Epigallocatechin-3-gallate local pre-exposure application prevents SHIV rectal infection of macaques

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Abstract

Epigallocatechin-3-gallate (EGCG), a natural and major ingredient of green tea, has been shown to have anti-inflammation and anti-HIV-1 properties. We demonstrated that the intrarectal administration of EGCG could protect rhesus macaques from repetitive, intrarectal challenges with low-dose $SHIV_{SF162P3N}$. This protection has a per-exposure risk reduction of 91.5% (P=0.0009; log-rank test) and a complete protection of 87.5% (P<0.001; Fisher's exact test). All protected animals showed no evidence of systemic and mucosal SHIV infection as demonstrated by the absence of viral RNA, DNA and antibodies. In contrast, all controls became infected after repeated SHIV challenges (a median of 2.5 times, range of 1-8 times). Mechanistically, EGCG could block the binding of HIV-1 gp120 to CD4 receptor and suppress the macrophage infiltration/activation in the rectal mucosa of macaques. These data support further clinical evaluation and development of EGCG as a novel, safe and cost-effective microbicide for preventing sexual transmission of HIV-1.

Introduction

In the absence of a protective vaccine or a cure for HIV-1 infection, intervention with highly potent and cost-effective inhibitors that target at the initial sites of HIV-1 infection is an ideal

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strategy for slowing the AIDS pandemic. Currently, HIV-1 infection spreads predominantly through sexual transmission, in which the virus gains access to a new host through the penile, rectal or vaginal mucosal tissues. During sexual intercourse, HIV-1-infected men transmit the virus through their semen, which carries free-floating virus as well as the infected cells.[1] Semen contains amyloid fibrils that potently enhance HIV-1 or SIV/SHIV infectivity *in vitro*.[2-4] Therefore, to inhibit HIV-1 during sexual intercourse represents the most effective way in preventing transmission of the virus.

Microbicides targeting the early events in sexual transmission of HIV-1 have been extensively studied.[5-8] Several recent studies reported that pre-exposure prophylaxis with the antiretrovirals (ARVs) was effective in the prevention of SHIV transmission in non-human primate (NHP) model.[9, 10] These ARVs-containing microbicides were usually used in the formulation of topical gels.[7] However, the topical gel microbicides are associated with rectal mucosal damage, manifested as histological abnormality and induction of proinflammatory response.[11] In addition, ARVs-based microbicides are not effective on resistant HIV-1 strains and can induce viral resistance. Furthermore, semen can impair the antiviral efficacy of ARVs that act on intracellular targets at different steps of viral replication cycle.[4] As compared with ARVs or other chemical drugs, the use of natural products for prevention of HIV-1 mucosal transmission might have some advantages, as they have low toxicity, minor side-effects, and are cost-effective.

The consumption of green tea has many physiological and pharmacological health benefits. Mounting evidence has shown that epigallocatechin-3-gallate (EGCG), a natural and major ingredient of green tea, not only has anti-inflammatory and anti-oxidative effects, but also possesses antiviral abilities against diverse families of viruses, including HIV-1.[12] *In vitro* studies have demonstrated that EGCG at a physiologic concentration could inhibit HIV-1 infection/replication in peripheral blood mononuclear cells (PBMCs) and macrophages.[13] In addition, EGCG could remodel seminal amyloid fibrils [14] and effectively suppress semen-mediated enhancement of HIV-1 replication.[15] EGCG can interfere with several aspects of the HIV-1 life cycle, including the destruction of virions,[16, 17] the inhibition of reverse transcriptase and integrase activities of the virus.[18, 19] Most conclusively, several independent studies have confirmed that EGCG can bind to the same molecular pocket on CD4 receptor as does HIV-1 gp120, thus preventing the initial attachment of the viruses to CD4+ T cells.[20-22] We recently reported that EGCG could also inhibit macaque SEVI (semen-derived enhancer of virus infection)-mediated enhancement of SIV/SHIV infection and degrade the formation of macaque semen-derived amyloid fibrils.[23] A most recent clinical study showed that EGCG is a well-tolerated and non-toxic therapeutic agent that can upregulate protective CC chemokines against HIV-1. [24] Taken together, these observations strongly argue for further studies, particularly *in vivo* investigations on the anti-HIV-1 effect of EGCG as an entry inhibitor. In the present study, we evaluated whether mucosal pre-exposure application of EGCG can prevent SHIV rectal infection of rhesus macaques.

Results

EGCG inhibits HIV/SIV/SHIV *in vitro*

The major active ingredients of green tea are polyphenolic compounds, known as catechins, which include EGCG, EGC, ECG, and EC. EGCG accounts for 50% of the total green tea catechins. We first examined whether EGCG and other green tea catechins have the ability to inhibit infections with different strains of HIV-1, SHIV and SIV in TZM-bl cells. Among the green tea catechins, EGCG was the most potent inhibitor of the viruses (Figure 1a). In addition, EGCG dose-dependently inhibited HIV-1 Bal replication in primary human macrophages (Figure 1b). Furthermore, EGCG could inhibit SHIV infection of primary lymphocytes and macrophages of macaques (Figure 1c). Evaluation of cytotoxicity revealed that the treatment of TZM-bl and PBMCs of macaques with the catechins for as long as 48 h at the dose as high as 100 μM had little toxic effect (Supplementary Figures 1 and 2).

