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Characterization of peripheral and mucosal immune responses in rhesus macaques on long-term tenofovir and emtricitabine combination antiretroviral therapy

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Abstract

Background—The goal of antiretroviral therapy (ART) is to suppress virus replication to limit immune system damage. Some have proposed combining ART with immune therapies to boost antiviral immunity. For this to be successful, ART must not impair physiological immune function.

Methods—We studied the impact of ART (tenofovir and emtricitabine) on systemic and mucosal immunity in uninfected and SIV-infected Chinese rhesus macaques. Subcutaneous ART was initiated 2 weeks after tonsillar inoculation with SIVmac239.

Results—There was no evidence of immune dysregulation as a result of ART in either infected or uninfected animals. Early virus-induced alterations in circulating immune cell populations (decreased central memory T cells and myeloid dendritic cells) were detected, but normalized shortly after ART initiation. ART-treated animals showed marginal SIV-specific T cell responses during treatment, which increased after ART discontinuation. Elevated expression of CXCL10 in oral, rectal and blood samples and APOBEC3G mRNA in oral and rectal tissues was observed during acute infection and was down-regulated after starting ART. ART did not impact the ability of the animals to respond to tonsillar application of polyICLC with increased CXCL10 expression in oral fluids and CD80 expression on blood myeloid dendritic cells.

Conclusion—Early initiation of ART prevented virus induced damage and did not impede mucosal or systemic immune functions.

Keywords

antiretroviral therapy; mucosal immunity; peripheral immunity; SIV

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Introduction

Antiretroviral therapies (ART) need to control virus replication thereby limiting associated pathogenesis, which could be combined with immune therapies to boost immunity while replication is suppressed. Thus, ART must not interfere with immune functions. The similarities in disease pathogenesis, immunology and physiology (i.e. drug metabolism) between experimental simian immunodeficiency (SIV) infection in nonhuman primates (NHPs) and HIV infection in humans provide an excellent model to study the biology of HIV infection, including potential adverse effects of ART on immune function¹⁻³.

ART is effective in NHPs³⁻⁸. The nucleotide reverse transcriptase inhibitors (NRTIs) tenofovir and emtricitabine are highly effective and generally well-tolerated and the combination of both is used as the NRTI backbone of numerous ART regimens to treat HIV⁹⁻¹⁴. The tenofovir/emtricitabine regimen can control SIV replication in macaques^{4,8,15-21}.

We investigated whether long-term ART impacts immunity. To minimize SIV-associated effects, we initiated ART 14 days after tonsillar inoculation with SIVmac239²²⁻²⁴. Treated uninfected animals, and uninfected and infected animals not receiving ART were included as controls. There was no evidence of immune dysfunction as a result of ART, but virus-induced changes that likely contribute to the onset and spread of infection were apparent. In primary infection via the tonsillar route, CXCL10 expression was elevated in oral, rectal and blood samples and increased APOBEC3G (A3G) levels were detected in mucosal tissues. CXCL10 and A3G decreased on ART. We observed early infection-related loss of central memory CD4+ and CD8+ T cells and myeloid dendritic cells (mDCs) in blood, which normalized after initiation of ART. ART did not impair the animals' ability to respond to polyICLC applied to the tonsils. Thus, tenofovir/emtricitabine ART does not appear to adversely affect mucosal and systemic immune functions in macaques and reinforces the idea that commencing ART early in infection can limit virus-induced damage to the immune system.

Materials and Methods

Animals and Treatment

Adult male Chinese rhesus macaques (*Macaca mulatta*) were housed at the Tulane National Primate Research Center (TNPRC, Covington, LA). All animal studies were performed in accordance with federal laws and regulations and institutional policies, including the approval by the Animal Care and Use Committee of the TNPRC (OLAW Assurance #A4499-01), which has received continued full accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC #000594). Animals were housed and cared for in compliance with the regulations detailed under the Animal Welfare Act²⁵. All animals received environmental enrichment and were clinically monitored daily. Animals were pair- or group-housed when possible. For surgical and sampling procedures animals were anesthetized with ketamine hydrochloride (10mg/kg i.m.) followed by appropriate analgesics for pain and discomfort (buprenorphine 0.25 mg/kg i.m.), which was carefully monitored. Upon study termination or when signs of advanced stages of simian AIDS were present (IACUC approved endpoint criteria), animals were euthanized using methods consistent with the recommendation of the American Veterinary Medical Association Guidelines on Euthanasia.

