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Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T cell activation following therapy

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Abstract

Objective—HIV-1 replication and microbial translocation occur concomitant with systemic immune activation. This study delineates mechanisms of immune activation and CD4 T cell decline in pediatric HIV-1 infection.

Design—Cross-sectional and longitudinal cellular and soluble plasma markers for inflammation were evaluated in 14 healthy and 33 perinatally HIV-1-infected pediatric subjects prior to and over 96 weeks of protease-inhibitor-containing combination antiretroviral treatment [ART]. All HIV-1-infected subjects reconstituted CD4 T cells either with suppression of viremia or rebound of drug-resistant virus.

Methods—Systemic immune activation was determined by polychromatic flow cytometry of blood lymphocytes and ELISA for plasma soluble CD27 [sCD27], soluble CD14 [sCD14], and tumor necrosis factor [TNF]. Microbial translocation was evaluated by limulus amebocyte lysate assay to detect bacterial lipopolysaccharide [LPS] and ELISA for anti-endotoxin core antigen IgM antibodies. Immune activation markers were compared to viral load, CD4% and LPS by regression models. Comparisons between healthy and HIV-1 infected or between different viral outcome groups were performed by non-parametric rank sum.

Results—Microbial translocation was detected in healthy infants but resolved with age ($P<0.05$). LPS and sCD14 levels were elevated in all HIV-1 infected subjects ($P<0.05$ and $P<0.0001$, respectively) and persisted even if CD4 T cells were fully reconstituted, virus optimally suppressed, and lymphocyte activation resolved by ART. Children with CD4 T cell reconstitution but viral rebound following ART continued to display high levels of sCD27.

Conclusions—Microbial translocation in pediatric HIV-1-infection is associated with persistent monocyte/macrophage activation independent of viral replication or T cell activation.

Keywords

HIV-1; microbial translocation; monocyte/macrophage; systemic immune activation; children and adolescents; lipopolysaccharide; inflammation

Introduction

HIV-1 infection develops with acute viremia and rapid depletion of CD4 T cells within mucosal-associated lymphoid tissues [MALT], particularly in gut lymphoid compartments [1-3]. HIV-1-induced disruption of MALT results in intestinal fibrosis and translocation of microbial products across the intestinal mucosa into the peripheral circulation producing high plasma levels of lipopolysaccharide [LPS] and bacterial DNA[4-6] that persist throughout chronic HIV-1 infection[7]. Microbial translocation appears related to systemic leukocyte activation and elevated plasma levels of inflammatory proteins[5,8,9], although whether or not microbial translocation and consequent inflammation are direct causes of progression to AIDS or merely sequelae of HIV-1 infection seems to differ among cohorts[5,7-9].

Chronic HIV-1 infection is characterized by progressive activation of CD4 and CD8 T cells, as well as CD14 monocyte/macrophages[5,10-14], and gradual depletion of peripheral naïve and memory CD4 T cells. Microbial translocation is implicated in systemic T cell activation associated with HIV-1 infection[8] and idiopathic CD4 lymphocytopenia[15], providing evidence that circulating LPS or other microbial products may contribute to acquired and/or

primary immunodeficiency. The premise that microbial translocation is a causative factor for systemic T cell activation, CD4 T cell decline, and disease progression is supported by studies of natural SIV infection of sooty mangabeys that develop high levels of viral replication in the absence of systemic T cell activation, CD4 T cell decline, or microbial translocation[16-18].

In HIV-1 infection, systemic immune activation drives viral replication generating a vicious cycle of viremia, immune activation, and CD4 T cell attrition that ultimately results in AIDS [14]. Effective combination antiretroviral treatment [ART] rapidly suppresses viremia, restores peripheral CD4 T cells, reduces lymphocyte activation, and restores most immune functions [19]. However, ART fails to fully restore memory T cells in MALT[20,21] or completely reverse microbial translocation even following complete suppression of viremia[5]. A unique group of individuals receiving ART reconstitute T cell immunity but fail to control viral replication due to the development of drug resistant virus[22]. This discordant outcome is not uncommon among HIV-1-infected children who develop improved T cell function despite viral rebound[23,24]. Persistent viral replication following ART in conjunction with immune reconstitution in HIV-1-infected children provides an opportunity to study the correlates of pathogenesis, including systemic immune activation and microbial translocation, in the presence of high viral replication in a human host that might be modeled by SIV infection in sooty mangabeys[22-25].

