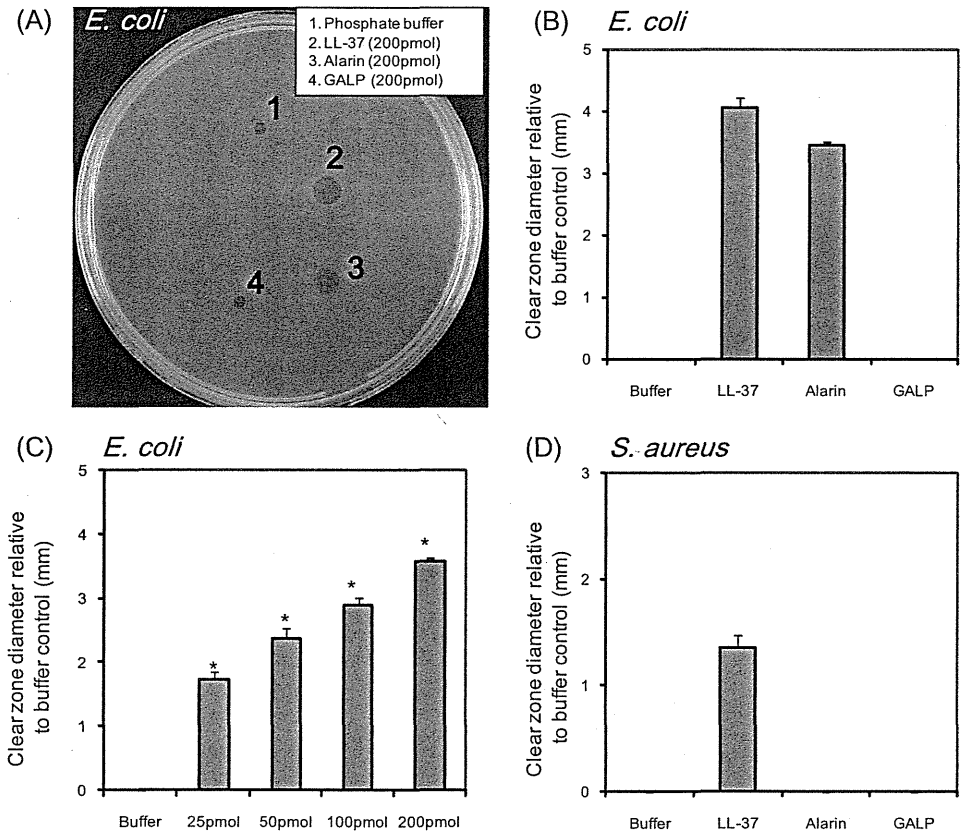

Fig. 1. Antimicrobial activities of LL-37, alarin and GALP against *E. coli* ATCC43827 (ML-35) and *S. aureus* ATCC25923 by radial diffusion assay. (A) Bacterial culture plate used for radial diffusion assay of LL-37, alarin and GALP. (B) Clear zone diameter (mm) depicting antimicrobial activity of LL-37, alarin and GALP at 200 pmol. (C) Dose-dependent antimicrobial activities of alarin against *E. coli* ATCC43827 (ML-35) and (D) dose-dependent antimicrobial activities of alarin against *S. aureus* ATCC25923. Zone diameter (mm) represents the antimicrobial activity of various concentrations of alarin relative to buffer control. An increase in the zone size caused by alarin is obtained by subtracting the diameter of the buffer control well (3 mm). Statistical significance is expressed as $^*p < 0.05$ (one-way ANOVA with Dunnett's Multiple Comparison Test) versus buffer control. Each bar represents the mean ± SD of data ($n = 4$).