EGCG has little toxicity on rectal mucosa

To determine the *in vivo* protective effect of EGCG on SHIV rectal infection of macaques, we first assessed the toxicity of EGCG on rectal mucosa of macaques and mice. We demonstrated that the rectal application of 5 mM EGCG for 10 min had little toxic effect on the rectal mucosa of macaques, as shown by the normal microstructures of epithelium, lamina propria, and intestinal glands in the rectum mucosa and submucosa (Supplementary Figure 3a) and lack of fecal blood (Supplementary Table 1). We also showed that EGCG had little effect on the expression of inflammatory cytokines and toxicity on the rectal mucosa of mice which is evidenced by normal intestinal microstructures, stool consistency, and lack of fecal blood (Supplementary Table 2 and Figure 3).

EGCG protects macaques from rectal SHIV infection

We next examined the protective effect of EGCG on repetitive and intra-rectal challenges with low-dose $SHIV_{SF162P3N}$ in macaques (Figure 2a). The assessment of the plasma SHIV RNA over the course of the study showed that all animals (8 out of 8) in the control group were infected, which required a median of 2.5 times of challenges (range from 1 to 8 times, Figure 2b). In contrast, only 1 out of 8 animals in the EGCG group became infected (Figure 2c). Evaluation of SHIV RNA and proviral DNA in PBMCs and the biopsied specimens (rectum and inguinal lymph nodes, LNs) at week 20 postinfection revealed infection in control animals, while no evidence of infection in 7 out of 8 EGCG-treated animals (Supplementary Figure 4).

Because gut-associated lymphoid tissue (GALT) is an important target organ of HIV-1 and may serve as a key viral reservoir, we also examined SHIV proviral DNA and RNA in the gastrointestinal tract (GI) tissues, lymph nodes (LNs) and intraepithelial lymphocytes (IELs) of study animals necropsied at week 36 postinfection. As shown in Figure 3 and Supplementary Table 3, SHIV RNA and proviral DNA were detected in all analyzed tissues (GI tissues, LNs and IELs) from control animals. In contrast, only one animal (WRE08) in EGCG group had detectable SHIV RNA and DNA in the GI tissues, LNs and IELs. In addition to the plasma viral loads, we also examined plasma antibody against SHIV in the study animals. Consistent with the viral load data, while all control animals were positive for

SHIV-specific antibody, only one animal (WRE08) in EGCG group had detectable SHIV antibody (Supplementary Table 3). This animal had the highest CD69 and HLA-DR expression on CD4+ and CD8+ T cells in the first two weeks post-infection (Supplementary Figure 5). In addition, there was a significant decrease of CD4+ T cell numbers in blood of this animal at week 3 after SHIV infection (Supplementary Figure 6).

Statistically, the EGCG treatment afforded a protective efficacy of 91.5% [1-(hazard ratio), P=0.0009, log-rank test] reduction in the per-exposure acquisition risk as compared with control (Figure 4). The complete protection for macaques in the EGCG group was 87.5% (P=0.001, Fisher's exact test).

Mechanisms of EGCG-mediated *in vitro* and *in vivo* anti-HIV activities

We first studied the mechanisms of the *in vitro* findings that the pre-treatment of the target cells (TZM-bl, primary macaque lymphocytes and macrophages) with EGCG potently inhibited HIV/SHIV/SIV infection. As shown in Figure 5a, b, EGCG pretreatment of T cells could block the binding of CD4 antibody to CD4 receptor. We also observed that the incubation of CD4$^+$ T cells with EGCG blocked the binding of FITC-conjugated HIV-1 gp120 to the cells (Figure 5c). To determine *in vivo* mechanism of EGCG-mediated protection of macaques from SHIV rectal infection, we examined the effect of EGCG on the infiltration and activation of T cells and macrophages in rectal mucosa (Figure 6a). EGCG rectal administration significantly reduced the infiltration of CD68$^+$ macrophages (P<0.01), but not CD3$^+$ T cells in rectal mucosa (Figure 6b, c). In addition, EGCG pretreatment suppressed the local immune activation as shown by the diminished expression of CD163$^+$ cells (P<0.05) and HLA-DR$^+$ cells (P<0.001) in rectal mucosa (Figure 6d, e).

Discussion

Worldwide, sexual intercourse is currently the predominant mode of HIV-1 transmission, in which unprotected anal sex is riskier than unprotected vaginal sex.[25] The rectum is a particularly susceptible entry site for HIV-1 or SHIV infection, as it is composed of only a single layer of columnar epithelium. In addition, the rectal mucosa contains numerous CD4-bearing target cells (CD4$^+$ T cells, macrophages and DCs).[26] The risk of HIV transmission during anal intercourse is estimated to be around 18 times greater than that in vaginal intercourse, according to the results of a meta-analysis.[27] Therefore, the interventions to target the rectal site are critical in preventing sexual transmission of HIV-1. In this study, we demonstrate for the first time that EGCG can protect 7 out of 8 macaques from repeated intra-rectal challenges with SHIV, resulting in a per-exposure risk reduction of 91.5% (P=0.0009 by the log-rank test) and a complete protection of 87.5% (P<0.001 by Fisher's exact test). All protected animals showed no evidence of systemic and mucosal SHIV infection as evidenced by the absence of viral RNA, DNA and antibodies. Interestingly, EGCG failed to protect one animal from SHIV infection. This animal appeared to be highly susceptible to SHIV challenge, as it became infected after only one-time of exposure to the virus. In contrast, all control animals except one required 2 or more repeated SHIV challenges (Figure 2b and 2c). In examining the immune activation markers (CD69 and HLA-DR) on CD4+ and CD8+ T cells of all infected animals, we found that this

unprotected animal had the highest CD69 expression on CD4+ and CD8+ T cells among all infected monkeys in the first two weeks post-infection (Supplementary Figure 5).

