CCAAT/Enhancer Binding Protein β Is a Major Mediator of Inflammation and Viral Replication in the Gastrointestinal Tract of Simian Immunodeficiency Virus-Infected Rhesus Macaques

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The gastrointestinal tract (GIT) is a major target of infection with human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Chronic GIT disease and inflammation are common sequelae to HIV/SIV infection. Nonetheless, the molecular mechanisms that cause and maintain GIT dysfunction remain unclear. We investigated the contribution of CCAAT/enhancer-binding protein β (C/EBP β) to GIT disease and viral replication in jejunum and colon collected at necropsy from 12 SIV-infected (group 1), or 10 uninfected macaques with chronic diarrhea (group 2), and 9 uninfected control macaques (group 3). All group 1 and 2 macaques had chronic diarrhea, wasting, and colitis, but group 1 animals had more severe lesions in the jejunum. C/EBP β gene expression increased significantly in colon of groups 1 and 2 and in jejunum of only group 1 macaques compared with controls. In group 1 animals, CEBP β expression was localized predominantly to macrophages and occasionally lymphocytes. Chromatin immunoprecipitation assays confirmed the binding of C/EBP β and p65 to the SIV long terminal repeat region in colonic lamina propria cells, suggesting a mechanistic link between inflammation and activation of viral replication *in vivo*. This is the first *in vivo* study describing the transcriptional changes and immunophenotypic localization of C/EBP β in the GIT of SIV-infected macaques. More importantly, these data provide a molecular mechanism for persistent inflammation and immune activation leading to increased SIV burden and GIT pathology in SIV-infected macaques and perhaps HIV-infected individuals. (Am J Pathol 2008, 173:106–118; DOI: 10.2353/ajpath.2008.080108)

Infection with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) in humans, leads to progressive immune deficiency because of massive loss of CD4⁺ T cells. This observation has been confirmed and extended using the macaque model of AIDS in which rhesus macaques are infected with simian immunodeficiency virus (SIV), a close relative of HIV.¹ Based on these studies, which have been confirmed in HIV-infected humans, it is clear that the most significant and rapid depletion of CD4⁺ T cells occurs in the gastrointestinal tract (GIT) during the first 14 to 21 days after infection.^{2–4} Associated with the loss of GIT CD4⁺ T cells are a variety of symptoms of GIT dysfunction that affect most HIV-infected patients. Interestingly, even in developed countries, ~9% of HIV patients on highly active antiretroviral therapy with normal peripheral CD4⁺ T-cell counts still presented with opportunistic GIT disorders.⁵

Several opportunistic infections caused by protozoal, viral, bacterial, and fungal agents as well as possible direct effects of HIV infection have been implicated as contributing to the pathogenesis of intestinal dysfunction.^{6–9} Although the structural components of the GIT have not been reported to be targets for HIV/SIV, we and others have described signs of intestinal dysfunction during the first few weeks after SIV infection in macaques even before the appearance of opportunistic infection.^{10–13} Although the pathogenesis of this enteropathy remains unclear, there is ample evidence that severe intestinal immune dysfunction can lead directly to marked structural and functional abnormalities of the GIT.^{14,15}

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More recently, the acute depletion of mucosal CD4⁺ T cells and the immune dysfunction that is associated with HIV/SIV infection has been linked with disruption of the intestinal barrier and microbial translocation that would cause chronic immune activation facilitating viral replication and the development of AIDS.¹⁶ The specifics of the interplay between immune dysfunction caused by destruction of intestinal CD4⁺ T cells and immune activation (both of which occur in HIV/SIV infection) in mediating GIT dysfunction are poorly understood. Accordingly, these observations underscore the need to investigate the various immunological signaling pathways that mediate this interaction so that we can better understand the underlying pathogenic mechanisms to elucidate therapeutic strategies that can complement existing highly active antiretroviral therapy regimens.

Although the etiology of GIT dysfunction in HIV-infected patients remains complex, there is ample evidence to suggest that HIV/SIV infection results in the modulation of circulating levels of several host-derived factors. Among these, proinflammatory cytokines released by infected lymphoid cells and other inflammatory cells may be expected to play a central role because they can induce inflammation¹⁷ and at the same time influence viral replication.^{18,19} Increased production of interleukin (IL)-6, RANTES, IL-10, tumor necrosis factor- α , IL-8, and interferon- γ , at least at the mRNA level, in the intestinal mucosa of HIV-infected patients and SIV-infected rhesus macaques may play a role in the development of GIT dysfunction.^{20,21} We have previously reported up-regulation of IL-6 in the GIT of SIV-infected rhesus macaques with chronic diarrhea.²² IL-6 is a cytokine that has been reported to facilitate the transition from acute to chronic inflammation.²³ Increases in IL-6 mRNA were also accompanied by constitutive activation of p-STAT3 and increases in SOCS-3 mRNA.²² Expression of p-STAT3 was localized to CD68-expressing macrophages and scattered CD3-expressing lymphocytes in the GIT of SIV-infected rhesus macaques with chronic diarrhea.²²

Besides activating STAT3, IL-6 also induces the expression of CCAAT/enhancer binding protein β (C/EBP β) either through a tethering mechanism involving STAT3 and CUG binding protein²⁴ or through the Ras-Raf-MAPK signaling pathway.²⁵ The C/EBP family of transcription factors include transcription activators C/EBP α , C/EBP β , and C/EBP-related protein 1, and negative regulators such as C/EBP γ , liver-enriched transcriptional inhibitory protein (LIP), and C/EBP-homologous protein 10 (CHOP-10).^{26,27} C/EBP β is an intronless gene and the presence of an internal ribosome entry site facilitates the production of several variants with activation or inhibitory functions.²⁸ C/EBP β also described as nuclear factor (NF)-IL6, NF-M, liver transcriptionally enriched activator protein (LAP), IL-6-D-element binding protein/EBP, and C/EBP-related protein 2, has three reported variants in the human: 46-kDa full-length, 41-kDa LAP, and 16-kDa LIP.²⁹ LAP, similar to the full-length C/EBP β activates transcription and shows reduced expression levels in normal tissues.^{30,31} However, inflammatory stimuli such as bacterial lipopolysaccharide (LPS), IL-6, IL-1 β ,

