Persistence of Gut Mucosal Innate Immune Defenses by Enteric α-Defensin Expression in the Simian Immunodeficiency Virus Model of AIDS

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Gastrointestinal (GI) complications, including diarrhea, weight loss, and enteric opportunistic infections, occur frequently in therapy-naive HIV-infected patients (1, 2). HIV infection causes severe CD4+ T cell depletion in gut-associated lymphoid tissue (GALT) early in infection, which contributes to impaired T cell responses against pathogens at gut mucosal sites (3). Studies on HIV pathogenesis have been focused on the impairment of HIV-specific immune response and its role in the development of AIDS (4, 5). However, innate immune defenses play a critical role in the protection of GALT against pathogens, and impairment of the innate immune system may accelerate disease progression. A better understanding of the mucosal innate defenses during HIV infection will provide insights into the mechanisms of HIV enteropathogenesis.

The GI epithelium is an important source of innate immune response and provides a functional barrier against enteropathogenic bacteria, viruses, and parasites. Paneth cells (PC), located at the base of intestinal crypts, are key players in the innate immune defense of the intestinal mucosa and express an array of host defense proteins and peptides, including α-defensins (6). Human PC produce two known α-defensins, human defensin (HD)-5 and HD-6, whereas rhesus macaque PC express several highly diverse enteric α-defensins (7) termed rhesus enteric α-defensins (REDs), of which six have been reported (8, 9). The role of defensins has been well established in determining the composition of the small bowel microbiota (10) and in protecting the intestinal crypt epithelium against colonization by microorganisms (11, 12). Anti-HIV activity of various α-, β-, and θ-defensins has also been reported, but their role in controlling HIV infection is unknown (13, 14). The loss of α-defensin during HIV infection may lead to overgrowth of enteric pathogens, resulting in GI dysfunction.

The SIV-infected rhesus macaque model is most suitable for the study of HIV-associated enteropathy (15). Rapid depletion of CD4+ T cells in GALT correlates with active viral replication during primary SIV infection (16). Effects of the SIV infection on enteric innate immune responses in the SIV model have not been fully explored. We hypothesize that mucosal innate immune defenses, including α-defensin expression and secretion, play an important role in protecting the host against intestinal microbial pathogens in SIV infection and may locally compensate for the loss of T cell-mediated immune responses. Therefore, we sought to determine RED expression during SIV infection by analyzing intestinal tissues from rhesus macaques at different stages of infection with and without antiretroviral therapy (ART). Our data showed that RED mRNA expression increased in response to SIV infection as a function of increased PC numbers. SIV-infected animals treated with ART also maintained RED expression. A
marked reduction in RED protein levels was observed in gut mucosa of animals with advanced SIV disease, coinciding with increased incidence of opportunistic infections. Our findings highlight the importance of α-defensins in protection against mucosal pathogens despite severe CD4+ T cell depletion.

Materials and Methods

Rhesus macaques, SIV infection, and therapy
Peripheral blood and intestinal tissue samples from 44 colony-bred rhesus monkeys (Macaca mulatta) were evaluated (17). Animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Samples from six healthy, SIV-negative animals served as negative controls. Thirty-eight animals were i.v. inoculated with 10–100 animal infectious doses of pathogenic SIVmac251 and were euthanized during primary SIV infection (n = 13), chronic infection (n = 9), or advanced infection with simian AIDS (SAIDS; n = 9). Nine SIV-infected animals received 9-(R)-2-(phosphonooxy)propyl adenine (tenofovir disoproxil fumarate or PMPA; N. Bischoffberger, Gilead) monotherapy once daily at 10–30 mg/kg body weight, for 6–132 wk, beginning at 6 (n = 5) or 10 (n = 4) wk post-SIV inoculation (18). Animals receiving ART were euthanized between 12 and 142 wk postinfection (6–132 wk posttherapy). Longitudinal jejunal biopsy samples were collected by upper endoscopy and peripheral blood samples by venipuncture (19).