EGCG has been reported to have *in vitro* antiviral effect on different subtypes, strains and clinical isolates of HIV-1 in primary human CD4^{+} T cells and macrophages.[13] We showed that EGCG had broad inhibitory effect on different strains of HIV-1, SIV and SHIV in TZM-bl cells, macaque lymphocytes and macrophages (Figure 1). Studies from different laboratories have demonstrated that EGCG exhibits antiviral activity at multiple steps of HIV-1 infection/replication cycle, including the inhibition of HIV-1 reverse transcriptase,[18] protease,[16] and proviral genome integration.[19] Importantly, EGCG-mediated HIV-1 inhibition appears to be its interference with HIV-1 entry into the target cells. EGCG can directly disassemble HIV-1 virion,[16, 17] block HIV-1 gp120 binding to CD4 molecule on T cells,[20, 22, 28] and degrade the seminal amyloid fibrils (a semen-derived enhancer of virus infection, SEVI) that reportedly enhances HIV-1 infection/transmission.[2, 4, 14, 29] We found that EGCG specifically reduced the surface expression of CD4 receptor, but not CD3 receptor on human T cells (Figure 5a), suggesting that EGCG blocks the binding of CD4 antibody to CD4 receptor (Figure 5b). Also we showed that EGCG could block the binding of FITC-conjugated HIV-1 gp120 to CD4 receptor (Figure 5c). More importantly, we demonstrated that *in vivo* application of EGCG could suppress the macrophage infiltration/activation in the rectal mucosa of macaques. The protective effect of EGCG was also observed in a recent human study, showing that EGCG oral administration upregulated the CC chemokines (MIP-1α/β and RANTES) against HIV-1. [24] These combined *in vitro* and *in vivo* observations provides a possible mechanism for EGCG-mediated rectal protection from SHIV infection.

These combined anti-HIV-1 actions of EGCG should reduce the risk of developing emergence of resistant viruses during the course of treatment. Furthermore, EGCG may confer an additional advantage over ARVs as it can counteract semen-mediated enhancement of HIV-1 infection. [23, 29] We recently reported that EGCG counteracted macaque SEVI-mediated enhancement of SIV/SHIV infection of macaque PBMCs.[23] This finding is clinically relevant and important, as it has been reported that the anti-HIV-1 activity of a variety of potential microbicides could be compromised by the presence of semen or SEVI amyloid fibrils.[4] Therefore, in order to identify the best candidates for the prevention of HIV-1 sexual transmission, it is necessary to evaluate the anti-HIV-1 efficacy of the ARVs-formulated microbicides in the presence of semen.[30-32]

EGCG is generally considered a natural product with minimal toxicity. Clinically, EGCG has been found to be safe and well tolerated by the study subjects and the reported adverse events were rated as mild events.[33, 34] EGCG administration is safe in healthy individuals, at oral dose of 800 mg per day over a time period of 4 weeks, which is equivalent to about 8-16 cups of green tea a day.[35] Ullman et al reported that purified EGCG at the doses up to 1600 mg per day were generally well-tolerated and safe to use clinically.[36] Oral application of EGCG in mouthwash formulation at the dose of 35-87 mM for 2 min daily for 7 days had little systemic side effects.[37] A recent phase I clinical trial showed that the drug formulation of EGCG, Polyphenon E, is a well-tolerated, non-toxic therapeutic agent with no significant adverse effects at up to 1600 mg per day for 14 days.[24] Our evaluation of cytotoxicity in the

cell cultures showed that EGCG at doses up to 50 μM had little cytotoxicity to TZM-bl cells and the primary macaque PBMCs. It was reported that the viability of HIV-infected human lymphocytes and macrophages was not significantly inhibited by EGCG over a range of 1–100 μM.[13] As compared to the concentrations of EGCG used in the cell cultures, higher dose (5 mM) of EGCG was administrated in the animal experiments. The rationale for use of this EGCG dose was based on the consideration that the macaque rectum contains multiple factors (feces, mucus, a number of microbial flora and metabolites) that could dilute and interfere with EGCG, reducing its antiviral activity. Nevertheless, we showed that 5 mM EGCG had little toxicity and inflammatory effects on rectal mucosa of macaques and mice, as evidenced by the normal rectal epithelium structure and lack of inflammatory cytokine induction after the EGCG application (Supplementary Figure 3, Tables 1 and 2). The dosage of the FDA approved green tea extract Polyphenon E ointment (Veregen) for topical treatment of genital warts is about 100~300 mM.[38, 39]

Because sexual intercourse is a primary route of HIV-1 transmission worldwide and vaginal mucosal surface is a major site of the heterosexual transmission, future studies to determine the protective effect of EGCG on SHIV vaginal infection are also necessary. By extrapolating, it is likely that the same benefits of EGCG shown in the SHIV rectal infection model might apply to the prevention of vaginal SHIV infection. This supposition, however, will need to be confirmed experimentally given the differences between the rectal and vaginal transmission of HIV-1. [26] The fact that EGCG is very stable in acidic solution,[41] a condition similar to vaginal environment (pH range of 3.8 to 4.5), supports the future studies on use of EGCG for prevention of intravaginal HIV-1 transmission. Importantly, it is necessary to develop EGCG formulations that have stable, effective and long-lasting action on inactivation of HIV-1 in vaginal lumen and rectum. For example, EGCG in a buffered gel or film should be able to maintain an acidic pH in the presence of semen.