Effects of microbial translocation in HIV-1-infected infants and children may be unique due to inherent differences in gut flora and LPS responses during early development[26-28]. Robust thymic output in younger HIV-1-infected individuals[29] may compensate for CD4 T cell activation and attrition induced by microbial translocation, although systemic activation of monocytes, macrophages, microglia or other cell types potentially contribute to HIV-1-associated illnesses such as neurological disorders, dementia, cardiovascular events or cancer [13,30-32]. Determining the causes and consequences of inflammatory HIV-1 complications is particularly critical for infants and children who may survive, due to effective antiretroviral therapies, for several decades with chronic inflammation.

Methods

Study Design

Prospectively stored plasma and cryopreserved peripheral blood mononuclear cells were obtained from a cohort of HIV-infected infants and children enrolled in a clinical trial of protease inhibitors plus two nucleotide reverse transcriptase inhibitors as previously described. [23,24,33]. Briefly, all HIV-1-infected subjects were infected perinatally and viral and immune outcomes were known. The current study included 33 of the 40 subjects who displayed T cell immune reconstitution and completed 96 weeks on the study. The protocol was approved by University of Florida and University of South Florida/All Children's Hospital Institutional Review Boards. Control subjects were volunteer healthy infants and children with no underlying medical conditions, recruited over the same time frame according to a separate protocol approved by both institutions. Subjects with gastrointestinal conditions were excluded. Whole blood samples were collected, using phlebotomy, in sterile Vacutainer™ (Becton Dickinson) acid citrate dextrose tubes, and processed within 12 hours[34-36]. PBMC and plasma samples were stored at -180° C in liquid nitrogen or at -80° C, respectively, in non-pyrogenic polypropylene cyrovials (Nunc Cryotubes™).

Healthy and HIV-1 infected subjects ranged in age from 0.4 to 15 years (median = 1.4; 25^{th} / 75^{th} quartiles = 0.4 / 13.0) and 0.2 to 17.7 years (median = 6.6; 25^{th} / 75^{th} quartiles = 4.5 / 11.6), respectively ($P>0.05$, Mann-Whitney U test). Viral load, CD4% and CD4 CD45RO% were analyzed at 0, 4, 12, 48 and 96 weeks post-ART. Prior to ART, HIV-1 infected subjects had plasma viral loads ranging from 2.9 to 6.1 $\log_{10}$ HIV-1 RNA copies/ml (median = 4.6

log_{10}). By 24 weeks of ART, all subjects experienced increased CD4% either with viral suppression [<400 viral RNA copies/ml plasma] that was sustained for 96 weeks or viral rebound [>400 viral RNA copies/ml plasma] (24 week median = 3.9 log_{10} copies/ml; 96 week median = 4.0 log_{10} copies/ml)[23,24,33].

Measurement of CD4 and CD8 T cell activation

Polychromatic flow cytometry analysis was performed with fluorescence-tagged monoclonal antibodies described previously[29,37] and a multiparameter LSR2 flow cytometer incorporating the cytometer setup and tracking program and Diva software (BD Biosciences, San Jose, CA, USA). Activated effector/memory CD4 T cells were defined as CD3 CD4 cells expressing CD45RO. [38]. Activated CD3 CD8 T cells were defined as $CD45RA^{+}$ $CD27^{-}$ $CD11a^{++}$ (bright), a phenotype associated with HIV-1 disease progression [29,39-41]. The frequency of recent thymic emigrant T cells was determined by performing T cell receptor excision circle [TREC] analysis as previously described[29].