All animals were antibody (Ab) negative to simian type D retrovirus, simian T lymphotropic virus type 1 and SIV at study enrollment. 12 animals were inoculated with 2000 TCID₅₀ SIVmac239 on the tonsils (TNPRC stock virus propagated in SEB-stimulated rhesus

PBMCs; “SIVmac239 RhPBMC 7/29/94” dribbled across the tonsils in 0.2ml). ART was administered for 34 weeks between 2 and 36 weeks post challenge and consisted of a daily subcutaneous injection of tenofovir (PMPA, 9-[2-(Phosphonomethoxy)propyl]adenine, 20mg/kg/day) and emtricitabine (beta-2',3'-dideoxy-3'-thia-5-fluorocytidine, FTC, 40mg/kg/day). PolyICLC treatment (0.5ml, 1mg/day on two consecutive days, Hiltonol®, Oncovir, Washington, DC) was applied over the palatine tonsils and the back of the tongue. All animals received two polyICLC treatments during ART (weeks 28 and 32 post-challenge) and two treatments after ART (weeks 44 and 48 post-challenge). The animals and treatments were listed in Supplementary Digital Content 1.

Sample collection and cell isolation

Immune responses were monitored in blood, mucosal fluids and oral and rectal biopsies. Peripheral lymph nodes (LNs) and tonsils were collected at necropsy. Samples were transported by overnight courier service (blood at room temperature, fluids and tissue samples on ice) and processed immediately after arrival. Cells from peripheral blood, LNs, and tonsils were isolated as described²⁶. Plasma samples were collected and stored as described²⁶.

Mucosal fluids were collected by insertion of a foam pad (approx. size 1×0.5 cm) in the oral or rectal cavity for 5min, after which the swab was placed into a tube containing 1ml PBS/1% FCS/penicillin-streptomycin. After overnight shipment, the mucosal fluids were spun at 1100g, 4°C for 10min and the supernatant was aliquoted and stored at -80°C until analysis.

For the collection of oral biopsies the oral cavity was exposed by placing gauze behind both the upper and lower canines with retraction of the gauze. Alligator forceps were used to obtain 1 mm pinch biopsies of the buccal mucosa. Rectal biopsies were sampled by placing sterile lubricant on the distal end of a vaginal speculum, which was then gently advanced through the anus into the rectum to visualize the mucosa. Alligator forceps were used to collect 1.5 mm pinch biopsies of rectal mucosa. Up to 20 mucosal biopsies were taken at one time point from the oral or rectal site. After overnight shipment, mucosal pinch biopsies were washed twice in PBS (Invitrogen), incubated overnight at 4°C in RNAlater (Qiagen, Valencia, CA) then stored at -80°C until isolation of RNA.

Viral load and SIV Ab determination

Plasma SIV RNA was determined by quantitative RT-PCR²⁷ and SIV-specific Abs were measured by ELISA²⁸. Neutralizing Ab (nAb) activity against SIVmac251 was determined in the plasma collected at baseline and 52 weeks post-infection¹⁷.

Flow Cytometry

Leukocytes in blood and tissues were characterized by polychromatic flow cytometry. T cell subsets were identified as described¹⁷. DC subsets were identified within the Lin⁻HLA-DR⁺ population using FITC-conjugated anti-Lineage Abs (CD3, clone SP34; CD14, clone M5E2; CD20, clone 2H7, all BD Biosciences) with HLA-DR-PerCP-Cy5.5 (clone G46-6), CD123-PE (clone 7G3), CD11c-PE-Cy7 (clone 3.9, all BD Pharmingen), CD80-biotin (clone L307.4, BD Pharmingen) Abs followed by streptavidin-APC-Alexa650 (Invitrogen). Isotype Ig controls were included in all experiments and typically gave mean fluorescence intensities (MFIs) of <1 log. All samples were acquired on a BD LSRII (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Antigen-specific T cells were detected using intracellular cytokine staining (ICS)^{29,30}. Aldrithiol-2 (AT-2)-inactivated SIVmac239 (300ng/ml p27, lot #P4148, AIDS and Cancer Virus Program, NCI-Frederick, Frederick, MD) was used to stimulate SIV-specific T