Microbiological and parasitological examination of intestinal mucosa
Intestinal specimens collected prior to SIV infection, at the onset of an episode of diarrhea and at necropsy, were examined for the presence of Salmonella, Shigella, Yersinia, Pleistomonas, Aeromonas, and Campylobacter species. Fecal specimens were screened for the presence of parasites and bacterial overgrowth using both a saline and an iodine wet preparation. The formaldehyde acetate sedimentation procedure was used for the detection of OVA and parasites. The detection of Cryptosporidium oocysts and Giardia cysts was performed by direct immunofluorescence assay using the Merifluor Crypto and Giardia assay (Meridian Bioscience, Cincinnati, OH). Lesions and occult opportunistic infections were identified by microscopic examination of jejunal tissue sections stained with H&E. Clinical findings were obtained from health records at the California National Primate Research Center.

Measurement of viral burden
Viral burden in plasma and intestinal tissue samples was determined by real time RT-PCR analysis, as previously described (20). A change in the SIV loads was presented as n-fold difference compared with the lowest level of expression, as previously described (ΔΔ cycle threshold method) (20). The SIV-infected cells in intestinal tissue samples were detected and localized using in situ hybridization (ISH) assay (16). Formalin-fixed paraffin-embedded tissue sections were hybridized with digoxigenin (DIG)-labeled SIV gag-specific RNA probe (SIV239, clone from C. Brown, catalogue L3-2100SP, Lofstrand Labs, Gaithersburg, MD) at 56˚C for 15 h and processed. The SIV-infected cells stained with NBT-5-bromo-4-chloro-3-indolylphosphate (Roche) in Levamisole and counterstained with nuclear fast red, were visualized using a light microscope. The percentage of SIV RNA+ cells was also analyzed by real-time RT-PCR (Table I). A change in the SIV RNA load was presented as back-transformed least squares means.

Localization of RED protein in intestinal tissue

The cellular localization of SIV protein was determined by enzymatic immunohistochemistry (IHC) using the BioGenex i6000 Automated Staining System and BioGenex IHC reagents (BioGenex, San Ramon, CA). Tissue sections were incubated overnight at 4˚C with a 1:800 dilution of rabbit anti-HD-5 serum (courtesy T. Ganz, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA) and stained using diaminobenzidine (BioGenex, San Ramon). The RED protein-expressing cells with red-brown color were visualized under light microscope. The percentage of crypts with HDS protein was also analyzed.

Results

SIV replication and histopathologic changes in gut mucosa
Thirty-eight SIV infected rhesus macaques were evaluated for the presence of SIV-infected cells and pathologic changes in gut mucosa compared with six uninfected healthy rhesus macaques. SIV-negative control animals showed no obvious GI pathology or clinical signs (Table I). Animals with primary or chronic SIV infection exhibited lymphohfollicular hyperplasia or lymphadenopathy and splenomegaly, but had no evidence of pathologic changes or gastroduodenal ulceration (Table I). Animals with SAIDS exhibited multiple GI complications, including gastrecterocolitis, nonresponsive diarrhea, and cholecystitis, as well as dehydration, anemia, and pulmonary complications. One SIV-infected animal with rapid disease course (rapid progressor [RP]) developed SAIDS at 12 wk postinfection and had GI complications. A long-term SIV-infected nonprogressor (LTNP) animal suffered from Pneumonysissimicola infection of the lungs and anemia at the time of necropsy, but experienced only mild GI dysfunction.

Viral burden in gut tissue of SIV-infected animals was evaluated by ISH as well as by real-time RT-PCR (Table I). SIV RNA was detected in lymphocytes and macrophages in the gut mucosa and was localized to the villus tips and cryptopatches (isolated lymphocytic aggregations). A few infected cells were detected in lamina propria surrounding the base of the crypts (Figs. 1, 2). SIV+ cells were not frequent in the villus epithelium, with the exception of animal 26333, which had the majority of its infected cells located in the epithelial layer. Highest numbers of infected cells were seen during primary SIV infection (ISH score = 0–6, median 3.0) or during SAIDS (ISH score = 0–6, median 3.4). Low levels of SIV RNA+ cells were observed in the gut mucosa of therapy-naïve animals with chronic SIV infection, as well as in the LTNP (ISH score = 0–2, median 1.3). In contrast, the RP animal had high numbers of SIV-infected jejunal lymphocytes (ISH score = 6).