Measurement of soluble markers for immune activation and microbial translocation

Plasma samples were diluted 1:4 in 0.15 M NaCl, heat inactivated at 65° C for 30 minutes, then used to measure soluble CD27 [sCD27] by PeliKine human soluble CD27 ELISA kit (RDI Fitzgerald Industries, Acton, MA, USA); soluble CD14 [sCD14] by Human sCD14 Immunoassay, (R&D Systems, Minneapolis, MN, USA); LPS levels by limulus amebocyte assay [LAL] Chromogenic Endpoint Assay (Lonza Group, Ltd, Allendale, NJ, USA); TNF (TNF superfamily, member 2; TNFα) levels by human TNF ELISA (BD Biosciences); and anti-endotoxin core antigen antibodies [EndoCAb] IgM levels by EndoCAb ELISA (Cell Sciences Inc., Canton, MA, USA). Specimens were handled in an endotoxin-free environment and controls were performed with all LAL assays to rule out endotoxin contamination.

Statistical analysis

Pearson's correlation and simple linear regression analyses were performed to determine the relationships between two variables. Cross-sectional comparisons of median clinical values between different groups of subjects were performed by Mann-Whitney U test. Longitudinal comparison between pre-ART and post-ART values was performed by Wilcoxon matched pairs test. Power analysis was performed based on a one-way ANOVA for three group comparisons. Sample size has an 80% power to identify a minimum detectable effect size of 0.95 S.D. in the group differences. Effects of continuous predictors and their interactions on CD4 percent were measured and tested for significance by using a multiple regression model. Analyses were performed with GraphPad Prism 5.0 software (La Jolla, CA) or SAS software (Cary, NC) and considered significant when P value was less than 0.05.

Results

Pre-therapy CD4 CD45RO T cell frequency and viremia differentially correlate with CD4 T cell decline

Increased frequency of effector/memory CD4 CD45RO T cells, reduced frequency of CD4 CD45RA naïve T cells and activation of CD8 T cells are associated with CD4 T cell decline and progression to AIDS in HIV-1 infection[29,42]. As expected, prior to ART, HIV-1-infected subjects displayed significant inverse correlations between overall CD4 T cell percent and frequency of CD4 CD45RO T cells ($P<0.0001$), frequency of CD8 CD45RA T cells with an activated $CD27^{-}$CD11a bright phenotype ($P<0.05$), or plasma viral load ($P<0.01$) (Figure 1A, 1C, and 1E). Pre-ART measures of T cell frequency, T cell activation, or viral load were similar between groups who ultimately suppressed [VS] or failed to suppress [VF] viral burden following ART (Figure 1B, 1D, and 1F).

To determine the effects of length of infection [LOI], which was identical to age as all subjects were infected perinatally, a multiple regression model was applied. CD4 decline was related inversely to the frequency of CD4 CD45RO cells, a correlation more pronounced in infants than children and adolescents (Figure 1G, $P<0.01$). Conversely, a trend in the relationship between viral load and CD4 T cell attrition was more pronounced among older rather than younger children (Figure 1H, $P=0.097$). These analyses indicated that age/LOI is a determining factor in the relationship between viral load, CD4 T cell activation and CD4 T cell decline.

Microbial translocation develops concomitant with multilineage leukocyte activation in HIV-1 infection

Microbial translocation develops coincident to systemic inflammation in some, but not all HIV-1-infected subjects[5,7,8]. Microbial translocation (LPS) was evident among all therapy naïve HIV-1 infected subjects, as well as a subset of healthy children (Figure 2A, $P<0.05$). Among healthy subjects, microbial translocation was more pronounced in infants (0-2 years), than in children or adolescents (>2-18 years) (Figure 2B, $P<0.05$). Levels of LPS in HIV-1 infected infants were comparable to healthy infants, while infected children had higher levels than healthy children ($P<0.01$). The monocyte/macrophage soluble activation marker sCD14 was detected in the plasma of all healthy and HIV-1-infected pediatric subjects. In our study sCD14 levels (median sCD14 = 20.4 ng/ml) were markedly lower in healthy pediatric subjects, including infants who had microbial translocation, than sCD14 levels reported for healthy adults[5,7] (Figure 2B and 2C). In contrast, sCD14 levels were significantly elevated (median sCD14 = 931.8 ng/ml) in HIV-1 infected pediatric subjects compared to healthy subjects (Figure 2C, $P<0.0001$). EndoCAb levels were significantly reduced in HIV-1-infected subjects (Figure 2D, $P<0.05$), although unrelated to LPS levels in HIV-1-infected or healthy children (Figure 2E or data not shown). Levels of sCD27, reflecting generalized lymphocyte activation, were elevated in HIV-1-infected subjects compared to healthy controls (Figure 2F, $P<0.0001$).