cells³¹. No-virus microvesicle preparations and 50nM phorbol 12-myristate 13-acetate and 1mg/ml ionomycin (both Sigma, St. Louis, MO) were used as controls. *Candida albicans* (ATCC, strain SC5413) was maintained at room temperature on yeast-peptone-dextrose agar plates (Sigma), and *Candida* yeast (which induce CD4+ and CD8+ responses³²) were amplified in Sabouraud dextrose broth (Sigma) overnight at 30°C. Viable yeast were counted by trypan blue exclusion and used in a *Candida*:PBMC ratio of 1:1. Amphotericin B (5µg/ml, Sigma) was added to prevent *Candida* overgrowth. IL-17-Alexa Fluor 647 Abs (clone eBio64CAP17, eBioscience, San Diego, CA) was added to the published Ab panel³⁰. Data were acquired (200,000 events in the CD3⁺ lymphocyte gate) using BD LSRII and analyzed using FlowJo software.

Luminex and ELISA

Chemokine and cytokine levels were measured in cell-free mucosal fluids using the monkey-reactive Beadlyte human 14-plex Detection System (Invitrogen). Data were acquired on a Luminex 200 instrument (Luminex, Austin, TX) and analyzed using StarStation software version 2.0 (Applied Cytometry Systems, Sacramento, CA). IFN-α, IFN-β (PBL Interferon Source, Piscataway, NJ, detection limit 25pg/ml) and CXCL10 (R&D, Minneapolis, MN, detection limit 15.1pg/ml) were detected by ELISA.

Detection of immune markers by real time PCR

Tissue samples were homogenized using a FastPrep bead mill homogenizer and lysing matrix D (MP Biomedicals, Irvine, CA). Total RNA was isolated with the RNeasy Mini Kit (Qiagen) and quantified on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Immune marker mRNA levels were determined¹⁷, with expression levels being calculated from normalized ΔC_T values using the following formula: fold change in gene expression = $2^{-\Delta\Delta C_T}$. Test groups were compared to the uninfected controls collected at the same time points, rather than to the respective baselines or different sample collection time for each animal, to control for the quality of RNA that might have been affected by long-term storage.

Sequencing the SIV-RT gene

Viral RNA was extracted from the plasma of animals EL42 and P427 collected at 35 weeks post infection (w.p.i.; 1 week before stopping ART) using the Qiagen Viral RNA Isolation Kit. The RT gene was sequenced³³ using primers RTamp5' (5'-TACTAAAGAATACAAAAATGTAGA-3') and RTamp3' (5'-CTCTGTGGATTGTATGGTACCCC-3') for first round PCR product amplification and SIVmacRT5' (5'-TGGAAAAGGATGGTCAGTTGGAGGA-3') and SIVmacRT3' (3'-CCGTGGCTTCTAATGGCTTGCCT-5') for nested PCR reaction.

Statistical analysis

All statistical calculations were performed utilizing GraphPad Prism software (San Diego, CA), version 5.02 for Windows. Non-parametric tests were used due to small sample size. When comparing two groups two-tailed Mann-Whitney U-test was used while comparison of more than two groups was performed using the Kruskal-Wallis test with Dunn's multiple comparison test. P-values were two-sided and considered significant when < 0.05 .

Results

Impact of early ART on viral loads and adaptive immunity

The effect of a continuous tenofovir/emtricitabine ART regimen on immune parameters was evaluated in infected and uninfected macaques (see table, Supplementary Digital Content 1).