or interferon- γ results in significant up-regulation of C/EBP β .³¹ In addition, activation of C/EBP β is also contingent on phosphorylation of serine or threonine residues catalyzed by various intracellular kinases.²⁹ LIP lacks most of the transactivation domain in the N-terminal region and functions as a dominant-negative inhibitor of C/EBP-mediated transcription of target genes.²⁸ Functionally active C/EBP β proteins have been reported to regulate transcription by binding to their cognate DNA binding elements either in association with other C/EBP isoforms or other transcription factors such as p65³² and p50 subunit of NF- κ B, CREB/ATF, AP-1, glucocorticoid receptor, hepatitis B virus X protein, and the retinoblastoma protein.³¹ Furthermore, the identification of C/EBP β functional responsive elements in the promoter region of IL-1 β ,³³ tumor necrosis factor- α ,³⁴ IL-6,³⁵ IL-8,³⁶ cyclooxygenase 2 (COX-2),²⁹ as well as chemokine receptors such as CCR2³⁷ and CCR5³⁸ throws more light on the proinflammatory properties of this transcription factor.

Additionally, the inclusion of three and up to five C/EBP β binding sites on the long terminal repeat (LTR) of HIV and SIV,³⁹ respectively, provides an opportunity for HIV and SIV to subvert normal immune regulation to its own advantage. Further, C/EBP β is essential for efficient HIV replication in macrophages but not in CD4⁺ T cells^{40–42} and has been reported to play an important role in regulating HIV/SIV replication in brain macrophages^{43,44} and alveolar macrophages.^{45–47} Although the GIT is well established as a major target for HIV/SIV replication, the role of C/EBP β as a mediator of inflammation and viral replication in this tissue has not been examined. In our earlier study, we observed that all SIV-infected macaques with high IL-6 and constitutive STAT3 expression had high mucosal viral loads.²² Although constitutive STAT3 expression in the GIT was associated with inflammation,²² it does not have a binding site on the HIV/SIV LTR. Therefore, in the present study, using the SIV-rhesus macaque model of AIDS we attempted to identify mechanisms by which IL-6 promotes not only GIT inflammation/disease but also viral replication with a special focus on the role of C/EBP β . The results of our study point to a significant increase in C/EBP β mRNA and protein expression as a key event. C/EBP β gene and protein expression was significantly increased in SIV-infected rhesus macaques with chronic diarrhea and was found to efficiently bind to the SIV LTR in infected cells isolated from the colonic lamina propria. We conclude that chronic uncontrolled intestinal inflammation, characterized by increased expression of C/EBP β may lead to persistent localized immune activation, disruption of the intestinal architecture and function, and facilitate viral replication that ultimately leads to chronic diarrhea, wasting, and death.

Materials and Methods

Animals and Tissue Collection

Tissues were collected from a total of 31 animals including 12 animals infected with pathogenic strains of SIV

Table 1. Animals, Inoculum, Viral Load, CD4⁺ T-Cell Count in Group 1 Macaques

Animal group and no.	Duration of infection (days)	Inoculum	CD4 count cells/ μ l*	Plasma viral copies/ml $\times 10^6$	Viral copies/ mg of total RNA $\times 10^6$, colon	Viral copies/ mg of total RNA $\times 10^6$, jejunum
SIV-infected with diarrhea (group 1)						
AJ82	232	SIVmac251	NA	NA	1.95	0.078
DD88	388	SIVmac239	50	1.2	26.89	3.35
CI65	377	SIVmac239	450	1.5	4.59	2.5
L441	170	SIVmac251 and 239	632	1.26	15.5	33.2
H405	232	SIVmac239	523	71.4	17,200	15,480
V205	973	SIVmac239	296	0.3	8.0	3.45
AT81	171	SIVsmG932	640	0.06	0.5	0.057
T56	1460	SIVmac251 and 239	56	360	213,000	16,380
DT56	265	SIVmac239	931	1.2	5.9	NA
DI28	81	SIVmac251	954	0.018	41	1.27
DE68	70	SIVmac239	275	1	59	NA
EB17	111	SIVmac251	935	3.6	2.7	NA

*The lower end of the normal range for CD4⁺ T cells/ μ l of blood in the rhesus macaques is 800 cells/ μ l of blood. NA, not applicable.

(group 1) that use CCR5 *in vivo* and 19 animals not infected with SIV. Of the uninfected animals, 10 had chronic diarrhea (group 2) and 9 did not (group 3). The animals in group 2 with chronic nonresponsive diarrhea of no known infectious etiology have been described and used as a model of inflammatory bowel disease.^{48,49}

It would be ideal to have a fourth group consisting of SIV-infected animals without diarrhea. Unfortunately, untreated SIV infection consistently leads to GIT dysfunction and diarrhea and hence it is not possible to include such a group. Jejunum and colon specimens were collected at necropsy from the 12 SIV-infected macaques with chronic diarrhea (group 1), 10 non-SIV-infected macaques with chronic diarrhea (group 2), and four uninfected control macaques (group 3) (Table 1). Chronic diarrhea and weight loss were major causes for euthanasia in all animals except those in group 3. In addition, pinch biopsies from jejunum and colon were collected from another five control macaques bringing the total number of control macaques (group 3) used in this study to nine. Colon specimens were collected for all 31 macaques. Jejunum specimens were available for 9 of 12 group 1, 6 of 10 group 2, and 9 of 9 group 3 control macaques. All animals in groups 1 and 2 were euthanized when they became unresponsive to treatment (eg, subcutaneous or intravenous fluids and antibiotics as appropriate based on a culture and sensitivity) or lost greater than 20% of their body weight. After euthanasia with an intravenous overdose of pentobarbital all animals received a complete necropsy and histopathological examination. All tissues were collected in RNAlater (Ambion, Austin, TX) for RNA quantification and confocal microscopy. According to the manufacturer, RNAlater protects both RNA and protein (by reversible inhibition of nucleases and proteases) in addition to preserving tissue architecture. Tissues were also collected in cryovials and snap-frozen by immersion in a 2-methylbutane/dry-ice mixture for protein extraction.