Together with the known low toxicity, anti-inflammation and anti-viral properties of EGCG, our results argue in favor for more *in vivo* studies to examine the persistence, metabolism and chemical stability of EGCG at the mucosal exposure sites. These investigations are crucial for further clinical evaluation and development of EGCG as a safe, cost-effective and naturopathic microbicide for preventing sexual transmission of HIV-1, particularly in resource-poor settings.

Methods

Animals

Sixteen adult male rhesus macaques were used to examine the protective effect of EGCG on prevention of SHIV rectal infection. The animals were obtained from the Kunming Institute of Zoology of Chinese Academy of Science. The animals were individually housed at the Animal Biosafety Level-III Laboratory at the Center for Animal Experiment of Wuhan University with AAALAC International accreditation (001274). All the animal protocols were approved by the Institutional Animal Care and Use Committee (approval number: 2015024) of Wuhan University in accordance with the NIH "Guide for the Care and Use of Laboratory Animals". All the details of animal welfare were carried out in accordance with the recommendations of the Weatherall report, "The Use of Nonhuman Primates in

Research". All the animals in this study were experimentally naïve at the beginning and negative for antibodies against the specific pathogens. All the procedures were performed under anesthesia with ketamine, and necessary efforts were taken to minimize suffering, improve housing conditions, and provide enrichment opportunities for the study animals.

Cells and viruses

TZM-bl cells with integrated firefly luciferase gene under the control of HIV-1 LTR were obtained from the NIH AIDS Research Program and grown in DMEM medium. Human and macaque PBMCs were prepared by Ficoll gradient centrifugation and cultured in RPMI medium containing 10% FBS, 100 units ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 2 mM L-glutamine and 20 U ml^{-1} interleukin-2. Human PBMCs were incubated with complete DMEM in gelatin flasks for 45 min at 37°C to collect monocytes. After detachment with 10 mM EDTA, monocytes were washed with DMEM and resuspended in complete DMEM and plated in 48-well plates. Monocytes differentiated into macrophages during *in vitro* culture. Macaque monocytes from PBMCs were cultured *in vitro* for 7 days when the cells became macrophages as described.[42] SIV_{mac251}, SIV_{mac239}, $SHIV_{KU-1}$, $HIV-1_{Bal}$, and $HIV-1_{NL4-3}$ strains were obtained from the NIH AIDS Research Program. The R5 $SHIV_{SF162P3N}$ derived from the $HIV-1_{SF162}$ primary isolate [43, 44] was a generous gift from Dr. Cecilia Cheng-Mayer (Aaron Diamond AIDS Research Center, New York, NY) and was propagated in phytohemagglutinin (PHA)-activated macaque PBMCs. The 50% tissue culture infectious doses ($TCID_{50}$) of $SHIV_{SF162P3N}$ was titrated in macaque PBMCs and the other virus stocks were titrated in TZM-bl cells.[44] Briefly, PHA-activated PBMCs ($2.5×10^5$/well) were infected with a serial dilution of the virus stock in quadruplicate, starting with a 1/5 dilution in RPMI medium containing IL-2. The p27 antigens in culture supernatant were measured by SIV p27 ELISA (XpressBio, Frederick, MD). The $TCID_{50}$ of the virus stocks were determined by the method of Reed and Muench.[45]

Rectal administration of EGCG and SHIV challenge

EGCG (Sigma-Aldrich, St. Louis, MO) was prepared as a stock solution (20 mM) in PBS. The protective efficacy of EGCG on the rectal SHIV infection was evaluated in a repeated exposure macaque model using the $SHIV_{SF162P3N}$, an extensively used SHIV strain in NHP model of sexual transmission.[46, 47] The animals were intramuscularly anesthetized with ketamine hydrochloride (10 mg kg^{-1}) plus xylazine hydrochloride (1 mg kg^{-1}). Anesthetized animals were maintained a ventral recumbent position and 2 ml of EGCG (5 mM) or PBS was administrated atraumatically into the rectal vault prior to the intrarectal inoculation with $SHIV_{SF162P3N}$ at a dose of 10 $TCID_{50}$.[44] Animals that were not infected after the first inoculation were treated with EGCG or PBS and inoculated with SHIV again once biweekly. All the experiments were performed under highly controlled conditions by the same personnel using the same virus stock, inoculum dose and methods. Animals were considered infected and the virus challenges were stopped following two positive plasma SHIV RNA results. The experiments were stopped when all control animals became infected.[9]

Plasma SHIV RNA determination

Plasma SHIV RNA was analyzed by real-time RT-PCR for SIV/SHIV gag gene as previously described.[9] Primer used were: forward 5′-

TGGAGAACAAAGAAGGATGTCAAA-3′, reverse 5′-CACCAGATGACGCAGACAGTATTAT-3′. Probe sequence was 6FAM-TTGGCACTAATGGAGCTAAGACCGAAAGTATT-BHQ1 (Gag-Btaq.2). Real-time PCR condition was: 95°C for 10 min, 40 cycles of 95°C for 30 sec and 60°C for 15 sec. Duplicate samples were analyzed and the limit of detection (LOD) was 200 SIV or SHIV RNA copies ml^{-1} plasma.