Viral burden in jejunal mucosa from 26 animals was measured by real-time RT-PCR. In concordance with the ISH findings, highest levels of SIV burden were found during primary and advanced stages of disease, whereas a reduction in the viral burden was noted...
## Table 1. Summary of clinical and pathological characteristics and microbial findings in study groups

<table>
<thead>
<tr>
<th>Clinicopathological markers</th>
<th>SIV Negative</th>
<th>Primary</th>
<th>Chronic</th>
<th>SAIDS</th>
<th>PMPA Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of infection (wk)</td>
<td>Uninfected</td>
<td>0.4–2</td>
<td>4–26</td>
<td>11–78</td>
<td>12–142</td>
</tr>
<tr>
<td>Length of therapy (wk)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>4–132</td>
</tr>
<tr>
<td>Age range (mo)</td>
<td>59–103</td>
<td>32–72</td>
<td>25–68</td>
<td>25–75</td>
<td>82–103</td>
</tr>
<tr>
<td>Weight range (kg)</td>
<td>6.3–8.2</td>
<td>2.7–7.1</td>
<td>2.8–4.4</td>
<td>2.5–6.2</td>
<td>4.6–5.1</td>
</tr>
<tr>
<td>Relative fold Δ in RED</td>
<td>(1.1)–0.9</td>
<td>(1.1–7.0)</td>
<td>1.4–6.8</td>
<td>1.3–11.4</td>
<td>(3.2)–0.5</td>
</tr>
<tr>
<td>Viral burden: relative fold Δ/(ISH)</td>
<td>UD(0)</td>
<td>5.3 ( \times 10^{-3} - 4.3 \times 10^7/(1–6)</td>
<td>1.0 ( \times 10^7 / (0–3)</td>
<td>UD: 3.0 ( \times 10^7/(0–6)</td>
<td>UD: 0.4 \times 10^3/(0–1)</td>
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### Clinical observations

- **SIV Negative**: Various commensal bacteria, Lymphofollicular hyperplasia, recurrent diarrhea, various commensal bacteria
- **Primary**: Lymphadenopathy, SM, Pn
- **Chronic**: Nonresponsive recurrent diarrhea, cachexia, LA, SM, choledochochistis, I/D, pulmonary acarisis, Pn, GEC, various commensal and pathogenic bacteria, anemia
- **SAIDS**: LA, renal tubular karyomegaly, SM, choledochochistis, I/D, GEC
- **PMPA Treated**: LA, gallbladder disease, SM, choledochochistis, I/D, GEC

### Intestinal pathologic changes

- **E; within normal limits**
- **E; acute to chronic, diffuse, mild to moderate**
- **E; subacute to chronic, diffuse, mild to moderate**
- **E; subacute to granulomatous, diffuse, mild to severe**
- **E; subacute, diffuse, mild to severe**

### Comorbidity factors (%)

- **Diarrhea**: 33%
- **Cachexia**: 0%
- **Lymphadenopathy**: 0%
- **Splenomegaly**: 0%
- **Enteritis**: 17%
- **Colitis**: 0%

### Secondary infections (%)

- **Trichomonas hominis**: 0%
- **Blastocystis hominis**: 17%
- **Campylobacter coli** or jejuni): 42%
- **Entamoeba coli**: 17%
- **α-hemolytic Streptococci**: 0%
- **Balantidium coli**: 0%
- **Coagulase + Staphylococcus**: 0%
- **Cryptosporidia**: 0%
- **Giardia**: 0%
- **Gram – rods, anaerobic**: 0%
- **Streptococcus viridans**: 0%

**Bold font designates values that differ significantly from other treatment groups \((p < 0.0395)\).**

**Determinations made by veterinary pathologists at the time of necropsy.**

**Determinations made by retrospective examination of formalin-fixed tissues.**

**Data for comorbidity factors and secondary infections were obtained directly from clinical records maintained at the California National Primate Research Center (Davis, CA). Microbial and parasitological examinations performed within 1 mo of necropsy were included within these estimations.**