The cytokine TNF, a key mediator of systemic inflammation, was undetectable among healthy subjects, but detected in 8 of 33 HIV-1-infected subjects prior to ART (Figure 3A). No difference in LPS levels between TNF(-) and TNF(+) subjects was identified (Figure 3B). Microbial translocation displayed a trend toward a positive correlation with activation of monocyte/macrophages (Figure 3C, $P=0.055$). In contrast, LPS levels showed no relationship to general lymphocyte activation measured by sCD27 levels or frequency of CD4 CD45RO T cells or activated CD8 T cells (Figure 3D-3F). Together, these results indicate that microbial translocation was independent of lymphocyte activation.

Microbial translocation or systemic inflammation may contribute to CD4 T cell attrition in HIV-1 infection. Prior to ART, plasma levels of LPS were unrelated to CD4 T cell levels (Figure 4A). Likewise, neither sCD14 nor sCD27 levels was associated with CD4% (Figure 4B and 4C). Overall, pre-ART levels of markers for microbial translocation or multilineage leukocyte activation were unrelated to CD4 T cell decline and similar between subjects who developed viral success or failure following ART (Figure 4D, 4E and 4F).

Microbial translocation and monocyte/macrophage activation both persist independent of ART-related CD4 T cell reconstitution

ART results in significant immune reconstitution and improved immune function in individuals who optimally control viral replication, as well as in subjects who develop viral rebound with drug resistant virus[24]. In longitudinal analyses, viral success subjects suppressed viremia below limits of detection by 24 weeks of ART (Figure 5A, $P<0.0001$) with concomitant improvement in CD4 T cell frequency (Figure 5B, $P<0.01$). In contrast, viral failure subjects experienced transiently reduced viral loads at 4 weeks post-ART that rebounded by 24 weeks

to an average >4.0 $\log_{10}$ copies per ml (Figure 5A), even though CD4 T cell frequencies improved significantly and persisted at levels >25% (Figure 5B, P<0.001).

Frequency of CD4 CD45RO T cells declined by 24 weeks of ART in both outcome groups to levels approaching those in healthy subjects (Figure 5C). Decline in CD4 CD45RO T cell frequency was significantly associated with increasing T cell receptor excision circles [TREC] (Figure 5D, P<0.0001). Plasma sCD27 levels remained unchanged by ART in viral failure subjects, but were reduced in viral success subjects (Figure 5E, P<0.05).

Pretherapy levels of sCD14 were unchanged at 24 weeks of ART, but declined by 96 weeks to significantly lower levels in both viral success (P<0.001) and viral failure (P<0.05) groups. Nonetheless, elevated sCD14 persisted among HIV-1 infected individuals for two years after initiation of ART at levels about 20-fold higher than healthy controls (Figure 5F). Likewise, microbial translocation was unchanged over 96 weeks of ART in viral success or viral failure subjects and remained well above levels detected in healthy controls (Figure 5G). No subject with undetectable TNF pre-ART developed measurable levels post-ART, while pretherapy plasma TNF, detected among 8 subjects, persisted for 96 weeks of ART (Figure 5H),. Elevated TNF had no association with CD4%, microbial translocation or monocyte macrophage activation and was independent of viral success or viral failure outcome (data not shown).

Longitudinal changes in viremia, immune activation and microbial translocation were compared over the course of treatment by linear regression to contemporaneous changes in CD4%. No significant associations between changes in CD4% and viral load, sCD27, sCD14 or LPS following 24 or 96 weeks of ART were identified. Decreasing frequency of CD4 CD45RO T cells was the only parameter related to sustained improvement in CD4 T cell frequency during ART in viral success and viral failure subjects (24 weeks: P<0.05; 96 weeks: P<0.001). (See Table S2, Supplemental Digital Content).