PCR amplification of proviral DNA and RNA

DNA and RNA was extracted from PBMCs, lymphocytes isolated from LNs and other GI tissues (Supplementary Table 3) with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA). Specimens were analyzed in duplicate with 200 ng of DNA to amplify SHIV proviral DNA with the same primers and probes as for the plasma viral load assay described above. At week 20 after first SHIV challenge, inguinal LN and rectal mucosal were biopsied and subjected to RNA and proviral DNA detection. Intraepithelial lymphocytes from rectum or colon tissues were enriched to increase the sensitivity of the PCR as reported.[9, 33]

gp120 binding assay

CD4$^+$ T cells were incubated with 50 μM of EGCG in RPMI-1640 medium for 10 min at 37°C. Large volume of cooled (4°C) PBS was then added immediately after incubation to halt the interaction between cells and EGCG. Cells were washed twice with PBS and aliquoted for two sets of experiments. In the first set of experiment, the cells were stained directly with PB-conjugated mAb against CD4 (Sk-3 clone) at room temperature subdue light for 45 min. PB-conjugated mouse IgG of unrelated specificity was used as a negative control. The stained cells were then examined by flow cytometry for CD4 expression. In the second set of experiments, the EGCG-treated CD4$^+$ T cells were incubated with recombinant HIV gp120 protein conjugated with FITC (20 μg ml^{-1}) for 1 h. The cells were washed with PBS and the fluorescence intensity of gp120-FITC bound to the surface of CD4$^+$ T cells was measured by the FACSCanto flow cytometer (BD Biosciences). Data were analyzed using the FlowJo data analysis software package (TreeStar Inc., USA).

Serology

Plasma SHIV-specific antibodies (IgG and IgM) of the study animals were measured by enzyme immunoassay (EIA) as instructed by the manufacturer (Genscreen™ ULTRA HIV Ag-Ab; Bio-Rad).

Immunohistochemistry

Autopsied specimens from the rectal tissues were obtained 4 days after the rectal administration of PBS or EGCG and fixed in paraformaldehyde. The sections were incubated with mouse monoclonal antibodies against CD3 (F7.2.38) or CD68 (DK25; Dako; Carpinteria, CA), CD163 (EDHu-1; AbD Serotec; Raleigh, NC), HLA-DR (LN3; eBioscience; San Diego, CA) overnight at 4°C. Normal mouse serum was used as the negative antibody control for the specificity of the immunohistochemistry. The whole slides were digitally scanned using BX53 microscopy (Olympus, Japan) with ProScan III

controller (Prior, UK). The tissue positivity for individual staining was assessed by Aperio ImageScope software (Leica Biosystems Inc., Buffalo Grove, IL).

EGCG toxicity analysis

The *in vitro* cytotoxicity of EGCG on the TZM-bl and PBMCs was determined by MTT assay or by flow cytometry using 7-amino actinomycin D (7-AAD). The *in vivo* toxicity of EGCG on the rectal mucosa was examined by assessing the mucosal pathology, inflammatory response, stool consistency and bleeding.[48, 49] Briefly, EGCG was applied to rectum of the mice and the macaques. Fresh stools were then collected at 0, 24, 48, and 72 h post EGCG administration for occult blood test. Stool consistency of mice were graded accordingly as previously described.[48] Inflammatory cytokines in the rectal tissues of the mice were measured by real time RT-PCR 72 h post-EGCG intrarectal administration. The impact of EGCG on rectal mucosal histology was analyzed by hematoxylin/eosin (HE) staining based on Obermeier's procedure.[50] The rectum was divided into 3 equal segments with different proximity to the anus (proximal, middle, and distal), cross sectioned, and fixed in 10% formalin overnight. The tissues were then embedded in paraffin and serially sectioned at a thickness of 5 μm. Histological analysis was performed and evaluated in a blinded fashion. Each score represented the mean of nine sections.