To limit cumulative and progressive immune damage due to infection, ART was initiated 2 weeks after infection. All animals reached peak viremia 2 weeks post tonsillar SIVmac239 inoculation (fig. 1A). Peak viral loads were comparable in infected animals that did ($6.0 \pm 2.9 \times 10^6$ RNA copies/ml) or did not ($2.8 \pm 0.9 \times 10^6$ RNA copies/ml) receive ART ($p=0.91$). All 5 treated animals initially responded to ART with viral loads dropping significantly between weeks 3 and 6 compared to untreated controls (fig. 1B and C). Three of the animals continued to respond to ART with plasma viral loads being maintained below 100 copies/ml during treatment (fig. 1C), while viral loads in the other 2 animals reached set point values comparable to untreated controls (fig. 1A). Due to the small numbers of animals we were unable to make statistical comparisons between the ART responders and transient responders. Upon stopping ART, 2 of the 3 responding animals had an initial rebound in viremia ($<10^4$ copies/ml), which returned to <400 copies/ml until euthanasia. The third ART responder maintained plasma viral RNA levels below threshold from the time of ART discontinuation through to euthanasia. Viremia in the 2 transient ART responders appeared unaltered upon ART discontinuation. Virus from the transiently responding animals carried the RT-sequence of the parental challenge virus as detected in the plasma collected at 35 w.p.i. and there were no amino acid changes at positions known to confer resistance to NRTIs (see table, Supplementary Digital Content 2)^{3,34,35}. Mamu-A*01, -A*02, -A*08, -A*11, -B*01, -B*03, -B*04, -B*08, -B*17 haplotypes were tested in all infected monkeys. Only FH22 expressed Mamu-A*01, but showed no evidence of enhanced virus control or delayed disease progression. There were no differences between the CD4 counts of treated and untreated animals (fig. 1D).

All infected animals developed SIV-specific plasma IgG (see table, Supplementary Digital Content 1). Anti-SIV nAbs were detected in all infected animals except for animals AA47 (fast progressor), EB50 (high viral load) and EL42 (transient responder) (see fig. A, Supplementary Digital Content 3), with similar titers in untreated and ART-treated animals (see fig. B, Supplementary Digital Content 3). SIV-specific TNF α - and IFN γ -producing T cells were most frequent in infected, non-treated animals, while the ART-receiving group had low responses during ART treatment (fig. 2A). SIV-specific IL-2 responses were most prominent earlier after infection, but SIV-specific IL-17 responses were not consistently detected (independent of ART) and were even detected in uninfected animals (fig. 2A). Comparable *Candida*-specific responses were seen (independent of SIV infection and ART), with responses decreasing over time (fig. 2B). Additionally, ART did not appear to significantly impact multifunctional T cell responses in SIV-infected animals (fig. 2C).

SIV-induced changes in blood T cell and DC subsets

No significant differences in the frequencies of T cell subsets (see figures, Supplementary Digital Content 4 and 5), mDCs, or plasmacytoid DCs (pDCs) (see figure, Supplementary Digital Content 6) were found between uninfected ART-naïve and -treated animals. To simplify the analyses and enhance the statistical power, the uninfected animals (ART treated and untreated) were consolidated into one control group. SIV-infected animals showed a significant decrease of central memory T cells in acute infection (fig. 3, $40 \pm 9\%$ decrease for CD4 $^{+}$ and $30 \pm 11\%$ for CD8 $^{+}$, 2 w.p.i. compared to baseline). Trends towards lower numbers of CD4 $^{+}$ central memory T cells (fig. 3) and higher frequencies of CD25 $^{+}$ Foxp3 $^{+}$ CD4 $^{+}$ Tregs were detected in untreated chronic infection (7.6 ± 2.1 -fold increase at 54 w.p.i. compared to baseline versus 2.9 ± 0.5 in the controls and 4.3 ± 0.7 in infected ART-receiving group). Animals with the highest frequencies of CD4 $^{+}$ Tregs had the highest viral loads (see figure, Supplementary Digital Content 4).

The percentages of Lin-HLA-DR $^{+}$ DCs remained stable during acute and chronic treated and untreated infection (fig. 4). However, CD11c $^{+}$ mDCs decreased in acutely infected animals ($25 \pm 5\%$ decrease 2 w.p.i. compared to uninfected animals, $p=0.009$), returning to

control levels shortly after initiation of ART but persisting in ART-naïve animals up to 8 w.p.i. (fig. 4). CD80 expression on mDCs was not altered by infection (fig. 4). Elevated CD123+ pDC percentages were detected in acute infection (2.01 ± 0.29 fold increase 2 w.p.i. compared to uninfected animals, $p=0.004$), which persisted in untreated infection up to 32 w.p.i. (fig. 4). ART-receiving animals also showed higher pDC frequencies in chronic infection (not significant).