Histopathology

GIT tissues were collected immediately after euthanasia and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μ m, and stained with hematoxylin and eosin (H&E) for analysis. Sections of jejunum and colon were examined in a blinded manner and inflammation was scored semiquantitatively on a scale of 0 to 3 as follows: 0, within normal limits; 1, mild; 2, moderate; 3, severe. In addition, the presence of crypt dilatation, villous blunting, diverticulosis, and amyloidosis were recorded (Table 2).

Quantitative Real-Time SYBR Green One-Step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Gene expression for C/EBP β in the jejunum and colon tissues was evaluated by the Quantitative Real-Time SYBR Green One-Step RT-PCR assay (QRT-PCR) (Qiagen Inc., Valencia, CA). Total RNA was extracted from both jejunum and colon samples using the SV Total RNA isolation kit (Promega Corp., Madison, WI) and a RNA sample representing the colon and jejunum from each macaque was assayed in triplicate wells. Each QRT-PCR reaction (25 μ l) contained the following: 2 $\times$ Master Mix without uracil-N-glycosylase (12.5 μ l), reverse transcriptase (0.25 μ l), target forward and reverse primer, and total RNA (200 ng) quantified spectrophotometrically based on A₂₆₀:A₂₈₀ ratios. Forward and reverse primer sequence for C/EBP β including β -actin were as follows. An ~111-bp region of C/EBP β was PCR amplified using 500 nmol/L of forward (5'-AACCTGGAGACGCAGCA-CAA-3') and reverse primer (5'-CTTGAACAAGTCCG-CAGGGTG-3') on the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA) using the following thermal cycling conditions: 50°C for

Table 2. Intestinal Histopathology in Groups 1, 2, and 3 Macaques

Animal group and no.	Intestinal histopathology*	
	Colon	Jejunum
SIV-infected with diarrhea (group 1)		
AJ82	2	3
DD88	1 and AMD	1 and AMD
CI65	1 and AMD	1 and AMD
L441	1	1
H405	3 and CD	2
V205	3	1 and AMD, VB
AT81	3	3 and VB
AT56	3 and AMD, CD	3
DT56	3 and CD	0
DI28	1	1
DE68 [†]	3 and CD	NA
EB17 [†]	1	NA
Non-SIV-infected with diarrhea (group 2)		
EI90	3 and CD	NA
EL45	3 and CD	NA
EC49	3 and CD	NA
EB12	3 and CD	NA
EM41	1	1
EL71	3 and CD, DV	0
EB27	3 and CD, DV	1
DJ15	3 and CD	1
CT77	2	1
EJ54	3	1
Uninfected controls (group 3)		
BV52	0	0
EH70	0	0
CB98	0	0
CF33	0	0
M302	0	0
CC96	0	0
R842	0	0
EL66	0	0
EH80	0	0

*Sections of jejunum and colon were examined in a blinded fashion and inflammation was scored semiquantitatively on a scale of 0 to 3 as follows: 0, within normal limits; 1, mild; 2, moderate; 3 severe. In addition the presence of crypt dilatation (CD), villous blunting (VB), diverticulosis (DV), and amyloidosis (AMD) were recorded.

[†]Macaques DE68 and EB17 were only used for the ChIP assay and their histopathology severity scores were not included for the statistical analysis.

NA, not applicable.

30 minutes, 95°C for 15 minutes followed by 40 repetitive cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. As a normalization control for RNA loading, parallel reactions to amplify an ~107-bp product representing β -actin mRNA were run in the same multi-well plate using 300 nmol/L forward (5'-AGGCTCTTCC-CAACCTTCCTT-3') and reverse (5'-CGTACAGGTCTT-TACGGATGTCCA-3') primer.

Quantification of gene amplification after RT-PCR was made by determining the threshold cycle (C_T) number for SYBR Green fluorescence within the geometric region of the semilog plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is

equivalent to a doubling of the amplified product of the PCR. The relative quantification of target gene expression across treatments was evaluated using the comparative C_T method. The ΔC_T value was determined by subtracting the β -actin C_T value for each sample from the target C_T value of that sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (ie, sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of target was determined by evaluating the expression, $2^{-\Delta\Delta C_T}$.

Immunoprecipitation and Western Blotting

Small 2.5-cm² pieces of colon from two group 1, and three group 2 and 3 animals were crushed using a disposable polypropylene pellet pestle and protein extraction was performed in a lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, 1 mmol/L Na₃VO₄, 1 μ g/ml leupeptin, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Approximately 500 μ g of total protein extract was first precleared with 5 μ l of normal mouse immunoglobulin for ~20 minutes and then immunoprecipitated with ~10 μ l of a mouse monoclonal antibody against C/EBP β (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C followed by incubation with 30 μ l (50% w/v) of protein G agarose beads (Invitrogen Corp., Carlsbad, CA) at 4°C for 4 to 5 hours. The supernatant was removed and transferred to a separate 1.5-ml microcentrifuge tube and immunoprecipitated using goat polyclonal antibody (~4 μ l) (Santa Cruz Biotechnology) for β -actin at 4°C overnight on a shaker. Immunoprecipitated C/EBP β and β -actin proteins were heat-denatured for 5 minutes at 100°C in sample loading buffer containing 62.5 mmol/L Tris-HCl, 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and bromophenol blue, resolved on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to 0.2- μ m nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with a rabbit polyclonal primary antibody against C/EBP β (LAP) (Cell Signaling Technology, Inc.), and rabbit polyclonal antibody against β -actin (Santa Cruz Biotechnology) followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Membranes were treated with Supersignal West-Femto or Pico chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL) for 5 minutes and the signal was developed by exposing the membrane to X-OMAT Kodak film (Eastman-Kodak, Rochester, NY) for 1 to 5 minutes. Immunoprecipitated C/EBP β proteins from groups 1 and 2 animals were run on one gel and the control animals on another gel. Hence the images shown in Figure 3A (see below) were obtained from two separate radiographs, cropped, aligned, and fused into one composite image.