Statistical analysis

The *in vitro* data were expressed as mean ± standard deviation (SD) of triplicate cultures and statistical significance was assessed by Student's *t* test. To protect the macaques from SHIV$_{SF162P3N}$ infection was analyzed using Cox proportional hazard models based on the exact partial likelihood for discrete time. The number of challenges was used as a discrete time scale. The hazard ratios with 95% confidence intervals (CI) for the per-exposure relative reductions of acquisition risk were calculated for EGCG group as compared to the control group. Protection against acquisition of infection was also analyzed by log-rank (Mantel-Cox) tests. The statistical analyses of complete protection for the study animals against SHIV challenge were performed by Fisher's exact tests. GraphPad Prism software (La Jolla, CA) was used for all statistical analyses. Significance was defined as *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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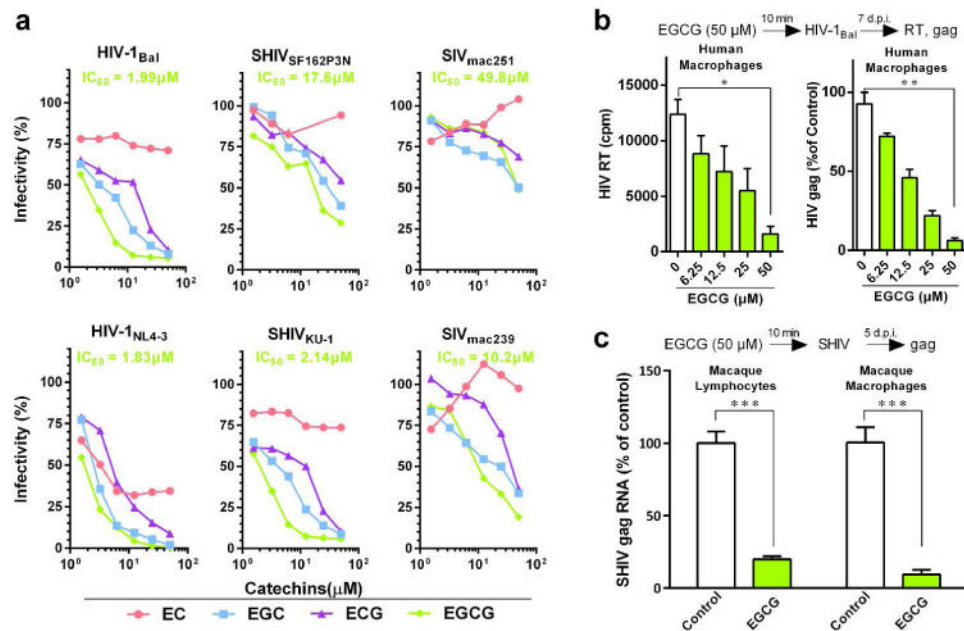
Figure 1.
EGCG inhibits viral infectivity of a broad spectrum of AIDS-related viruses. (a) TZM-bl cells were treated with the indicated concentrations of green tea-derived catechins (EC, EGC, ECG and EGCG) for 10 min prior to infection with different strains of HIV-1 (Bal, NL4-3), SHIV (SF162P3N, KU-1), or SIV (mac239, mac251). Viral infectivity was assessed by luciferase activity, which is expressed as a percentage relative to that of the control (untreated). The half maximal inhibitory concentration (IC_{50}) of EGCG is indicated, which was calculated based on the untreated control by the method of Reed and Muench. (b) Human peripheral blood monocyte-derived macrophages were incubated with the indicated doses of EGCG for 10 min prior to HIV-1$_{Bal}$ infection. Culture supernatant was collected on day 7 post-infection for HIV-1 reverse transcriptase (RT) assay. Cellular RNA was subjected to the real time RT-PCR for HIV-1 gag and GAPDH RNA. Data are expressed as HIV-1 RNA levels relative (%) to untreated control, which is defined as 100%. (c) Primary lymphocytes and macrophages from rhesus macaques were treated with or without EGCG (50 μM) for 10 min prior to SHIVSF162P3N infection. Intracellular gag RNA was measured by the real time PCR at day 5 post infection. Data are shown as mean ± SD, representative of three independent experiments with 3-4 replicates. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.