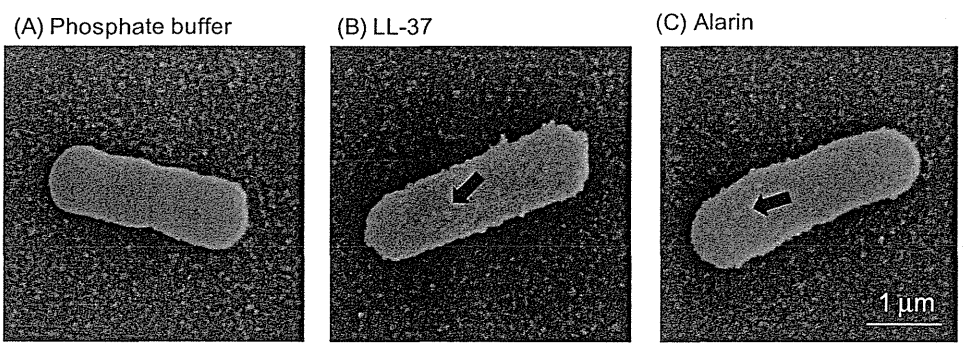

Fig. 2. Electron micrographs of *E. coli* ATCC43827 (ML-35) cells. (A) *E. coli* incubated with phosphate buffer showed normal surface of membrane; bacteria incubated with (B) 20 μM LL-37 and (C) 20 μM alarin showed blebs (black arrows) on the membranes. Scale bar, 1 μM.

1 h at 37 °C. LL-37 showed hemolytic activity to horse erythrocytes but not to alarin and GALP (Fig. 4).

4. Discussion

Alarin is differentiated from its alternative splicing form, galanin-like peptide (GALP) at the C-terminal region. Although alarin shares some similar functions with GALP, it is unknown if alarin has other more specific roles. Antimicrobial peptides are characterized as amphiphilic molecules and consist of basic amino acids. Because the C-terminal region of alarin contains some basic amino acids such as Arg and Lys, we postulated that alarin may have antimicrobial activity.

Results from the present study showed that alarin has antimicrobial activity but unlike the human cathelicidin, LL-37 which has a broader spectrum of antimicrobial activity, the antimicrobial activity of alarin is limited to the gram-negative bacteria, *E. coli*. This is not surprising as different susceptibilities of bacterial species against a particular peptide have been observed and this can be attributed to various reasons such as bacterial cell surface

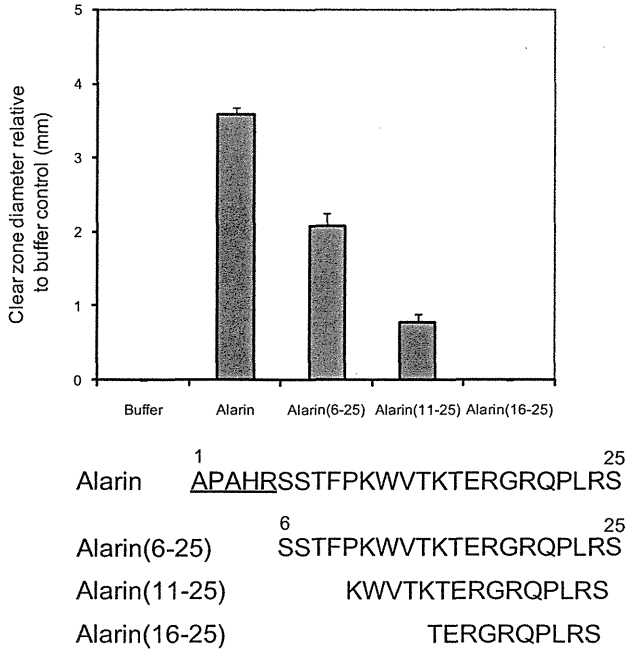

Fig. 3. Antimicrobial activities of alarin and its truncated peptides against *E. coli* ATCC43827 (ML-35) by radial diffusion assay. Clear zone diameter (mm) depicting antimicrobial activity of alarin and its truncated peptides at 200 pmol (upper panel) and amino acid sequences of the truncated peptides (lower panel). Conserved sequences of alarin and GALP are underlined.

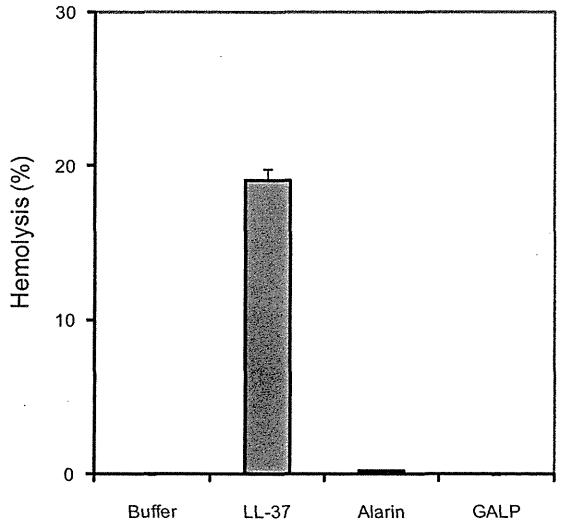

Fig. 4. Hemolytic activity of LL-37, alarin and GALP. Horse erythrocytes were incubated with each peptide (10 μM) for 1 h at 37 °C. Hemolytic activity is a measurement of the percentage of Tween 20-induced hemolysis. LL-37 showed hemolytic activity against horse erythrocytes but not alarin and GALP.