Lamina Propria Cell (LPC) Isolation and Chromatin Immunoprecipitation (ChIP) Assay

Given that the objective of using the ChIP assay is to demonstrate *in vivo* binding of C/EBP β to the SIV LTR in infected cells during inflammation, large number of cells carrying the SIV genome are required. However, isolating a pure population of SIV-infected cells from the GIT is also not technically feasible. Therefore we performed the assay using a heterogeneous population of LPCs (a mixture of T cells, plasma cells, stromal cells, macrophages, dendritic cells, and possibly neutrophils) assuming that interspersed in this population are SIV-infected cells. LPCs were isolated as previously described.^{2,50} Briefly, 1-cm intestinal segments were incubated with 1 mmol/L EDTA in Hanks' balanced salt solution for 30 minutes to separate the epithelial cells from the lamina propria. This was followed by a 1-hour incubation in RPMI 1640 containing 20 U/ml of collagenase while rapidly shaking at 37°C to isolate the LPCs from the stroma. LPCs were separated from red blood cells by centrifugation using lymphocyte separation medium (LSM; MP Biomedicals, Aurora, OH).

LPCs ($\sim 50 \times 10^6$) were suspended and cultured in 30 ml of RPMI 1640 supplemented with 25 mmol/L HEPES, 2 mmol/L glutamine, 5% fetal calf serum, and 100 U penicillin/streptomycin with or without recombinant human IL-6 (20 ng/ml) for 6 hours at 37°C in the presence of 5% CO₂. Isolation of LPCs from the GIT takes a few hours and during this period transcription factors such as C/EBP β may dissociate from their cognate binding sites on the DNA. To overcome this, we also treated the cells with rhIL-6 (20 ng/ml) so that the C/EBP β proteins would be activated and translocate to the nucleus and remain bound to the DNA. After incubation, nuclear proteins were crosslinked to genomic DNA with 1% formaldehyde for 10 minutes at room temperature. Cross linking was stopped by adding glycine to a final concentration of 0.125 mol/L and incubating for 5 minutes at room temperature. The contents of the flask were transferred to a 50-ml tube and centrifuged to pellet the cells. The cells were washed twice with ice-cold RPMI 1640 after which they were resuspended in 750 μ l of lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.0, protease inhibitor cocktail (Sigma Chemical Co.)]. The lysates were sonicated four to five times, at setting 4, for 10 seconds duration each with a 10-second break on ice between pulses using a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT) to produce DNA fragments of 300 to 1000 bp in length. Approximately 120 μ l of the sonicated lysate was diluted 1:10 in ChIP dilution buffer (16.7 mmol/L Tris-HCl, pH 8.0, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L NaCl, protease inhibitor cocktail) in three different 2.0-ml Dolphin microcentrifuge tubes (Light Laboratory Systems, Dallas, TX) and immunoprecipitations were performed overnight at 4°C with 4 μ g of polyclonal antibodies specific to C/EBP β (sc-150), p65 (sc-109) (Santa Cruz Biotechnology), and control rabbit immunoglobulin (Biomed Corp., Foster City, CA). A fourth tube was used as the input control. The immune

complexes were captured by incubation with MagnaBind Protein G beads (Pierce Biotechnology) for ~ 3 hours at 4°C and separated from the supernatant by magnetic separation. The beads were then washed sequentially for 5 minutes with 1 ml of the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.1), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 500 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8), and LiCl wash buffer (0.25 mmol/L LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0). Finally, the beads were washed twice with 1 ml of TE buffer (1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0). The immune-complexes were then eluted by adding 100 μ l of elution buffer (1% SDS, 100 mmol/L NaHCO₃) and incubating for 15 minutes at room temperature. After sedimenting the magnetic beads, the supernatant was collected and the elution was repeated. The supernatants were combined and the cross-linking was reversed by adding NaCl to a final concentration of 200 mmol/L and incubating overnight at 65°C. The remaining proteins were digested by adding proteinase K (final concentration, 40 μ g/ml) and incubation for 1 hour at 45°C. Genomic DNA fragments were recovered (MO BIO Laboratories, Carlsbad, CA), resuspended in Tris-HCl, pH 8.0 buffer (50 μ l), and analyzed by PCR using the primers specific to the SIV LTR (forward: 5'-GTCAGGCCTGTCA-GAGGAAG-3' and reverse: 5'-AGAGGGCTTTAAG-CAAGCAA-3'), for C/EBP β and p65, in a 25- μ l reaction containing either 5 μ l (DE68) or 20 μ l (EB17) of eluted DNA sample, 300 nmol/L dNTP, 300 mmol/L each primer, 1.25 U of *Pfx* Accuprime TaqDNA polymerase (Invitrogen Corp.). The reaction contents were heated to 95°C for 2 minutes for polymerase activation followed by 35 (DE68) and 40 (EB17) cycles at 94°C for 15 seconds, 56°C for 30 seconds. The amplified products were resolved on a 2.5% agarose gel. The amplified PCR product (~ 346 bp) was gel purified and its identity was further confirmed by nested PCR using a second set of primers (forward: 5'-AGATGAGACAGCAGGGACTTTCCA-3' and reverse: 5'-GCAGAGCGACTGACTACATAGCAA-3') to generate a 127-bp product.