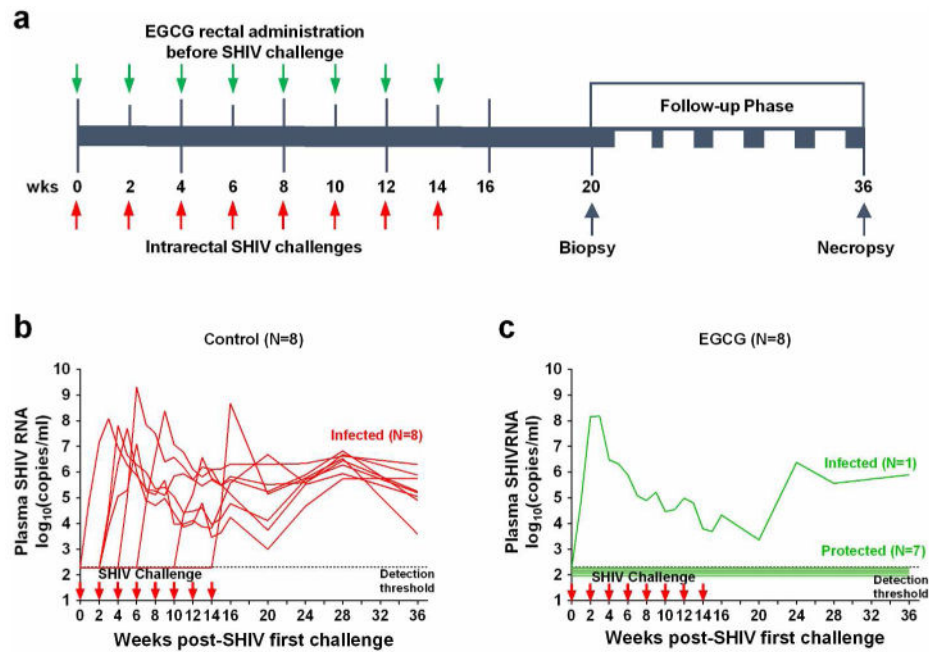
Figure 2.
EGCG protects macaques from intra-rectal SHIV infection. (a) Experimental design of EGCG protective effect on macaques. Sixteen male macaques were administered with 2 ml of 5 mM EGCG (8 animals) or 2 ml of PBS (8 animals) atraumatically in rectum 10 min prior to each $SHIV_{SF162P3N}$ challenge. All animals were rectally inoculated with $SHIV_{SF162P3N}$ (10 $TCID_{50}$) for up to 8 times or until infection occurred. All animals were biopsied at week 20 and necropsied at week 36 postinfection for the evaluation of SHIV RNA and proviral DNA in the multiple tissues. (b, c) Longitudinal assessment of the plasma SHIV RNA (copies ml^{-1}) levels in the animals with intrarectal pretreatment with PBS (control) or EGCG prior to SHIV challenges (up to 8 times or till infection occurred). Duplicate plasma samples were analyzed for SHIV RNA detection. Animals were considered infected and the virus challenges were stopped following two consecutive positive plasma SHIV RNA results.
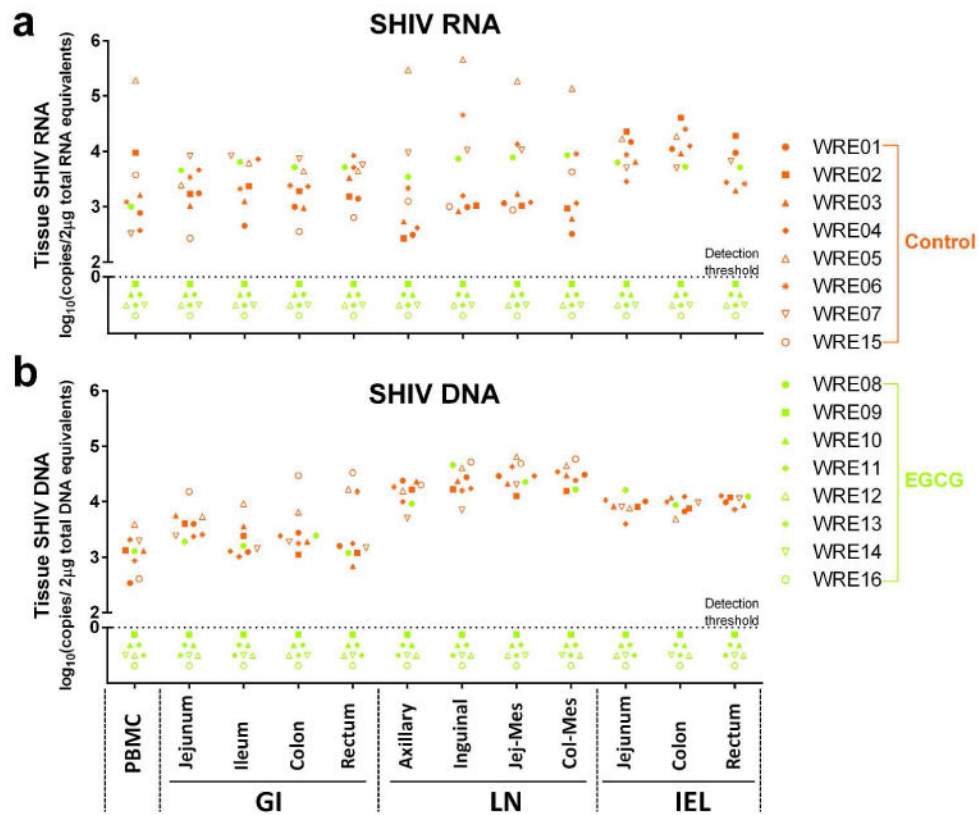
Figure 3.
SHIV RNA and DNA detection in multiple tissues necropsied at week 36 post first SHIV challenge. SHIV RNA (a) and DNA (b) assays in the indicated tissues from the animals of PBS control (red symbols, n=6~8) and EGCG group (green symbols, n=8) at necropsy. Log SHIV copies 2 μg^{-1} total genomic RNA or DNA equivalents are shown. Symbols represent individual animals and are pooled from three independent experiments. Triplication tissue samples were conducted in each independent experiment. The dot line: the detection threshold. GI: gastrointestinal tract; LN: lymph nodes; Jej-Mes: jejunal mesenteric; Col-Mes: colonal mesenteric; IEL: intraepithelial lymphocytes.
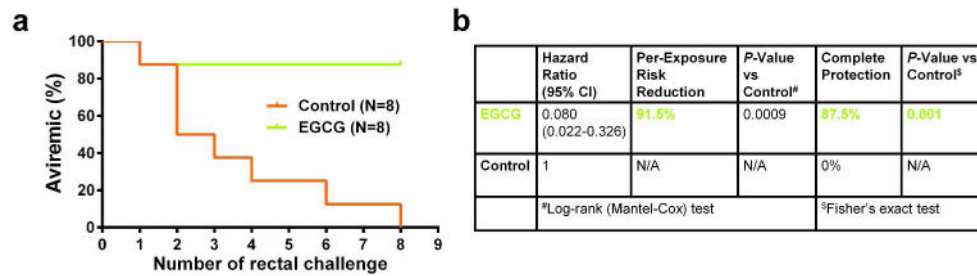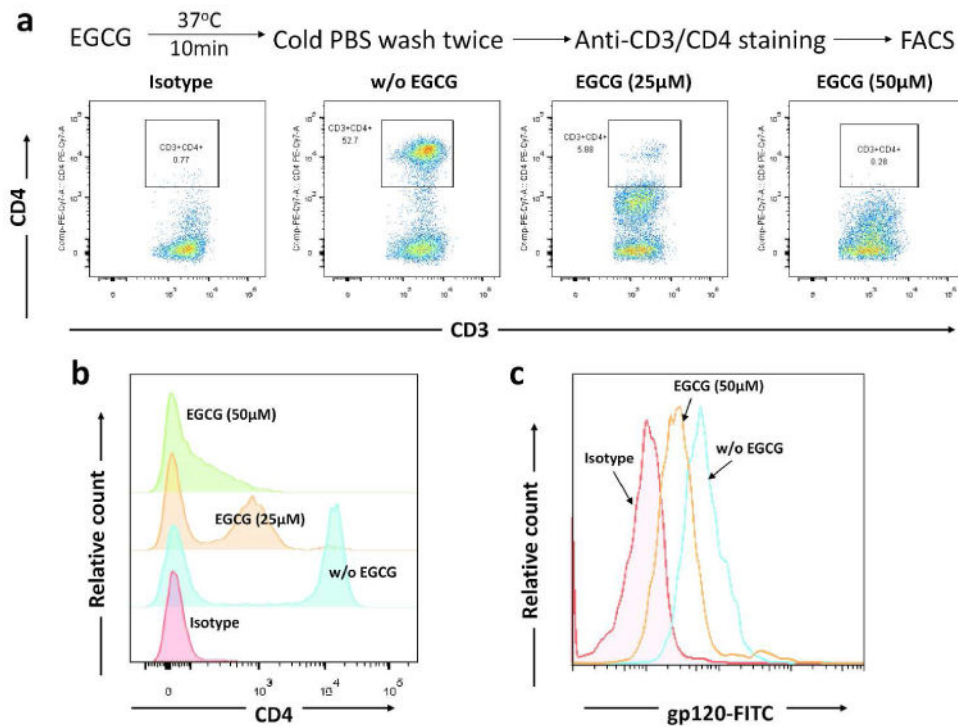
Figure 4.
Protective efficacy of EGCG local pre-exposure application on SHIV rectal transmission in macaques. (a) Kaplan-Meier plot demonstrating the protection in EGCG-treated animals (n=8) relative to PBS-treated animals (n=8). (b) Statistical analyses include the hazard ratio with 95% confidence interval and the per-exposure reduction of acquisition risk in each group, with P values reflecting log-rank tests and Fisher's exact test.