charge [24], genetic diversity [25], host ionic conditions [26] and peptide-to-lipid ratios [27]. The antimicrobial activity of alarin, however, is specific as alarin exhibited dose-dependent inhibition of the growth of *E. coli* and demonstrated potency equal to that of LL-37. We further showed by electron microscopy that alarin is capable of causing bacterial membrane blebbing, suggesting that membrane disruption could be one of its killing mechanisms. An effective antimicrobial peptide should be cytolytic and cell-penetrating but not hemolytic [28]. In general, hydrophobic interactions with eukaryotic cell membranes increase hemolytic activity [29]. Our results showed that alarin does not induce hemolysis of erythrocytes, suggesting that its hydrophobic interactions with the erythrocyte membranes are minimal. Hence, alarin can fulfill the desirable features of an effective antimicrobial peptide.

More interestingly, GALP, an alternative splicing form of alarin demonstrated no antimicrobial activity. However, deletion of the conserved amino acid sequence (APAHR) at the N-terminal region of alarin reduced its antimicrobial activity suggesting that APAHR remain essential for its antimicrobial activity. The C-terminal original sequence of alarin (6–25), which contains some basic amino acids are important for the antimicrobial activity of alarin as successive deletions reduced and eventually abrogated the antimicrobial activity of alarin. These results, hence, suggest that even though the C-terminal region of alarin confers its antimicrobial activity, the conserved N-terminal of alarin and GALP remains crucial for its maximum antimicrobial activity.

LL-37, defensins and histatins are some of the well-studied antimicrobial peptides which contain 37, 29–42 and ~32 amino acids, respectively [30]. With the discovery of its antimicrobial activity, alarin with a length of 25 amino acids is now one of the shortest antimicrobial peptides known. Results from this report show that alarin has antimicrobial potentials and should be considered for the development as a human therapeutic.

Acknowledgments

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Short Communication

Double Oral Administration of Emtricitabine/Tenofovir Prior to Virus Exposure Protects against Highly Pathogenic Simian/Human Immunodeficiency Virus Infection in Macaques

Tadashi Nakasone[1]*, Tsutomu Murakami[1], and Naoki Yamamoto[1,2]

[1]*AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan; and*
[2]*Department of Microbiology, National University of Singapore, Singapore 117597, Singapore*

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SUMMARY: In the absence of any effective vaccine against human immunodeficiency virus (HIV), current anti-retroviral drugs may be suitable for pre-exposure prophylaxis (PrEP). Previous large clinical trials showed that PrEP reduced HIV infection in high-risk populations. Emtricitabine/tenofovir (FTC/TDF) may be a suitable agent for PrEP. FTC/TDF PrEP efficacy was evaluated using a highly pathogenic simian/human immunodeficiency virus (SHIV) in a non-human primate model of AIDS, the SHIV-KS661c/cynomolgus monkey model. Double oral administration of FTC/TDF (20/30 mg/kg), at 24 h and a few minutes prior to exposure, completely protected 2/3 monkeys from infection. Interestingly, a single oral administration 2 weeks before viral exposure moderately rescued CD4 cells, although the data did not reach statistical significance. These results are consistent with previous primate studies and with recent clinical data.

UNAIDS estimated that 33.3 million people were living with human immunodeficiency virus (HIV) at the end of 2009 (1). The development of an effective vaccine against HIV is urgently needed and is critical to stop the spread of HIV. Although the STEP study was a disappointment in 2007 (2–4), the ALVAC/AIDSVAX study conducted in Thailand was more encouraging (5); however, efficacy remains uncertain and it may well be several more years before an effective HIV vaccine is available in the clnic.

Fortunately, the number of new HIV infections has decreased each year since 1996, and HIV prevalence among young people has also declined in many countries (1). Many interventions, such as safe sex education, condom use, risk reduction through the use of anti-retroviral drugs, and male circumcision, have contributed to this decline. Pre-exposure prophylaxis (PrEP) with anti-retroviral drugs is also considered a possible option to prevent infection with HIV (6–9). Recent preclinical and clinical studies demonstrated that PrEP significantly reduces virus infection and suggested that emtricitabine/tenofovir (FTC/TDF) may be a suitable pre-exposure prophylactic agent (10–16).