E: enteritis; Ex: exocytosis; GEC: gastroenterocolitis; I/D: inanition/dehydration; Id: lacteal dilatation; LA: lymphadenopathy; Lp: lymphopenia; NA: data are not available for this group; Pn: pneumonia; SM: splenomegaly; UD: undetectable levels (below 50 copies) of SIV RNA; vbf: villus blunting/fusion.
during chronic SIV infection (Fig. 3, Table I). Analysis of plasma samples from 17 animals by real-time RT-PCR showed that plasma viral loads correlated with tissue viral burden, with the highest levels seen during primary and SAIDS stages of infection and decreased burden during chronic SIV infection and ART (Supplemental Fig. 1). As previously demonstrated, CD4+ T cell depletion was observed in peripheral blood and was dramatically evident in GALT (Supplemental Fig. 1) (21).

**Increased expression of RED and PC numbers in response to SIV infection**

The location of RED mRNA-expressing cells, performed by ISH, was restricted to the base of crypts, and they were identified morphologically as PC based on presence of apical granules, basal nucleus, and pyramidal shape (Fig. 4A) (22). Longitudinal analysis of jejunal biopsy samples from six SIV-infected animals detected expression of RED mRNAs throughout the course of SIV infection (Fig. 4B). Our results indicated that RED mRNA was expressed in ∼98% of crypts in the jejunal mucosa of healthy uninfected animals and did not significantly change through the course of SIV infection (Fig. 5F). Variation in the intensity of RED immuno-reactivity did not correlate with stage of disease or viral burden.

Quantification of RED mRNA levels in gut mucosa demonstrated an increased expression of RED transcripts in SIV-infected, therapy-naive animals during primary (mean 2.8-fold increase), chronic (mean 3.7-fold increase), or SAIDS (mean 4.7-fold increase) stages of infection, as compared with uninfected controls (Fig. 3). A significant increase in RED expression was seen in SIV-infected, therapy-naive macaques as compared with uninfected controls and PMPA-treated animals (*p*, 0.04; Fig. 3), and was associated with more PC per jejunal crypt (Fig. 5D). Examination of H&E-stained gut tissue sections detected an average of 5 PC per crypt in SIV-negative controls, whereas SIV-infected animals had 10, 12, and 14 PC per crypt during primary, chronic, and SAIDS stages of infection, respectively. Collectively, our data suggested that SIV infection led to an increase in RED gene expression and expansion of PC numbers in jejunal mucosa that remained elevated throughout the course of SIV infection, independent of the magnitude of viral burden within the gut mucosal tissue.
Decreased RED protein levels correlate with enteric opportunistic infections and advanced SIV disease

IHC analysis was performed to determine the cellular localization of RED proteins. RED proteins were detected in jejunal tissues from uninfected and SIV-infected animals during the primary and chronic stages of infection, as well as in the LTNP animal (92% RED-positive crypts) (Fig. 6E–H). IHC analysis showed a reduction of RED protein levels in PC in SAIDS animals, including the animal with rapid disease progression (Fig. 6G, 6H). The percentage of RED⁺ crypts did not differ between SIV-ve control animals and SIV⁺ asymptomatic animals. However, a sharp decline in the percentage of RED⁺ crypts was observed in animals with SAIDS (Fig. 5E).

Although the primary structure of HD-5 differs greatly from known RED peptide sequences, a short region of highly conserved sequence identity exists between RED4, RED8, and HD5, shown as underlined, bold residues in the HD-5 sequence: ATCYCRTGRCATRESLSGVCEISGRLYRLCCR. The shared CRTGRC could be an epitope shared by these REDs that is recognized by anti–HD-5 Ab. The defensin immunoreactivity in the lumen appeared to be increased in the animals with SAIDS as compared with uninfected controls and animals during primary or chronic SIV infection (Figs. 5E, 6).

Serial gut tissue sections examined for either RED mRNA or protein, or SIV RNA demonstrated that RED protein was localized to dense granules and RED mRNA to the cytoplasm of corresponding cells in tissues from all animals. SIV RNA was not detected in PC, suggesting that PC did not support viral replication. A representative example of RED expression in an SIV-infected animal during primary acute stage of infection with high viral burden is shown (Fig. 5).