We used polyICLC to evaluate the innate responsiveness of the differently treated groups. No changes in viral loads were observed after polyICLC treatment when given during or after ART (fig. 1A). There was variability in responsiveness to polyICLC (i.e., not consistently responsive after each dose), but there were no significant differences between the groups. There were no changes in the frequencies of naïve, effector and central memory T cells, mDCs and pDCs in the blood (not shown), but there was transiently elevated expression of CD80 on mDCs 24h after the second and fourth polyICLC treatment (see figure A, Supplementary Digital Content 7). Additionally, CXCL10 protein levels in the oral fluids were increased 24-72h after the first and fourth treatments (see figure B, Supplementary Digital Content 7). IFN- α and - β protein levels in oral fluids and blood were unaltered. PolyICLC treatment did not affect antigen-specific T cell responses (fig. 2).

ART shuts down virus-induced mucosal immune responses

We also compared the mRNA expression of 10 innate immune modulators in the oral cavity versus the rectum and blood during acute and chronic infection ($\pm$ ART for the latter). No significant differences between uninfected ART-naïve and -treated animals were detected in the expression levels of the tested parameters in the oral, rectal, or blood samples (data not shown). Hence, data from the ART-treated and untreated uninfected animals were consolidated as the control group to increase the power of the statistical comparisons to the infected groups.

SIV-infected animals showed elevated levels of CXCL10 (85.1 ± 28.8 fold increase, $p=0.04$) and A3G (33.0 ± 22.0 fold increase, $p=0.006$) mRNA and a reduction of type I IFN mRNA expression (1.69 ± 0.14 fold reduction of IFN- $\alpha 2$, $p=0.001$ and 2.27 ± 0.42 fold reduction of IFN- β , $p=0.006$) in oral tissues 2 w.p.i. (fig. 5A). Oral CXCL10 and A3G mRNA levels declined with ART, while persistently elevated levels were observed in untreated chronic infection (fig. 5B). TNF- α and IL-10 mRNA levels increased in oral samples of acutely infected animals (fig. 5A, not significant). This trend was maintained in chronic untreated infection, but levels dropped to within normal ranges under ART (fig. 5B). Responses in blood paralleled the oral tissues. Elevated levels of CXCL10 mRNA were detected in acute infection in rectal tissue (fig. 5A) and CXCL10 protein expression in the blood plasma as detected by ELISA 1 w.p.i. (40 ± 8 pg/ml in the uninfected versus 127 ± 34 pg/ml in the SIV-infected; 3.18 ± 0.85 fold increase compared to uninfected, $p=0.005$). CXCL10 protein production in plasma was rapidly abrogated by initiation of ART and further augmented in the absence of ART (2.66 ± 0.83 fold increase in ART-treated animals versus 18.75 ± 8.39 in ART-naïve infected animals compared to uninfected controls, $p=0.003$).

Different responses were detected in rectal (versus oral) tissues: (i) CCL4 mRNA expression was significantly increased and returned to control levels under ART, (ii) TNF- α and IL-10 mRNA expression was not increased in acute or chronic infection, and (iii) A3G mRNA expression increased only minimally during acute infection.

Discussion

Given the widespread use of Truvada® (tenofovir/emtricitabine) and Atripla® (tenofovir/emtricitabine/efavirenz) as first line regimens to treat HIV^{11,36} and promising results from

studies using Truvada® for pre-exposure prophylaxis to prevent HIV transmission³⁷, understanding the interactions of these drugs with the immune system is of increasing importance.

We studied uninfected and SIVmac239-infected macaques to investigate the effect of tenofovir/emtricitabine ART on systemic and mucosal immune parameters. Based on studies showing that initiation of ART during acute HIV infection can preserve or increase antiviral immunity^{38,39}, ART was initiated early (14 d.p.i.) after tonsillar challenge with SIVmac239. Initiation of ART around the peak viremia poses a greater challenge and effective suppression of viral load may take longer compared to initiation of therapy during early chronic infection¹⁷. Inclusion of a protease or integrase inhibitor to the regimen should increase the effectiveness of the treatment⁴⁰.