Many FTC/TDF preclinical studies have been conducted in the context of PrEP in simian immunodeficiency virus (SIV)/monkey models and pathogenic CCR5-tropic simian/human immunodeficiency virus (SHIV)/monkey models (10–13). The present study evaluates the PrEP efficacy of FTC/TDF in a highly pathogenic and "CXCR4-tropic" SHIV/non-human primate (NHP) model of AIDS.

The monkey experiments were conducted at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBIO), Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization. The animals were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science (1987) under the Japanese Law Concerning the Protection and Management of Animals and were maintained in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of NIBIO, Japan. The IACUC of NIBIO and that of the National Institute of Infectious Diseases (NIID) of Japan approved the study. Both guidelines are in accordance with the recommendations of the Weatherall report "The use of non-human primates in research". The use of SHIV was also approved by the Institutional Advisory Committees for the Biosafety of Living Modified Organisms of NIBIO and NIID with the approval (Dai17-17) of the Japanese Minister of Education, Culture, Sports, Science and Technology (2005). Ethical standards incorporated into these guidelines and into our routine laboratory procedures included a maximum reduction in the number of animals, a psychological enrichment program, frequent contact with other animals (visual, auditory, and olfactory), and regular veterinary supervision and care.

Truvada® tablets (FTC/TDF) were purchased from Japan Tobacco Inc. (Tokyo, Japan). The tablets were ground and suspended in water immediately before use. Both FTC and TDF are nucleoside analogue reverse transcriptase inhibitors.

SHIV-KS661c, molecularly cloned from SHIV-C2/1, was used. The SHIV-C2/1 stock comprised plasma obtained by serum passages of p-SHIV (derived from

*Corresponding author: Mailing address: AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2737, Fax: +81-3-5285-1150, E-mail: nakabone@nih.go.jp

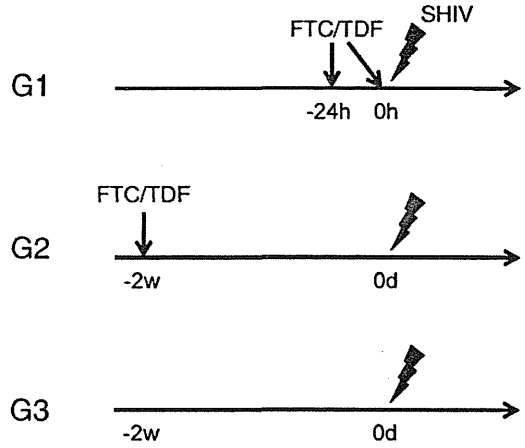

Fig. 1. Study design. Nine cynomolgus monkeys were divided into 3 groups (G1–G3), each comprising 3 animals. G1, double oral administration of FTC/TDF (20/30 mg/kg) 24 h and a few minutes before viral challenge; G2, single oral administration of FTC/TDF (20/30 mg/kg) 2 weeks before viral challenge; G3, naïve control monkeys. The drug was administered intragastrically via a nasal feeding tube under anesthesia. All animals were challenged intra-rectally with 10 times the AID_{50} of SHIV-KS661c and were monitored for more than 12 weeks.

SHIV-89.6) in cynomolgus monkeys (17–19). SHIV-KS661c was propagated in CEMx174 cells and was confirmed to be genetically identical to the major sequences of the parent virus. SHIV-KS661c can infect cynomolgus monkeys both intravenously and intra-rectally. The virus induces precipitous viremia and drastic CD4 cell depletion within 2 weeks of inoculation (19–26). SHIV-KS661c stocks were kept at −80°C and were thawed immediately prior to use.

The animal study design is shown in Fig. 1. Nine cynomolgus monkeys were enrolled and divided into 3 groups, each containing 3 animals. Group 1 was treated with a double dose of FTC/TDF (20/30 mg/kg) 24 h and a few minutes prior to viral exposure; group 2 was given a single dose of FTC/TDF (20/30 mg/kg) 2 weeks before viral exposure; and group 3 (naïve control group) was not treated with any drug. The study was divided into 2 research reports including the current manuscript and the previously published study (27); group 3 was the same in both papers. The drug was formulated in 3 ml of water and was administered intragastrically via a nasal feeding tube under anesthesia. Administration of the drug was followed by the administration of an additional 3 ml of water to wash out any compound remaining in the tube.