Advanced SIV disease was marked by a significant loss of RED protein in PC that coincided with an increase in the incidence of bacterial and protozoal infections (Table I). The infections included Fusobacterium, Bacteroides, α-hemolytic Streptococcus, Streptococcus viridans, coagulase-negative/positive Staphylococci, Corynebacterium, Pseudomonas maltophilia, Iodamoeba butschlii, Peptostreptococcus, and Lactobacillus species, each of which was found in one or more animals. Pentatrichomonas hominis was the...
most prevalent pathogen in SAIDS animals (seven of seven) despite treatment of five of these animals with metronidazole. There was a significant association with the loss of RED protein and the presence of Trichomonas infection of the large intestine in animals with SAIDS ($p \leq 0.0004$). Blastocystis hominis was the second most common infection (five of seven). This incidence may have been due to the ineffectiveness of most antiprotozoal medications against the organism. Entamoeba coli and Campylobacter were the third most prevalent pathogens in SAIDS animals (four of seven). E. coli are typically nonpathogenic, even in people with impaired immune functions. However, a breakdown of mucosal epithelium may enable its entry and colonization. Animals with SAIDS, including the RP animal, had 8 or more secondary infections and had nonresponsive, recurrent diarrhea even after metronidazole therapy. Two of these animals had high viral burden (ISH score = 5), but one animal had a very low viral burden (ISH score = 1).

Two animals with primary SIV infection had three or more secondary infections. The presence of RED protein in the PC of these animals was similar to that seen in uninfected animals. A high incidence of Campylobacter jejuni or C. coli was seen during the primary acute stage of infection, but was not evident during chronic SIV infection. Specific immunity, involving intestinal Ig (IgA) and systemic Abs, is required for healthy animals to control Campylobacter infection. The proinflammatory environment and eventual breakdown of the mucosal epithelial barrier during SAIDS could predispose animals to recurrence of Campylobacter infection due to entry and colonization.

**ART led to suppression of SIV replication and maintenance of enteric defensin expression**

We evaluated the effect of ART on viral replication and expression of RED peptides during SIV infection. The ART led to suppression of SIV loads in peripheral blood and in gut mucosal tissues (Table I). IHC and ISH analyses detected the expression of RED mRNA and protein in gut tissue samples from all SIV-infected animals receiving ART (Fig. 7). However, quantitative analysis of RED mRNA expression by real-time PCR showed that animals receiving ART had significantly decreased RED mRNA levels compared with therapy-naive SIV-infected animals, but similar to SIV-negative control animals (1.6-fold decrease, $p < 0.0003$) (Fig. 3). Our data showed that viral suppression induced by ART of SIV-infected monkeys might lead to the maintenance of RED protein expression that is comparable to uninfected healthy controls and better protection against enteric microbial infections. Thus, prevention of the loss of RED expression in the gut mucosa of these animals during ART would contribute to effective innate immunity at the mucosal site.