Discussion

HIV-1 infection results in systemic immune activation, which depends on virus replication and/or microbial translocation, and is resolved to an extent by antiretroviral therapy[5,9,14]. Systemic immune activation drives viral replication creating a cycle of T cell attrition. When combined with viral effects on thymic T cell output, dissecting the contribution of each disease variable to immune activation poses significant challenges. Our studies show that during the natural history of pediatric HIV-1 infection, T-cell activation and CD4 T-cell attrition are related to levels of viral replication depending on age/length of infection. In contrast, macrophage activation is related to both microbial translocation and effects of HIV-1 infection that persist even when viremia is suppressed below limits of detection. Sustained immune reconstitution by ART, independent of viral outcomes, induced concordant attenuation of CD4 and CD8 T cell activation[29], even though microbial translocation or macrophage activation persisted indicating that systemic immune activation in T cell versus monocyte/macrophage compartments can be dissociated based on distinct mechanisms.

While viral replication is the principle factor driving lymphocyte activation, microbial translocation is also implicated in systemic T cell activation in some, but not all, natural history studies of HIV-1 infection[5,7,8]. In contrast our study of perinatally-infected children found no association between microbial translocation and T cell activation or immune deficiency pre-ART, highlighting the complex relationships between different causes and consequences of immune activation. Following ART, reconstitution of CD4 T cells and increased TREC occurred in our cohort despite persistent microbial translocation indicating that thymic output, a major contributor to post-ART lymphocyte reconstitution[29,43,44], is independent of LPS-driven inflammation. Failure of ART to attenuate microbial translocation and LPS levels in

children, unlike ART treatment of HIV-1 infected adults[5], may reflect irreversible intestinal fibrosis induced by early viremia during infancy[45]. HIV-1-infection during infancy, compared to infection during adolescence or adulthood may have different effects on MALT due to normal processes of gut remodeling that occur during growth[46] when intestinal permeability is increased and adaptive immunity is still developing. Indeed, transient microbial translocation found in our study of healthy and HIV-1-infected infants resolved after 2 years of age only in healthy children, indicating that HIV-1 disrupted normal gut development. It would be interesting to further investigate the nature of microbial translocation in early infancy versus older HIV-1-infected children to determine if disruption of gut epithelial and endothelia tissues differs by age and illness. Microbial translocation, as a consequence of HIV-1 pathogenesis in adults, is inversely correlated with EndoCAb levels[5,47]. In our study, EndoCab levels were reduced among HIV-1 infected children, but no relationship between EndoCab and LPS levels was apparent. Developmental regulation of natural antibody production could impair the ability of perinatally-infected children to neutralize and clear circulating LPS during early infection.

The LPS detected in plasma of HIV-1 infected subjects is biologically active and inflammatory *in vivo*, reflected by the link with monocyte/macrophage activation, although healthy infants with similarly elevated LPS levels displayed no concomitant increase in monocyte/macrophage activation. HIV-1 might function as a co-factor for LPS-induced inflammation in infants. The overall extent of monocyte/macrophage activation found with HIV-1 infection could reflect direct activating effects of HIV-1 on monocytes[6,48,49] or priming by HIV-1, which sensitizes macrophages to subsequent activation with toll-like receptor [TLR] ligands including LPS[50]. Whether sCD14 detected in plasma is derived from monocytes or differentiated macrophages is unclear, but both scenarios are plausible. *In vivo*, macrophages are efficient targets for productive HIV-1 infection whereas monocytes are rarely infected [51,52], although binding of HIV-1 envelope proteins to CCR5 on monocytes induces cell signaling that enhances monocyte survival[53] and may induce activation and shedding of sCD14.

Lack of monocyte/macrophage responsiveness to LPS in healthy infants is characteristic of normal immune development[26,27]. For example, in atopic allergy, an immune hypersensitivity disorder, sCD14 levels are lower in neonates versus older children, even though neonates have increased frequency of $CD14^+$ cells[54]. Breast-feeding is another variable that regulates inflammatory versus tolerogenic responses to microbial translocation. Secretory IgA in breast milk can have significant effects on gut microbial flora through alterations in colonization and prevention of trans-mucosal sepsis, and other soluble milk components can mediate anti-inflammatory effects[28]. None of the neonates in our study was breast fed, which may itself contribute to immune activation. Clearly, systemic monocyte/macrophage activation is less pronounced during childhood and HIV-1 infection significantly disrupts normal immune homeostasis. Although ART for HIV-1-infected subjects in this study improved health with significant increases in height and weight and decreased prevalence of AIDS-defining illnesses[24], inability of therapy to resolve either microbial translocation or monocyte/macrophage activation may contribute to accelerated immune senescence with increased long-term morbidity and mortality from non-AIDS related inflammatory conditions [55].