Confocal Microscopy

To determine the cell types in the GIT expressing C/EBP β we performed double-label confocal microscopy. Tissues collected in RNAlater were first fixed in 2% paraformaldehyde for 4 hours, cryopreserved overnight in 30% sucrose solution, and embedded in Tissue Tek OCT (Sakura Finetechnical Company, Tokyo, Japan). Embedded tissues were sectioned at 10 μ m and stained with the appropriate primary and secondary antibody and also H&E to microscopically assess the histological status of the tissue. Briefly, slides were blocked with 100 μ l of blocking buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 3% bovine serum albumin, 10% normal goat serum, and 0.1% Triton X-100) for 1 hour followed by a 1-hour incubation at 4°C with rabbit polyclonal C/EBP β primary antibody (1:25 dilution, Santa Cruz Biotechnol-

ogy). The slides were washed three times in buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Triton X-100) followed by addition of goat anti-rabbit secondary antibody conjugated to Alexa Fluor 568 (1:1000, Invitrogen Corp.). This was followed by mouse anti-CD68 (IgG₁ macrophage clone KP1 at 1:20), or mouse anti-CD3 (IgG₁ clone F 7.2.38 at 1:10) (DAKO, Carpinteria, CA) at room temperature for 1 hour. The slides were washed three times and incubated for 1 hour with goat anti-mouse secondary antibody conjugated with Alexa Fluorophor 488 (1:1000). Confocal microscopy was performed using a Leica TCS SP2 confocal microscope equipped with three lasers (Leica Microsystems, Exton, PA). Individual optical slices represent 0.2 μ m and 32 to 62 optical slices were collected at 512 $\times$ 512 pixel resolution. NIH Image (version 1.62) and Photoshop (version 7.0; Adobe Systems, San Jose, CA) were used to assign colors to the three channels collected: Alexa 568 (Invitrogen Corp.) is red, Alexa 488 (Invitrogen Corp.) is green, and the differential interference contrast image is in gray scale. The three channels were collected simultaneously. Co-localization of antigens is demonstrated by the addition of colors as indicated in the figure legends.

Quantitation of Mucosal Viral Loads

Total RNA samples from all group 1 animals were subjected to a quantitative real-time TaqMan two-step RT-PCR analyses to determine the viral load in SIV-infected colon and jejunum samples. Briefly, primers and probes specific to the SIV LTR sequence were designed and used in the real-time TaqMan PCR assay. Probes were conjugated with a fluorescent reporter dye (FAM) at the 5' end and a quencher dye at the 3' end. Fluorescence signal was detected with an ABI Prism 7700 sequence detector (PE Applied Biosystems). Data were captured and analyzed with Sequence Detector Software (PE Applied Biosystems). Viral copy number was determined by plotting C_T values obtained from the colon and jejunum samples against a standard curve ($y = -3.351x + 40.377$) ($r^2 = 0.998$) generated with *in vitro*-transcribed

RNA representing known viral copy numbers. Plasma viral loads for 11 of 12 group 1 macaques were performed using the b-DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, NY).

Statistical Analysis

Gene expression for C/EBP β among groups were compared using the Kruskal-Wallis one-way analysis of variance by ranks for nonparametric independent group comparisons. When the results of Kruskal-Wallis one-way analysis of variance by ranks indicated a significant difference at the $P < 0.001$ level between the groups examined, Dunn's multiple comparison procedure was then used to identify the groups that differed at the $P < 0.05$ significance level. Histopathology severity scores for jejunum and colon in group 1, 2, and 3 animals were analyzed using Kruskal-Wallis one-way analysis of variance by ranks for nonparametric independent group comparisons. Within each group histopathology severity scores between colon and jejunum were analyzed using the Mann-Whitney test. The presence of other histopathological lesions such as villous blunting, crypt dilatation, and amyloidosis in the jejunum and colon of group 1 and 2 animals were analyzed using the two-tailed Fisher exact test.

Results

Intestinal Histopathology

Histological evaluation of H&E-stained sections of jejunum and colon (Table 2) revealed the presence of enterocolitis in most animals in groups 1 and 2 but not in group 3. When examining jejunum and colon separately, there did not appear to be much difference between groups 1 and 2 with regard to the presence of colitis (12 of 12 group 1 animals and 10 of 10 group 2 animals). However, the severity of the inflammation based on semi-quantitative histopathological grading criteria and blinded

Table 3. Comparison and Statistical Significance of Histopathological Lesions

Tissue	Animal and score details	Group 1	Group 2	Group 3	P value				
					G1 versus G2	G1 versus G3	G2 versus G3	Colon versus Jej – G1	Colon versus Jej – G2
Colon	No. of animals with colitis/total number of animals examined	10/10	10/10	0/7	NSD	<0.005	<0.0005	NSD	=0.004
	Total scores	21	27	0					
	Average scores	2.1	2.7	0					
	No. of animals with other lesions/total number of animals examined*	5/10	4/10	NA	NSD	NA	NA	NSD	NSD
Jejunum	No. of animals with enteritis/total number of animals examined	9/9	5/6	0/7	NSD	<0.0008	<0.005		
	Total scores	16	5	0					
	Average scores	1.78	0.83	0					
	No. of animals with other lesions/total number of animals examined*	4/10	0/6	NA	NSD	NA	NA		

*Lesions such as villous blunting, crypt dilation/abscesses, amyloidosis, diverticulosis, and so forth.
NSD, not significantly different; NA, not applicable.

analysis by a board certified pathologist (A.A.L.) suggested that differences do exist (Table 3 and see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Specifically, the inflammation in the GIT of group 1 animals was fairly uniform in the jejunum and colon whereas in group 2 animals the colon was much more severely affected ($P = 0.004$) (Tables 2 and 3). These differences, based primarily on the extent and severity of the inflammatory infiltrates were mirrored by the incidence of other intestinal lesions (Table 3) such as villous blunting, crypt dilatation, and abscess and amyloidosis in the jejunum of group 1 and not in group 2 animals. However, the presence of other intestinal lesions in the jejunum of group 1 macaques lacked statistical significance. Opportunistic pathogens such as cytomegalovirus and *Mycobacterium avium intracellulare complex* were detected in only 3 of 10 group 1 macaques (see Supplemental Table S1 at <http://ajp.amjpathol.org>). *Campylobacter coli*, *Campylobacter jejuni*, or *Shigella flexneri* were positively isolated from the colon at necropsy in 2 of 10 group 1 and 5 of 10 group 2 macaques (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Plasma and Mucosal Viral Loads and CD4⁺ T-Cell Counts

The viral loads in the plasma, jejunum, and colon of all of the SIV-infected macaques (group 1) are shown in Table 1 and were obtained at necropsy. All SIV-infected macaques (group 1) had substantial mucosal and plasma viral loads. Viral loads in the jejunum ranged from 0.057×10^6 to $16,380 \times 10^6$ copies/mg total RNA with a median of 3.35×10^6 copies/mg total RNA. The colon generally had higher viral loads than the jejunum for the same animal with a range from 0.5×10^6 to $213,000 \times 10^6$ copies/mg total RNA with a median of 11.75×10^6 copies/mg total RNA. Peripheral CD4⁺ T-cell counts obtained at necropsy were available for 11 of 12 group 1 macaques (Table 1). Of the 12 group 1 macaques, at least 8 had low CD4⁺ T-cell counts.