Figure 5.
Effect of EGCG on binding of anti-CD4 antibody and gp120 to CD4 T cells. (a) Peripheral blood mononuclear cells were incubated with indicated doses of EGCG for 10 min. The reaction was then terminated with cold PBS. Cells were washed and then stained with anti-CD3 and anti-CD4 antibodies and subjected to flow cytometry analysis. The data shown are representative of three experiments with cells from three different donors. (b) Overlapping of the CD4 expression in PBMCs pretreated with indicated concentrations of EGCG. (c) Interference of binding of HIV-1 gp120 to CD4$^+$ T cells. Purified CD4$^+$ T cells were incubated with or without (w/o) 50 μM of EGCG. The cells were then washed with PBS and further incubated with recombinant HIV-1 gp120 protein conjugated with FITC for 2 h. The binding of gp120 to cells was indicated by intensity of FITC, which was examined by flow cytometry. Data are representative of three independent experiments.
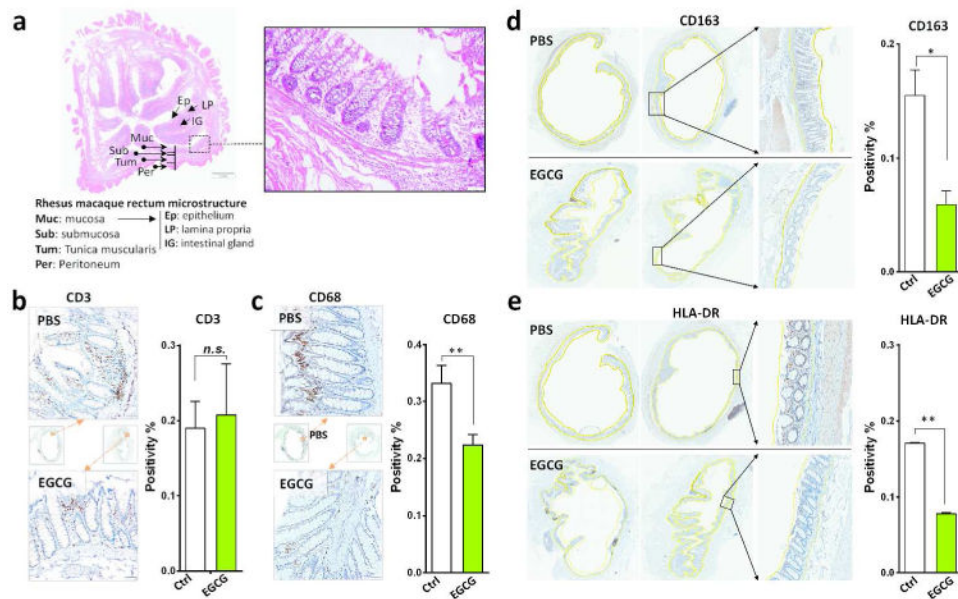
Figure 6.
EGCG suppresses macrophage infiltration and immune activation in the rectal mucosa of SHIV-infected macaques. (a) Morphological observation of the microstructure of the rhesus macaque rectum. Architecture of the rectal mucosa of rhesus macaques as examined by hematoxylin and eosin staining. Magnification ×200. (**b-e**) Immunohistochemistry staining of the cell infiltration and chronic immune activation in rhesus macaques intra-rectally treated with PBS or EGCG. EGCG (5 mM, 2 ml) or PBS (2 ml) was delivered to the rectum of rhesus macaques for 10 min prior to the challenge with $SHIV_{SF162P3N}$ (10 $TCID_{50}$). Rectal tissues from two macaques were collected at autopsy (96 h post-infection). Cell infiltrates were demonstrated by immunohistochemistry staining with anti-CD3 (b) or anti-CD68 (c) antibody. Tissue activation was examined by staining with anti-CD163 (d) or anti-HLA-DR (e) antibody. The positivity of mucosal tissue for $CD3^+$, $CD68^+$, $CD163^+$, and $HLA-DR^+$ cells in the rectum mucosa were quantified using the Aperio Image Scope software. The solid yellow lines (d, e) were used to designate regions, including the epithelium, lamina propria and intestinal glands of the rectal mucosa and submucosa, for algorithm analysis. Dotted yellow lines excluded the enclosed regions for the calculation. The positive cells within the mucosa and submucosa were counted per high-power field. At least two cross-sectioned rectal segments with different proximity to the anus were scanned and analyzed. Original magnification ×200. Data are shown as mean ± SD, which were analyzed using the 2-tailed Student's *t*-test. *$P<0.05$ and **$P<0.01$.