All monkeys were then challenged intra-rectally with 10 times the AID_{50} (50% animal infectious dose) of the highly pathogenic SHIV-KS661c. The general condition of the animals, including appetite, activity, and body weight, was carefully observed. Blood chemistry, complete blood cell counts, absolute CD4 cell counts, and plasma virus RNA copy number were measured frequently for over 12 weeks. Finally, the monkeys were sacrificed for virological analysis and analysis of the CD4 population in lymphoid tissues (LTs).

Plasma viral loads were evaluated using real-time reverse transcriptase polymerase chain reaction (RT-PCR) with a TaqMan probe as reported previously (19–23). Briefly, viral RNA was extracted from the plasma and purified using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA). For quantitative analysis, TaqMan technology (Applied Biosystems, Foster City, Calif., USA) was used with primers and probes targeting the SIVmac239 *gag* region. Viral RNA was amplified using a QuantiFast Probe RT-PCR Vial Kit (Qiagen) with TaqMan primers and probes. The fluorescence intensity of the RT-PCR product was monitored quantitatively using an Opticon 2 instrument (formerly MJ Research; Bio-Rad, Hercules, Calif., USA). The plasma viral load, measured in duplicate samples, was assessed using a standard curve prepared using control RNA. To assess the RNA recovery rate, 10^5 copies of SHIV-KS661c were extracted and purified using the same kit in parallel with the experimental samples. The recovered RNA was also amplified in parallel with the RNA recovered from the experimental samples. The limit of detection was approximately 500 RNA copies/ml.

The absolute CD4 cell count in peripheral blood (PB) was measured as described previously (19–23). Briefly, 50 μl of whole blood was incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource, Camarillo, Calif., USA), phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton Dickinson, Franklin Lakes, N.J., USA), or peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton Dickinson). After erythrocyte lysis using FACS lysis solution (Becton Dickinson), the cells were analyzed with reference beads (Beckman Coulter, Fullerton, Calif., USA) in a FACSCalibur cytometer (Becton Dickinson) using Cell Quest software (Becton Dickinson). The lymphoid cells used for flow cytometric analysis were prepared from thymus, spleen, and lymph node (LN) tissues obtained at necropsy. The cells were stained with the same 3 antibodies described above, and the CD4 population was measured.

The double administration of FTC/TDF (20/30 mg/kg), approximately 24 h and a few minutes before viral exposure, was protective in 2/3 monkeys (Fig. 2, G1). The 2 monkeys protected showed no evidence of CD4 cell depletion during the course of the experiment. We confirmed that anti-HIV-1 and anti-HIV-2 antibodies were not induced in these 2 monkeys (data not shown) and therefore concluded that the animals were completely protected from infection. The unprotected monkey in the same group showed moderate CD4 cell depletion and a moderate peak and set-point viral load in blood. A single administration of FTC/TDF (20/30 mg/kg) approximately 2 weeks before viral exposure failed to protect all 3 monkeys in group 2 (Fig. 2, G2); however, 1 monkey showed moderate CD4 cell depletion (>100 cells/μl) and lower peak viremia following an undetectable set-point viremia. In group 3 (naïve control; Fig. 2, G3), 2 monkeys showed CD4 cell depletion and high peak viremia that were accompanied by moderate to high set-point viremia. One of 2 monkeys with severe CD4 cell depletion and high viremia was euthanized due to AIDS. Unexpectedly, 1 monkey in group 3 showed moderate CD4 cell depletion (<300 cells/μl) and low set-point viremia (10^3–10^4 copies/ml). No abnormal findings were observed in any other monkey during the course of the experiment.