Mucosal Gene Expression for C/EBP β Is Significantly Increased in the GIT of SIV-Infected and Noninfected Macaques with GIT Inflammation

To verify that the occurrence of diarrhea in SIV-infected macaques was associated with a marked expression of C/EBP β , QRT-PCR was performed using total RNA extracted from jejunal and colonic mucosal samples from all macaques. Individual fold changes in gene expression calculated as described in the Materials and Methods for C/EBP β in all three groups are shown in Table 4. Figure 1, A and B, shows averaged group wise fold differences for C/EBP β in the colon and jejunum, respectively. In the colon, C/EBP β gene expression in group 1 animals was significantly increased compared with group 2 and normal controls ($P < 0.05$) (Figure 1A). Further, C/EBP β gene expression in group 2 ani-

Table 4. Individual Fold Differences in C/EBP β Gene Expression

Group	Animal no.	C/EBP β *	
		Colon	Jejunum
SIV-infected with diarrhea (group 1)	AJ82	3	4
	DD88	8	12
	CI65	11	11
	L441	1.12	1.1
	H405	5	3
	V205	5	5
	AT81	26	18
	AT56	13	4
	DT56	3	NA
	DI28	3	3
	EI90	4	NA
	EL45	4	NA
	EC49	6	NA
Non-SIV infected with diarrhea (group 2)	EB12	6	NA
	EM41	2	2
	EL71	7	1.3
	EB27	3	5
	DJ15	5	2
	CT77	2	5
	EJ54	3	3
	BV52	1	2
	CB98	2	1.3
	CF33	2	3
Control animals (group 3)	M302	5	3
	CC96	1.4	1
	R842	3	2

*Fold changes in the relative gene expression was determined by evaluating the expression, $2^{\Delta\Delta CT}$. Colon and jejunum specimens from EH70 (group 3) were not used for QRT-PCR analysis. NA, not applicable.

mals was also significantly increased compared with normal controls ($P < 0.05$) (Figure 1A). In the colon of group 2 animals, a statistically significant correlation between histopathology severity scores (Table 3) and C/EBP β gene expression ($r^2 = 0.69$) ($0.005 < P < 0.0005$) was observed (Tables 2, 3, and 4).

In the jejunum, C/EBP β gene expression in group 1 macaques differed significantly from group 2 and normal controls (Figure 1B) whereas there was no statistical difference between groups 2 and 3. This can be explained by the fact that the inflammation associated with SIV infection in group 1 animals affects the small and large intestine equally whereas in group 2 animals the colon was more severely affected than the jejunum (Table 2). Further, in the jejunum of group 2 animals, a statistically significant correlation was also observed between the low histopathology severity scores and the low C/EBP β gene expression values ($r^2 = 0.56$) ($0.05 < P < 0.025$) (Tables 2, 3, and 4).

Lamina Propria Mononuclear Cells Express High Levels of C/EBP β in the Colon of SIV-Infected and Non-SIV-Infected Macaques with Diarrhea

Although the RT-PCR assay demonstrated expression of C/EBP β mRNA in tissue homogenates from jejunum and colon, it provided no information on the specific cell types expressing C/EBP β . Accordingly, we performed confocal

Figure 1. Relative abundance in gene expression of C/EBP β detected using quantitative real-time SYBR green one-step RT-PCR. **A** and **B** represent colon and jejunum, respectively, from SIV-infected animals with diarrhea, non-SIV-infected with diarrhea, and uninfected control macaques. The fold differences in gene expression were calculated as described in the Materials and Methods. The relative fold increases for each group was summed and averaged to obtain a single figure that is shown on top of each bar graph. The error bars represent average fold difference for each group $\pm$ SEM. Animal groups that differ statistically ($P < 0.05$) are indicated using horizontal brackets.

microscopic analysis on colon tissue from three SIV-infected macaques with diarrhea (H405, AT56, DI28), two non-SIV-infected macaques with diarrhea (DJ15, EL45), and two uninfected control macaques (EH70, BV52). The photomicrographs shown in Figure 2 are representative of the three groups. In SIV-infected (Figure 2A) and non-infected macaques (Figure 2B), numerous CD68⁺ macrophages expressed C/EBP β . A small number of CD3⁺ T cells in the lamina propria were also found to express C/EBP β (Figure 2D) in the SIV-infected macaque. However, in the non-SIV-infected macaque with diarrhea, CD3⁺ cells expressing C/EBP β were much more frequent (Figure 2E). C/EBP β was mainly localized to the cytoplasm of macrophages and lymphocytes with few cells exhibiting nuclear localization. In contrast to animals with chronic diarrhea, in normal controls there were very few cells expressing C/EBP β (Figure 2, C and F). Further, unlike group 1 and 2 macaques, in the control animal, C/EBP β signal was very weak and increased laser strength had to be used to detect any signal.

C/EBP β Protein Expression Is Increased in the GIT of SIV-Infected Rhesus Macaques and Can Bind to the SIV Long Terminal Repeat

To identify the different isoforms of the C/EBP β protein expressed in the GIT during inflammation we performed immunoprecipitation/Western blotting in two group 1 and three group 2 and 3 animals. A significant increase in C/EBP β protein expression was observed in the colon of group 1 and 2 animals. As shown in Figure 3A, two strong bands with molecular weights of ~ 46 and ~ 41 kDa, respectively, were observed in both group 1 and 2 animals. These bands correspond to the full-length (46 kDa) and truncated transcriptionally active variant of C/EBP β (41 kDa) referred to as liver-enriched transcription activating protein (LAP). The C/EBP β variant known as liver transcriptionally enriched inhibitory protein (LIP, ~ 16 kDa) was not observed. In contrast, no C/EBP β protein expression was observed in normal controls (Figure 3A).