Although all 5 treated animals initially responded to ART, 2 monkeys showed increasing viral loads that reached set point values comparable to untreated controls. While the animal numbers are very small, we did examine the raw data of the ART responding versus poor-responding animals and there was no difference in the parameters being measured. As a result, we included them in the analyses, since (although the virus did not respond to the drugs) the animals' immune systems were exposed to the drugs.

It is unclear why, despite persistent viremia and drug therapy, no mutations were observed in the poor ART responding animals. Previous studies examining tenofovir resistance in macaque models showed that prolonged treatment of SIV-infected animals with tenofovir monotherapy lead to the emergence of K65R RT mutants^{18,41,42} which often coincided with or was followed by the development of additional compensatory mutations in the RT (i.e. K64R, N69S, I118V, and S211N). The emergence of K65R viral mutants did not always lead to an increase in viremia, as some animals were able to suppress K65R viremia to low or undetectable levels for many years due to the development of strong CD8⁺ cell-mediated immune responses⁴¹. Since we did not monitor the levels of tenofovir in the plasma during the ART-treatment, we cannot exclude the possibility that the drugs were cleared more rapidly in the two poor ART responders, rendering doses suboptimal or the PMPA prodrug was not efficiently phosphorylated into the active form.

One limitation of the present study was the wide range of viremia in untreated animals, thereby possibly making statistically significant differences even more difficult to observe. Of note, when we looked at the immune parameters of each animal relative to viral loads there was no patterns of differences evident.

The examination of uninfected animals treated or not with ART revealed no indication of peripheral or mucosal immune dysfunction as a result of ART, even at necropsy (see table, Supplementary Digital Content 8 and figure, Supplementary Digital Content 9). However, we observed several virus-induced changes in acute infection. i.e. loss of central memory T cells and mDCs, and mobilization of pDCs, which were restored by ART to levels similar to those seen in control animals. Other studies also reported the early loss of memory T cells^{43,44} and mDCs⁴⁵⁻⁴⁸. Barratt-Boyes et al.⁴⁹ observed a mobilization of pDCs into the blood 3 d.p.i. followed by a significant loss within 14 days after i.v. inoculation with SIVmac251, despite evidence of a profound mobilization of pDCs into blood and recruitment to LNs. We detected increased levels of pDCs from 2 w.p.i., which remained elevated in untreated infection. Different routes of infection (tonsillar versus i.v.), virus strain (SIVmac239 versus SIVmac251) and methods of pDC quantification (frequency versus absolute counts) could account for the differences seen between the studies.

To obtain a better understanding of the unique attributes of mucosal immunity induced by virus and how long-term ART can affect them, we compared the expression of innate

markers in the oral cavity as the site of viral inoculation to those at distal sites. We observed differences in the mRNA expression of innate mediators in acutely infected animals between the oral and rectal site. Type I IFN mRNA expression was reduced in oral tissue but was slightly elevated in rectal samples. In infant macaques infected orally with SIV, several IFN- α subtypes were rapidly induced in lymphoid tissues but only slightly in oral and gastrointestinal mucosal surfaces⁵⁰ indicating that there are differences between distinct anatomical sites in the innate response to virus. The tissue specific variation can be partly explained by the different cellular composition at each site. Similar to our results, data from a larger and more detailed study of early innate immune responses in the infant macaque model of oral SIV infection showed the induction of pro-inflammatory cytokines and the relative lack of antiviral type I IFN responses in oral (gingiva) and mucosal (esophagus and colon) tissues⁵¹. Interestingly, despite the lack of IFN- α response in mucosal tissues, the IFN-inducible genes Mx and CXCL10 were markedly increased in the gingiva and the esophagus of animals with detectable virus replication⁵¹. In our study, CXCL10 up-regulation was driven by acute SIV replication in multiple anatomical sites (oral, rectal and peripheral blood). Several reports showed elevated levels of CXCL10 following oral inoculation of macaques with pathogenic SIV⁵¹⁻⁵³ indicating that CXCL10 could have an important role in pathogenesis. CXCL10 is a potent chemotactic factor for multiple cell types^{54,55} and increased levels of CXCL10 at the site of inoculation may enhance recruitment of and viral spread to target cells. Furthermore, CXCL10 stimulates HIV-1 replication *in vitro*⁵⁶ and systemically heightened levels of CXCL10 could contribute to enhanced viral replication in blood. ART down-regulated CXCL10 expression to levels seen in uninfected controls indicating that active viral replication was responsible for the enhanced expression. We detected increased expression of A3G in acute infection in oral and blood samples, which persisted in untreated infection but remained at control levels in ART-treated animals. A study examining ART-responsive genes in HIV infected individuals also showed that CXCL10 and A3G expression is abrogated after successful ART⁵⁷.