After over 12 weeks of observation, all animals were

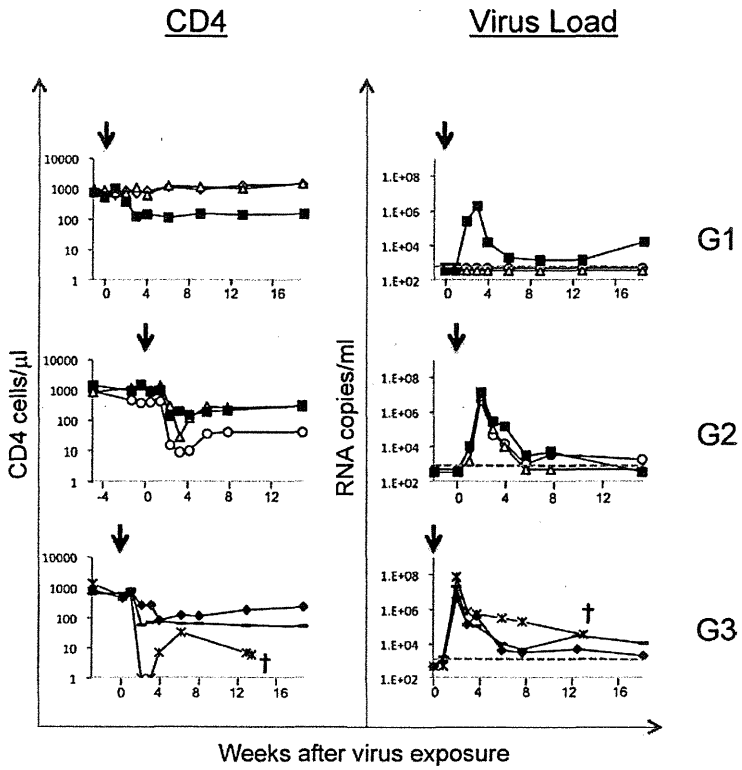

Fig. 2. Effect of PrEP on CD4 cells and viral load in peripheral blood (PB). Absolute CD4 cell counts (cells/μl) and virus RNA copy numbers (copies/ml) are shown for PB and plasma, respectively, over the course of the study. Arrows indicate intra-rectal viral challenges. Dash indicates undetectable levels (< 500 copies/ml) in this system. One naïve control monkey (cross in G3) showing severe CD4 cell depletion and high set-point viremia was euthanized due to AIDS.

sacrificed and the CD4 cell population in LTs was analyzed. Macroscopic findings generally suggested that thymus and LNs were atrophic in monkeys showing severe CD4 lymphocytopenia (< 100 cells/μl) at necropsy; however, no atrophic tissues were found in monkeys not presenting with severe CD4 lymphocytopenia.

The CD4 cell population in LTs was analyzed by flow cytometry and expressed as the CD4 cell/CD3 cell ratio (%) (Fig. 3). In group 1, CD4 cells were conserved in the LTs of 2 monkeys (>24%) but showed moderate depletion in the PB of the third monkey. The total CD4/CD3 ratio in group 1, including PB, was significantly higher ($P < 0.01$) than that of group 3. In group 2, the PB CD4 cells of 1 monkey showed no depletion and were well conserved in LTs (>30%). Interestingly, 2 monkeys with moderate PB CD4 cell depletion had normal thymus CD4 counts (30% and 31%) despite depletion in spleen and LNs (2–11%). Even though no significant difference was found between groups 2 and 3 ($P > 0.05$), the total number of CD4 cells in group 2 appeared greater than in group 3. In contrast, 2 monkeys in group 3 showed severe CD4 cell depletion in all LTs (0–8%), although the CD4 cell counts of another monkey were conserved in all LTs (15–48%).

In this study, the PrEP efficacy of FTC/TDF was evaluated using a highly pathogenic SHIV in a NHP model of AIDS. The study revealed that the double oral administration of FTC/TDF (20/30 mg/kg), 24 h and a few minutes before viral challenge, completely protected 2/3 monkeys from infection. Although no significant difference between groups 2 and 3 could be detected, the total number of CD4 cells in group 2 appeared greater than in group 3. Thus, we could not conclude that the single administration of FTC/TDF 2 weeks before virus challenge had no preventive effect. The median intracellular half-life of TDF was estimated to be approximately 150 to 180 h, and TDF was still detectable in some patients 14 and 28 days after the administration of the last dose (28,29). The long half-life of TDF might account for the results seen in group 2.