To examine if the C/EBP β protein was biologically active, binding of C/EBP β and p65 to the SIV LTR in group 1 animals was additionally evaluated by ChIP assay. Sonicated chromatin was precipitated with antibodies against C/EBP β or p65. Nonimmune rabbit IgG was used as control. The region of the SIV LTR pulled down by immunoprecipitation was identified by PCR using SIV LTR-specific primers described in Materials and Methods. In both SIV-infected macaques (DE68 and EB17), in the absence of any treatment (Unt), antibodies against C/EBP β precipitated the C/EBP β -containing LTR sequence (Figure 3B). Even though a very faint band was observed in the lanes representing precipitated protein-DNA complexes using the control IgG and p65, this band was very weak in intensity compared with the intense PCR product obtained after precipitation using the anti-C/EBP β antibody. However, in the second SIV-infected animal (EB17) the LTR sequence was precipitated only by the anti-C/EBP β antibody. The p65 and the control rabbit IgG failed to precipitate the SIV LTR sequence in this animal. Further, in the SIV-infected macaque (DE68), after 6 hours of treatment with 20 ng/ml of rhIL-6, antibodies against both p65 and C/EBP β precipitated the C/EBP β - and p65-containing LTR sequence, respectively (Figure 3B). Lastly, based on histopathological examination, the colonic lamina propria of macaque DE68 was characterized by severe lymphoplasmacytic and histiocytic colitis whereas macaque EB17 had mild colitis (Table 2).

Discussion

The GIT contains enormous numbers of activated CD4⁺ T lymphocytes of the memory phenotype that makes it a major target for infection with HIV/SIV resulting in rapid CD4⁺ T-cell depletion in this tissue.^{2,3,4,50,51} In addition to rapid and selective loss of mucosal CD4⁺ T cells, infection with HIV/SIV is characterized by chronic immune activation and GIT inflammation^{16,52–58} that may contribute to disruption of the intestinal epithelial barrier enabling translocation of GIT bacteria and their compo-

Figure 2. Immunophenotype of C/EBP β -positive cells in the colon of a SIV-infected macaque with diarrhea (H405, **A** and **D**), non-SIV-infected macaque with diarrhea (DJ15, **B** and **E**), and an uninfected control macaque (EH70, **C** and **F**). All panels involve double labels with C/EBP β (red) and either CD68 for macrophages (**A–C**) or CD3 for T cells (**D–F**), which appear green. For each panel the individual channels [green for CD3 or CD68, red for C/EBP β , and gray for differential interference contrast to reveal tissue architecture] appear on the **left** with a larger merged image on the **right**. Co-localization of labels appear yellow. Note that in SIV-infected animals with diarrhea (**A** and **D**) most of the cells positive for C/EBP β are CD68 $^{+}$ macrophages whereas in the non-SIV-infected animal with diarrhea (**B** and **E**) both CD68 $^{+}$ macrophages and CD3 $^{+}$ T cells are C/EBP β $^{+}$. Interestingly, in the normal control animal (**C** and **F**) few C/EBP β $^{+}$ cells were observed and the staining is very faint.

nents (eg, LPS).⁵⁹ Bacterial LPS, a known activator of several proinflammatory cytokines and their signaling transcription factors (eg, NF- κ B, C/EBP β , NFAT, AP-1), was shown to be elevated in the circulation of HIV/SIV-

infected humans and macaques during all stages of infection⁵⁹ and implicated as a major cause for chronic immune activation. The transcription factors activated, directly or indirectly by LPS, in addition to regulating

Figure 3. A: Levels of C/EBP β (LAP) protein are increased in the inflamed colonic mucosa of SIV-infected and noninfected rhesus macaques with chronic diarrhea. Representative expression of full-length (46 kDa) and the truncated (41 kDa) LAP isoforms of C/EBP β along with β -actin protein in colon samples taken from two SIV-infected macaques (G1), three non-SIV-infected macaques with chronic diarrhea (G2), and three control animals (G3) are shown. **B:** ChIP assay of transcription factors present at the SIV LTR in SIV-infected LPCs isolated from the GIT of SIV-infected rhesus macaques. ChIP assays were performed using lysates prepared from LPCs. The protein-DNA complexes were recovered using antibodies specific for each transcription factor (p65 and C/EBP β) and the binding was confirmed by PCR using SIV LTR-specific primers, as described in the Materials and Methods. All ChIP reactions were processed from the same chromatin isolated from the LPCs of each individual macaque.

immune responses and inflammation also have binding sites on the LTR of HIV and SIV. Consequently, although immunological activation in response to inflammatory stimuli (eg, bacterial, viral, fungal, parasitic infections) is necessary to elicit an immune response, paradoxically this event may function as a trigger to drive viral replication and disease progression in HIV/SIV-infected individuals. A clear understanding of the cellular and molecular pathways by which various inflammatory stimuli promote immune activation and viral replication in the GIT is required to facilitate the development of novel therapeutic strategies.

Earlier, using the rhesus macaque model of AIDS we demonstrated that SIV-infection and other causes of intestinal inflammation resulted in dysregulation of the IL-6-JAK-STAT3-SOCS-3 pathway and that the entire GIT was impacted in SIV infection in contrast to intestinal inflammation of other causes.²² Although IL-6 is known to mediate chronic inflammation, immune activation,²³ and HIV replication *in vitro*,¹⁹ the potential mechanism(s) promoting viral replication in the GIT remain unexplored. In the present study, jejunum and colon from SIV- and non-SIV-infected rhesus macaques with chronic diarrhea were examined to assess the role played by C/EBP β , a proinflammatory and proviral transcription factor activated by IL-6, IL-1 β , and interferon- γ in the development of GIT inflammation/disease. Our findings suggest that C/EBP β , known to be activated in response to IL-6 and other proinflammatory cytokine signaling in the GIT may not only be a mediator of chronic diarrhea and inflammation accompanying HIV/SIV infection but also may facilitate HIV/SIV replication.