In addition, we detected increased expression of CCL4 mRNA in rectal tissue. The production of beta-chemokines by CD8+ T cells was reported in naive and vaccinated macaques, the largest number of beta chemokine-secreting cells being in the rectal mucosa⁵⁸. CCL4 is one of the major HIV-suppressive factors produced by non-cytotoxic CD8+ T cells⁵⁹ and the increase in the rectal site (following tonsillar challenge) may contribute to the overall innate antiviral response.

As had been reported for short-term tenofovir monotherapy initiated very early (24h or 48h p.i.) in SIVmac239-inoculated macaques^{60,61}, we observed low to background levels of SIV-specific T cell responses during ART. However, SIV-specific CD4+, and to a lesser extent CD8+, T cell responses re-bounded after ART discontinuation suggesting that early ART intervention preserved antigen-specific T cells before they could be affected by the virus. Decrease of HIV-specific cytotoxic T cell responses⁶²⁻⁶⁴ and decay of both CD4+ and CD8+ T memory cell responses under ART has been reported⁶⁵ indicating that maintenance of HIV-specific memory T cells requires antigen persistence. *Candida*-specific T cells were not affected by ART or chronic infection, which is not surprising since the animals (except AA47) remained healthy overall. Connick et al. detected an increase in *Candida*-specific lymphoproliferative responses in HIV-infected individuals after 48 weeks on ART initiated in acute/recent infection, followed by a decrease after treatment interruption⁶⁶. *Candida*-specific lymphoproliferative responses and cytokine secreting capacity of T cells might not be congruent and it is likely that ART was initiated later than 2 w.p.i. with HIV thereby not allowing ART to rescue virus-induced effects.

The limited sample sizes in the study were a limitation to potentially identifying changes in immune functions as a result of ART. However, although we might have missed more subtle

(but significant) changes, it is clear that there were not dramatic differences in the parameters measured as a result of ART exposure. Additionally, due to limited oral and rectal sample collection that could be obtained under survival surgery without endangering the health of the animals we performed more extensive analyses on blood samples. Further studies should include the assessment of mucosal CD4+ and CD8+ T cell responses and DC subsets especially closer examination of oral and rectal CD4+ T cells (i.e. $\alpha\beta\gamma$ T cells), regulatory T cell subsets such as CTLA-4 and IDO-expressing cells and different DC subsets in ART-treated individuals.

In conclusion, tenofovir/emtricitabine ART does not adversely affect mucosal and systemic immune functions in uninfected and SIV-infected macaques. Early virus-induced changes including loss of blood central memory T cells and mDCs and elevated oral, rectal and blood CXCL10 expression were detected, which were rapidly restored by ART to control levels. These data highlight the fact that commencing ART early in infection can help avoid some virus-induced damage to the immune system that might allow immune therapies more chance at boosting potent immune responses to more effectively control virus replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Plasma viral loads and changes in CD4+ T cells counts in untreated and ART-treated animals

(A) Plasma SIV RNA levels were determined by quantitative real time RT-PCR. The ART treatment period is indicated by the shaded grey box. Each symbol indicates an individual animal. Arrows indicate time-points of polyICLC application. Average geometric mean viral loads ($\pm$ SEM) are shown for (B) all ART-treated animals and (C) ART-responding animals (excluding the two transient ART responders). Asterisks indicate significant differences between ART-naïve and ART-treated animals; * $p < 0.05$, ** $p < 0.01$. (D) Absolute CD4+ T cell counts are shown for individual animals over time. AA47 in the untreated group had lower CD4 counts at baseline, showed rapid, progressive loss of CD4+ cells after infection and succumbed to AIDS-related pneumonia 33 w.p.i.