Our results raise one question: is the NHP AIDS model used in the study appropriate for PrEP evaluation? Appropriate animal models provide important tools for obtaining insights into disease prevention and treatment. The SHIV-KS661c/cynomolgus monkey model was developed as a NHP AIDS model to evaluate anti-HIV vaccine candidates and anti-HIV microbicides (17–20). The model has been used to evaluate several anti-HIV candidate drugs such as mycobacterium-based vectors, vaccinia-based vectors, DNA-based vectors, adenovirus-based vectors, and monoclonal antibodies (21–23). SHIV-KS661c is a molecular clone virus derived from SHIV-89.6. Although SHIV-KS661c uses both human CXCR4 and human CCR5 as co-receptors, it is predominantly a CXCR4-tropic virus (18). Upon viral infection of monkeys, high-peak viremia, moderate set-point viremia, and severe CD4 cell depletion occur within several weeks (24–26). On the contrary, $SHIV_{SF162p3}$ used by Garcia-Lerma et al. is CCR5-tropic

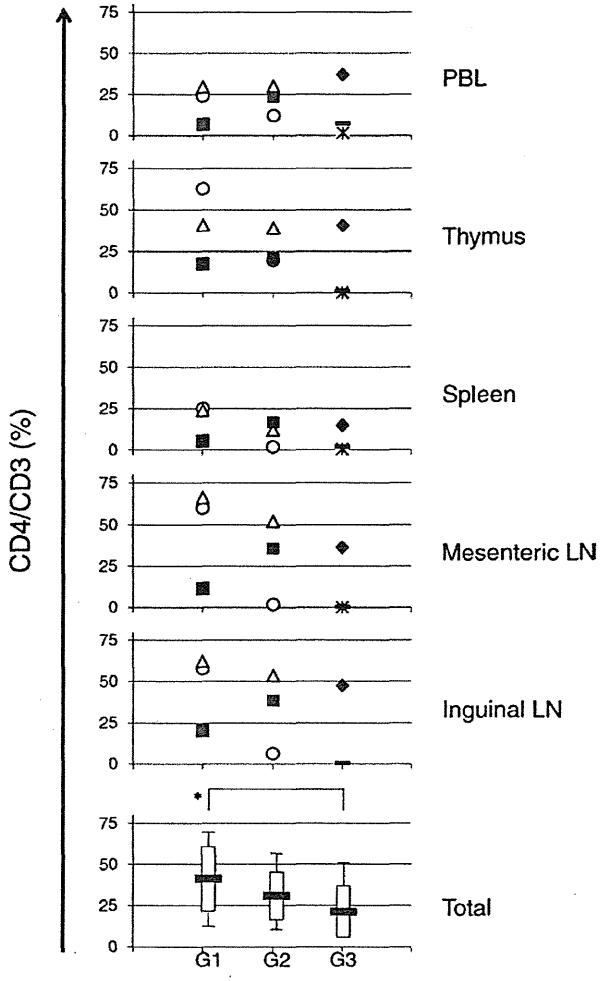

Fig. 3. Effect of PrEP on lymphoid tissues (LTs). The CD4 cell populations in LTs at necropsy are shown as the CD4 cell/CD3 cell ratio (%). The CD4/CD3 ratios in PB at necropsy are also shown. The total CD4/CD3 ratios assembled from 5 compartments and analyzed using a one-sided Student's t test are shown at the bottom. Narrow bars indicate ranges from minimum to maximum. Boxes indicate standard deviation. Bold bars indicate mean values. $^*P < 0.01$. PBL, peripheral blood lymphocytes; LN, lymph node.

and does not cause such a rapid CD4 cell depletion (10–13). Therefore, we suggest that both SHIV-KS661c and SHIV-89.6 are highly pathogenic SHIVs. CCR5-tropic HIV populations are generally present throughout the entire course of infection and are predominant during the acute and asymptomatic stages (30); however, CXCR4-tropic and dual-tropic populations emerge during the chronic stage or end stage, which are usually characterized by CD4 cell depletion (31). The frequency of this emergence was recently reported to be low (15%) in individuals infected with HIV subtype C but to be relatively high (60–77%) for other HIV subtypes (32). Transmission of CXCR4-tropic HIV has been reported in some cases (33), although the transmission of CCR5-tropic HIV is the most prominent. Furthermore, there are concerns that the incidence of CXCR4-tropic HIV has increased in several countries (34,35). The introduction and distribution of CCR5 antagonists for HIV treatment may help the emergence of CXCR4-tropic HIV (36–38). These findings support the idea that blocking both CCR5 and CXCR4 is needed to prevent HIV transmission. Thus, the SHIV-KS661c/cynomolgus monkey is a suitable model for the evaluation of PrEP, particularly because of its CXCR4 tropism.