The activity and expression level of C/EBP β is regulated by a number of inflammatory agents including LPS and a range of cytokines such as IL-6, IL-1 β , and tumor necrosis factor- α .^{30,31,59} Interestingly, C/EBP β expression is detectable in the intestine even under uninduced conditions.³¹ The latter observation is supported by the present study because C/EBP β mRNA, but not protein,

was detected in the jejunum and colon of group 3 animals (Figures 1 and 3). However, in group 1 and 2 animals with chronic diarrhea and intestinal inflammation, C/EBP β mRNA expression was significantly increased in the colon compared with group 3 controls. In the jejunum, elevations in C/EBP β were only found in SIV-infected animals with diarrhea. These animals also had more severe histopathological lesions in the jejunum compared with the other groups (Tables 2 and 3). Further, group 1 macaques showed significant elevation of C/EBP β mRNA expression relative to group 2 macaques suggesting that SIV infection has a particularly dramatic impact on expression levels of C/EBP β . Although the factors that trigger this marked increase in C/EBP β expression remain unclear, the high mucosal viral loads in group 1 animals could be a major driving force. Additionally, increased translocation of bacterial LPS from a leaky intestinal epithelial barrier recently reported to occur in chronic HIV-infected humans and SIV-infected macaques⁵⁸ can strongly induce the expression of C/EBP β .^{31,59} Increased expression of C/EBP β has also been detected in peripheral blood mononuclear cells of HIV-infected patients³⁸ and has been strongly associated with increased viral replication in HIV patients with pulmonary tuberculosis⁴⁵ and in the development of AIDS dementia.⁶⁰ Interestingly, Silver and colleagues⁶¹ had earlier reported a strong association between GIT inflammation (ulcerative colitis) and high systemic viral burden with a dramatic reversal of both high viral burden and low CD4⁺ T-cells counts after surgical resection of inflamed areas of the colon.⁶¹ This study clearly demonstrates an association between inflammation and viral burden and shows an increased accumulation of HIV-1-infected cells within the inflamed mucosa. The significantly increased expression of C/EBP β observed in the GIT of SIV-infected rhesus macaques offers a molecular pathological mechanism linking GIT inflammation and immune activation with increased viral burden in SIV-infected macaques and conceivably in HIV-infected individuals.

To determine the cell types expressing C/EBP β we then used multilabel confocal microscopy with cell-type-specific and anti-C/EBP β antibodies. C/EBP β expression was restricted to the mononuclear cell population present in the lamina propria. Using cell-specific surface markers such as CD3 and CD68 we found both lymphocytes (CD3) and macrophages (CD68), both of which are targets for SIV infection, to be expressing C/EBP β . In the non-SIV-infected macaque with diarrhea the expression of C/EBP β was equally distributed between macrophages and lymphocytes (Figure 2, B and E). However, in SIV-infected macaques, C/EBP β expression was detectable predominantly in macrophages (Figure 2, A and D). The reason for this difference may be related to the selective destruction of CD3⁺CD4⁺ T cells by SIV that would not occur in group 2 animals. In contrast to group 1 and 2 animals, very little C/EBP β expression was observed in control (group 3) animals. The strong expression of C/EBP β by intestinal macrophages in SIV-infected animals is significant because, macrophage numbers increase tremendously during inflammation primarily because of the increased trafficking of monocytes that express CD4, CCR5, and CXCR4⁶² from the blood into the inflamed GIT mucosa and submucosa.⁶³ C/EBP β is a strong transactivator of the CCR5 gene (approximately seven binding sites),³⁸ the HIV/SIV LTR (approximately three to five sites)³⁹ in addition to regulating the expression of several proinflammatory genes such as COX-2,²⁹ iNOS, and so forth. Hence the direct consequences of persistently elevated C/EBP β would likely be the generation of more HIV/SIV receptor-binding sites, target cells, increased viral burden, reservoir cells, immune activation, and progression to AIDS.

Given the fact that the GIT is a major site of viral replication and that most HIV/SIV-infected individuals develop GIT inflammation/disease, it is of considerable interest to identify host cellular factors that promote HIV/SIV replication in this pivotal tissue. The presence of three and up to five putative binding sites for C/EBP β in the 5'-LTR of HIV and SIV,³⁹ respectively, further prompted us to investigate the role of C/EBP β proteins in regulating viral replication in the GIT. Using the ChIP assay we observed that in SIV-infected LPCs isolated from an inflamed colon, both p65 and C/EBP β may either independently or synergistically bind to the SIV LTR (Figure 3B). These findings provide a plausible link between viral replication, inflammation, and immune activation. Similar *in vivo* binding of C/EBP β in cooperation with p65 and p50 to the LTR has been demonstrated in the brain of SIV-infected rhesus macaques.⁴⁴ Although these data do not indicate if this binding is stimulatory or inhibitory, the presence of high viral loads in the jejunum and colon of group 1 animals (Table 1) together with the strong expression of the 46- and 41-kDa transcription activating isoforms (LAP) (Figure 3A) suggests a stimulatory role. Aspirin and its *in vivo* metabolite sodium salicylate at pharmacological concentrations has been shown to selectively inhibit C/EBP β activation by blocking C/EBP β phosphorylation catalyzed by a number of kinases, that includes p90 ribosomal S6 kinase (RSK), which preferentially phosphorylates C/EBP β at Thr266.^{64,65} Based on

our findings it is logical to hypothesize that incorporation of anti-inflammatory drugs targeting C/EBP β might improve antiretroviral treatment outcomes. Collectively, our findings suggest that increased expression of C/EBP β helps maintain a proinflammatory environment locally in the GIT. Such an inflammatory milieu, if allowed to persist could be expected to facilitate damage to the mucosal barrier, thus setting up a positive feed forward mechanism involving viral replication, inflammation, increased GIT microbial translocation, and immune activation.

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