**Discussion**

One of the most common causes of mortality among ART-naive, HIV-infected individuals is the incidence of opportunistic infections. The innate immunity plays an important role not only during the initial stages of HIV infection, prior to the development of a virus-specific adaptive immune response, but also throughout the course of infection by protecting the host against various pathogens. Our study examined the role of mucosal innate defense in HIV infection by analyzing changes in the expression of rhesus enteric α-defensins in gut mucosa of SIV-infected rhesus macaques in the context of viral replication and CD4+ T cell depletion. Our findings showed an increased level of RED gene expression associated with an increase in PC numbers at all stages of SIV infection in therapy-naive SIV-infected animals. This suggests that REDs may be an important component of the gut mucosal innate immune defense that persists through primary and chronic stages of SIV infections.
Several mechanisms may contribute to the upregulation of REDs in the PC during SIV infection and their loss during the advanced stages of infection. Severe loss of CD4+ T cells including CD4+ Th17 cells in gut mucosa during primary HIV and SIV infections has been well documented (15, 23). This CD4+ T cell loss is not reversed despite the development of virus-specific humoral and cellular responses (3). The immediate and long-term impact of mucosal CD4+ T cells is not fully defined. It has been demonstrated that SIV-induced loss of CD4+ Th17 T cell in gut mucosa of SIV-infected rhesus macaques led to impaired Th17 CD4+ T cell responses to the Salmonella typhimurium infection in the gut mucosa and resulted in increased systemic bacterial translocation (24). Thus, the gut mucosal CD4+ T cell depletion may lead to incomplete control of enteric pathogens and increased microbial translocation from the lumen. An increase in RED gene transcription may be a mechanism by which the host mucosal response contains the microbial burden. It is also reasonable to propose that increased RED expression may partly compensate for the immune defects generated in the gut mucosa by SIV infection. Whereas the number of REDs in rhesus macaque PC far outnumbers that in human PC, identification of individual defensins is beyond the scope of this study. Their antimicrobial and antiviral activity may be crucial in providing protection to crypt epithelial progenitor cells as well as T cells in the neighboring regions. Further characterization of PC-derived RED peptides will be important to gain better understanding of the PC-mediated mucosal innate defense.

Increased microbial translocation due to the breakdown of the gut epithelial barrier in SIV infection may also impact enteric defensin gene expression and peptide levels and warrants further investigation. Disruption of the integrity of the intestinal epithelium in HIV and SIV infections has been well documented and is linked to decreased nutrient digestion and malabsorption and increased microbial translocation (24, 25). Increased expression of genes associated with inflammation and immune activation in the gut mucosa during HIV and SIV infections has been reported (21). Histopathologic changes of villus atrophy and crypt hyperplasia along with the mucosal CD4+ T cell loss may contribute to mucosal inflammation and inability to control microbial burden. Our findings of increased PC numbers in the gut mucosa suggest that the impairment or loss of adaptive immune responses and the integrity of the villus epithelial barrier secondary to SIV infection may result in the need for a compensatory increase in crypt innate responses resulting in expansion of the PC compartment. In a mouse model of inflammatory bowel disease, gut mucosal inflammation and disruption of PC homeostasis with hyperplasia have been reported that were associated depletion of PC gene products and secretory granules (26). ART is effective in suppressing viral loads and reducing inflammation in the gut mucosa. We found that animals receiving ART had RED expression comparable to the uninfected controls and low to almost no incidence of opportunistic infections in the GI tract as compared with untreated animals, indicative of better mucosal immune functions (27). It is possible that this may reduce PC secretion of innate proteins, thus

**FIGURE 6.** Expression of RED protein and presence of eosinophilic granules in gut mucosal PC during SIV infection. RED protein was detected by IHC using HD-5 Ab (left column, original magnification ×100). Eosinophilic secretory granules were visualized by conventional H&E staining (right column, original magnification ×200). Intestinal tissues were collected from SIV-negative controls (A, B) and macaques during primary (C, D), chronic (E, F), and SAIDS (G, H) stages of SIV infection. Localization of immunoreactivity within PC granules (I, original magnification ×600).

**FIGURE 7.** Localization of RED mRNA and protein in mucosa of SIV-infected rhesus macaques receiving PMPA therapy. Expression of RED protein (A) and mRNA (B) was detected in intestinal tissues from SIV-infected, PMPA-treated animals. Representative samples shown were obtained from four animals that began therapy at 6–10 wk post-SIV infection. Original magnification ×200.
The authors have no financial conflicts of interest.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Plasma Viral loads, Peripheral Blood CD4+ T cell counts and Jejunal Mucosa CD4+ T cell % in SIV infected animals and SIV negative controls. (A) Animals in the chronic stage of SIV infection had lower viral loads than during the primary stage (set point). PMPA significantly reduces viral loads in infected macaques. (B) SIV infection reduces CD4+ T cell numbers in peripheral blood (C) A dramatic loss of CD4+ T cells were observed in all stages of infection compared to uninfected controls. incomplete CD4+ T cell restoration observed in both PBMC and LFL. * indicated significant differences between groups.