In this study, single high-dose intrarectal (IR) viral challenge was chosen, although multiple low-dose intravaginal (IVAG) viral challenges would be more representative of HIV infection. A study design involving multiple low-dose viral challenges would have required a larger number of animals per group compared to a study design involving single high-dose inoculations. The majority of HIV infections occur though the vagina, while some are acquired via the rectum or orally. Therefore, IVAG challenge may be a more suitable study design than IR challenge; however, IVAG infectivity depends upon the menstruation cycle (39–41). Regulation of the menstrual cycle so that all study subjects menstruate at the same time is difficult to achieve. Moreover, the narrower and smaller vaginal cavity of cynomolgus monkeys compared to that of rhesus monkeys made it difficult to choose IVAG inoculation. For these reasons, a single high-dose IR challenge was chosen in the present study.

Clinical practice with anti-retroviral therapy clearly suggests that a single drug is not enough to control HIV replication. Recently, Karim et al. reported that a single drug such as TDF gel succeeded in protecting women from vaginal HIV infection (13,42). This is very encouraging; however, the PrEP efficacy of the TDF gel is not very high. A combination of anti-HIV drugs should be used for PrEP and called highly active anti-retroviral prevention or PrEP plus anti-retroviral therapy. Recent mathematical modeling suggested the latter would lower circulating HIV drug resistance (43).

In conclusion, this is the first report demonstrating that FTC/TDF prevents infection with a highly pathogenic and CXCR4-tropic SHIV in a preclinical monkey experimental model. These results support the recent clinical PrEP efficacy of FTC/TDF.

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Conflict of interest None to declare.

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Tadashi Nakasone, Sei Kumakura, Michiko Yamamoto, Tsutomu Murakami & Naoki Yamamoto

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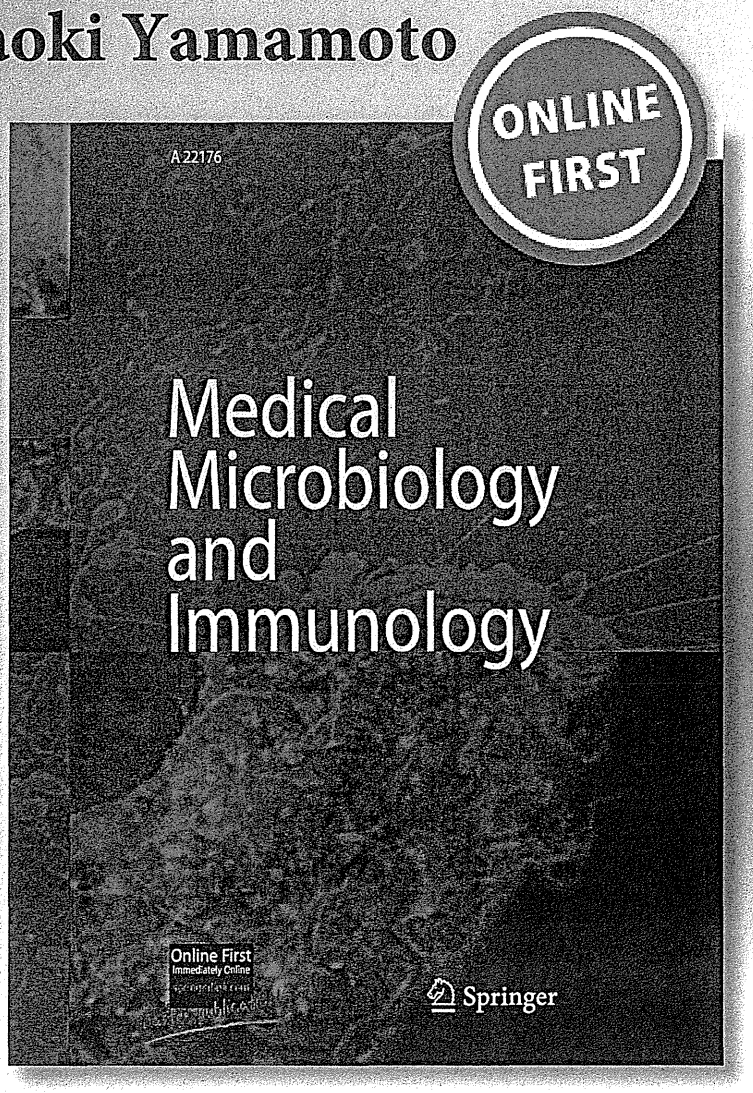

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