Figure 2. T cell immune responses to SIV- and *Candida albicans* in PBMCs during and after ART cessation

SIV- and *Candida albicans*-specific immune responses were detected in PBMCs by ICS at 26, 34, 51 and 61 w.p.i. PBMCs were gated based on forward/side scatter characteristics and subsequently on CD3+CD4+ or CD3+CD4- cells with doublet discrimination. Average percentages ($\pm$ SEM) of TNF- α -, IFN- γ -, IL-2- and IL-17 single positive (A) SIV-specific CD3+CD4+ (upper panel) and CD3+CD4- (lower panel) T cells, (B) *Candida*-specific CD3+CD4+ (upper panel) and CD3+CD4- (lower panel) T cells are shown. Results are from 8 animals in the uninfected group (white bars), 4 in the SIV+ART- group (except for week 26, n=5; grey bars) and 5 in the SIV+ART+ group (black bars). (C) The percentages of single positive, double positive (IFN- γ /IL-2, IFN- γ /IL-17, IFN- γ /TNF- α , IL-2/IL-17, and IL-2/TNF- α), triple positive (IFN- γ /IL-2/IL-17, IFN- γ /IL-2/TNF- α) and quadruple positive (IFN- γ /IL-2/IL-17/TNF- α) CD4+ and CD4- cells specific for SIV or *Candida* are shown for the SIV-infected animals ($\pm$ ART) at the 34 week time point.

Figure 3. Longitudinal assessment of the dynamics of CD4+ and CD8+ T cell subset frequencies in the blood during and after ART

Polychromatic flow cytometric analysis was performed to identify naïve, central and effector memory and regulatory T cells in PBMCs. PBMCs were gated on small lymphocytes and subsequently on CD3+ cells with doublet exclusion. CD4+ or CD8+ cells were then forward gated based on the expression of CD28 and CD95. Naïve T cells were defined as CD28+CD95-, central memory T cells (TCM) as CD28+CD95+ and effector memory T cells (TEM) as CD28-CD95+ cells. FoxP3+CD25+ regulatory T cells (Treg) were identified in the CD4+ gate. Average fold changes ($\pm$ SEM) of the frequency of the indicated T cell subset normalized on the baseline (three baseline time points were collected from each animal and the average was calculated and set as 1) are shown for uninfected animals (black, n=8), SIV-infected ART naïve animals (red, n=5 from baseline until week 32 and n=4 from week 36 until week 54), SIV-infected ART-treated animals (green, n=5). Asterisks indicate significant differences compared to uninfected animals; * $p < 0.05$, ** $p < 0.01$.

Figure 4. Longitudinal assessment of the dynamics of DC subset frequencies in the blood during and after ART

DCs were detected in PBMCs using polychromatic flow cytometry by gating on forward/side scatter characteristics and subsequently on lineage- (CD3, CD14, CD20 negative) HLA-DR+ cells with doublet discrimination. In the lineage-HLA-DR+ DC gate mDCs were identified as CD11c+ and pDCs as CD123+. Numbers designate the average fold changes ($\pm$ SEM) of the frequency of the indicated DC subset normalized on the baselines (two baseline time points were collected from each animal and the average was calculated and set as 1) for uninfected animals (black, n=8), SIV-infected ART-naïve animals (red, n=5 from baseline until week 32 and n=4 from week 36 until week 54) and SIV-infected ART-treated animals (green, n=5). Asterisks indicate significant differences compared to uninfected animals; * p < 0.05, ** p < 0.01.

Figure 5. Changes in the mRNA expression of innate and effector genes

Shown are normalized average fold changes ($\pm$ SEM) of mRNA expression of innate and effector genes assessed in the oral and rectal mucosa as well as in PBMCs of infected macaques compared to uninfected controls at two different time points after infection. (A) 2 weeks post inoculation – uninfected animals (white bars, n=8), SIV-infected animals (black bars, n=10, except for PBMCs with n=6). (B) 35-36 weeks post inoculation – uninfected animals (white bars, n=8), SIV-infected ART-naïve group (grey bars, n=4), SIV-infected ART-treated group (black bars, n=5). Asterisks indicate significant differences between the groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.