, Shimada S. The role of Langerhans cells in the sexual transmission of HIV. J Dermatol Sci 2005;40:147–55.
[41] Ogawa Y, Kawamura T, Matsuzawa T, Aoki R, Gee P, Yamashita A, et al. Antimicrobial peptide LL-37 produced by HSV-2-infected keratinocytes enhances HIV infection of Langerhans cells. Cell Host Microbe 2013;13:77–86.
[42] Duggal NK, Emerman M. Evolutionary conflicts between viruses and restriction factors shape immunity. Nat Rev Immunol 2012;12:687–95.
[43] Manel N, Hogstad B, Wang Y, Levy DE, Unutmaz D, Littman DR. A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. Nature 2010;467:214–7.
[44] Cao W, Bover L, Cho M, Wen X, Hanabuchi S, Bao M, et al. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. J Exp Med 2009;206:1603–14.
[45] Lederman MM, Offord RE, Hartley O. Microbicides and other topical strategies to prevent vaginal transmission of HIV. Nat Rev Immunol 2006;6:371–82.
[46] Kawamura T, Cohen SS, Borris DL, Aquilino EA, Glushakova S, Margolis LB, et al. Candidate microbicides block HIV-1 infection of human immature Langerhans cells within epithelial tissue explants. J Exp Med 2000;192:1491–500.
[47] Kawamura T, Gulden FO, Sugaya M, McNamara DT, Borris DL, Lederman MM, et al. R5HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. Proc Natl Acad Sci U S A 2003;100:8401–6.
[48] Kawamura T, Koyanagi Y, Nakamura Y, Ogawa Y, Yamashita A, Iwamoto T, et al. Significant virus replication in Langerhans cells following application of HIV to abraded skin: relevance to occupational transmission of HIV. J Immunol 2008;180:3297–304.
[49] Ogawa Y, Kawamura T, Kimura T, Ito M, Blauvelt A, Shimada S. Gram-positive bacteria enhance HIV-1 susceptibility in Langerhans cells, but not in dendritic cells, via Toll-like receptor activation. Blood 2009;113:5157–66.
[50] Aoki R, Kawamura T, Goshima F, Ogawa Y, Nakae S, Nakao A, et al. Mast cells play a key role in host defense against herpes simplex virus infection through TNF-alpha and IL-6 production. J Invest Dermatol 2013;133:2170–9.

Tatsuyoshi Kawamura graduated and received the M.D. degree from Yamanashi Medical University in 1990. He was at the Department of Dermatology, Yamanashi Medical University,Yamanashi, Japan, from 1990 to 1998 and received the Ph.D. degree in 1998. Between 1998 and 2002, he was a post doctoral fellow at the National Cancer Institute, Bethesda, Maryland. He won the Fellows Award for Research Excellence at the National Institute of Health in 2002. He has been an assistant professor in the Department of Dermatology, University of Yamanashi, Yamanashi, Japan, since 2002. His current interests include Langerhans cell immunity, HIV infection and skin cancer.

EFdA, a Reverse Transcriptase Inhibitor, Potently Blocks HIV-1 *Ex Vivo* Infection of Langerhans Cells within Epithelium

Journal of Investigative Dermatology (2014) **134,** 1158–1161; doi:10.1038/jid.2013.467; published online 2 January 2014

TO THE EDITOR

Despite increasing access to antiretroviral drugs, sexual transmission of HIV-1 remains a significant public health threat. A recent clinical trial, CAPRISA 004, of a vaginally administered microbicide using a nucleoside reverse transcriptase inhibitor (NRTI), tenofovir (TDF), has demonstrated that 1% TDF gel reduced HIV-1 acquisition by an estimated 39% overall (Abdool Karim *et al.*, 2010), indicating a potential utility of NRTI-based microbicides. In the VOICE study, however, a once-daily dosing regimen with TDF gel failed to demonstrate protective effects in at-risk women. These studies demonstrate the need to develop additional more potent microbicide candidates to potentially increase the activity to protect women from HIV-1 transmission.

We previously reported that a series of 4′-substituted NRTIs have excellent antiviral properties (Ohrui, 2006), and through optimization of such 4′-substituted NRTIs, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) was found to exert extremely potent activity against a wide spectrum of HIV-1 strains including highly multidrug-resistant clinical HIV-1 isolates, with favorable *in vitro* cell toxicities (Nakata *et al.*, 2007; Ohrui *et al.*, 2007). EFdA inhibited HIV-1 replication in activated peripheral blood mononuclear cells with an EC_{50} of 0.05 nM, a potency several orders of magnitude greater than any of the current clinically available NRTIs (Michailidis *et al.*, 2009). As the prevalence of new infections with drug-resistant HIV-1 variants could increase in the coming years (Nichols *et al.*, 2011), EFdA may be useful as a topical microbicide.

Langerhans cells (LCs) are dendritic cells located, among other sites, within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs in inner foreskin are primary target cells for HIV-1 infection, providing a plausible explanation for why circumcision greatly reduces the probability of acquiring HIV-1 (Ganor *et al.*, 2010; Zhou *et al.*, 2011). LCs also express CD4 and CCR5, but not CXCR4, and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells following contact with pathogens (Lederman *et al.*, 2006). Indeed, epidermal LCs are readily infected *ex vivo* with R5-HIV-1, but not with X4-HIV-1, and initiate and promote high levels of infection upon interactions with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013), consistent with previous epidemiologic observations that the majority of HIV-1 strains isolated from newly infected patients are R5-HIV-1 strains (Zhu *et al.*, 1993). Thus, LCs likely have an important role in disseminating HIV-1 soon after exposure to the virus.

To understand how HIV-1 traverses skin and genital mucosa, an *ex vivo* model was developed in which resident LCs within epithelial tissue explants obtained from suction blisters are exposed to HIV-1 and then allowed to emigrate from the tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV-1 infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). As expected, when epidermal tissue explants were pretreated with various concentrations of TDF, EFdA, and CCR5 inhibitor, maraviroc (MVC), prior to R5-tropic HIV-$1_{Ba\text{-}L}$ exposure, HIV-1 infection of resident LCs within epidermis as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells was decreased in a dose-dependent manner (Figure 1a and c; for detailed methods, see Supplementary Material). The blocking was confirmed by repeated experiments using skin explants from three additional randomly selected individuals (Figure 1b and d). Strikingly, although the blocking efficiency of TDF or MVC even at 5,000 nM was partial, EFdA demonstrated complete blocking of R5-HIV-1 replication in LCs as well as subsequent virus transmission from emigrated LCs to $CD4^+$ T cells at doses of 100–5,000 nM (Figure 1a–d). Furthermore, EFdA blocked *ex vivo* virus infection of LCs as well as subsequent virus transmission when two strains of R5-HIV-1, HIV-$1_{JR\text{-}FL}$ and HIV-1_{AD8}, were utilized in experiments ($n=3$, Supplementary Figure S1 online).

Similar to the results in epidermal LCs, preincubation of monocyte-derived LCs (mLCs) with 100–5,000 nM of EFdA completely blocked HIV-1 replication in mLCs as well as subsequent virus transmission from mLCs to cocultured $CD4^+$ T cells, whereas both TDF and MVC at the same doses only partially inhibited the transmission (Figure 2a and b; for detailed methods, see Supplementary Material online).

Abbreviations: EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LC, Langerhans cell; mLC, monocyte-derived LC; MVC, maraviroc; NRTI, nucleoside reverse transcriptase inhibitor; TDF, tenofovir

Accepted article preview online 11 November 2013; published online 2 January 2014