CD27, a member of the tumor necrosis factor receptor family and its ligand, CD70, are predominantly expressed by lymphocytes[56]. Lymphocyte activation in both the T cell and B cell compartments results in high levels of the soluble protein in the plasma and can be used to measure systemic immune activation in HIV-1 infection[56]. The divergence in sCD27 levels in post-ART viral success versus viral failure subjects may be linked to qualitative differences in HIV-1 phenotype and co-receptor utilization. HIV-1 isolated from subjects who

suppress viremia primarily use only CCR5 co-receptor for target cell entry, while HIV-1 from viral failure subjects use both CCR5 and CXCR4 co-receptors before and after ART[33]. Persistently elevated sCD27 demonstrates that replication of drug-resistant HIV-1 induces some component of lymphocyte activation in spite of improved CD4 T cells counts and normal immune function[24], indicating that systemic immune activation and its consequences are complex and multifactorial.

Discordant outcomes following ART represent an important paradigm in HIV-1 pathogenesis, which may be modeled by high levels of SIV replication in its natural host[18]. However, our study demonstrates fundamental differences between discordant therapy outcome in HIV-1 infection and natural SIV infection of sooty mangabeys in that CD4 T cell pathogenesis is uncoupled from microbial translocation in HIV-1 infection. In sooty mangabeys, viremia fails to induce intestinal fibrosis or microbial translocation which is related to the overall lack of CD4 T cell pathogenesis or immune deficiency in this model[5,17]. In contrast, HIV-1-infected children experience increased naïve CD4 T cell counts and TREC levels after ART regardless of viral outcome, suggesting that persistently elevated LPS two years into therapy does not adversely impact thymic output[29,57,58]. Post therapy viruses do not induce the same degree of T cell activation as wild type virus and are associated with higher TREC levels. In the discordant response group, post therapy drug resistant viruses are less fit for replication in the thymus resulting in a high proportion of naïve T cells in the peripheral blood[59,60]. Thus, improved CD4 T cell frequency in post-ART discordant response subjects is more likely a consequence of attenuated viral pathogenesis than changes in gut pathogenesis or microbial translocation. Similar to adults who continue failing ART regimens[22], discordant response in children and adolescents was associated with evidence of CD4 T cell declines by 96 weeks, although the proportion of CD45RO CD4 T cells failed to increase, an indication that robust thymic output persisted.

Our finding that microbial translocation is not a major factor in HIV-1 immunodeficiency in infants, children and adolescents is similar to a study of HIV-1 subtype A and D infections in African adults, who progressed to AIDS in the absence of HIV-1-specific microbial translocation and chronic HIV-1-associated inflammation[7]. Nonetheless microbial translocation is clearly an important component of innate immune inflammation in some HIV-1-infected subjects. In our study, both outcome groups developed post-ART declines in sCD14 levels, but monocyte/macrophage activation failed to normalize in either group, even following two years of ART, indicating that microbial translocation contributes to sustained macrophage activation that can be independent of high level viral replication. Prolonged systemic monocyte/macrophage activation may contribute to HIV-1-associated inflammatory conditions including dementia, increased risk for cardiovascular events and thrombophilia or enhanced angiogenesis in malignancy[13,30-32]. The extent of monocyte/macrophage activation and resulting inflammatory conditions may differ in perinatally-infected children compared to infected adults due to greater infectivity and HIV-1 replication in neonatal or cord blood-derived monocyte/macrophages[61,62]. Considering that perinatally-infected neonates will likely survive several decades with ART, the implications of persistent microbial translocation and monocyte/macrophage activation should be a major focus of investigation in coming years.

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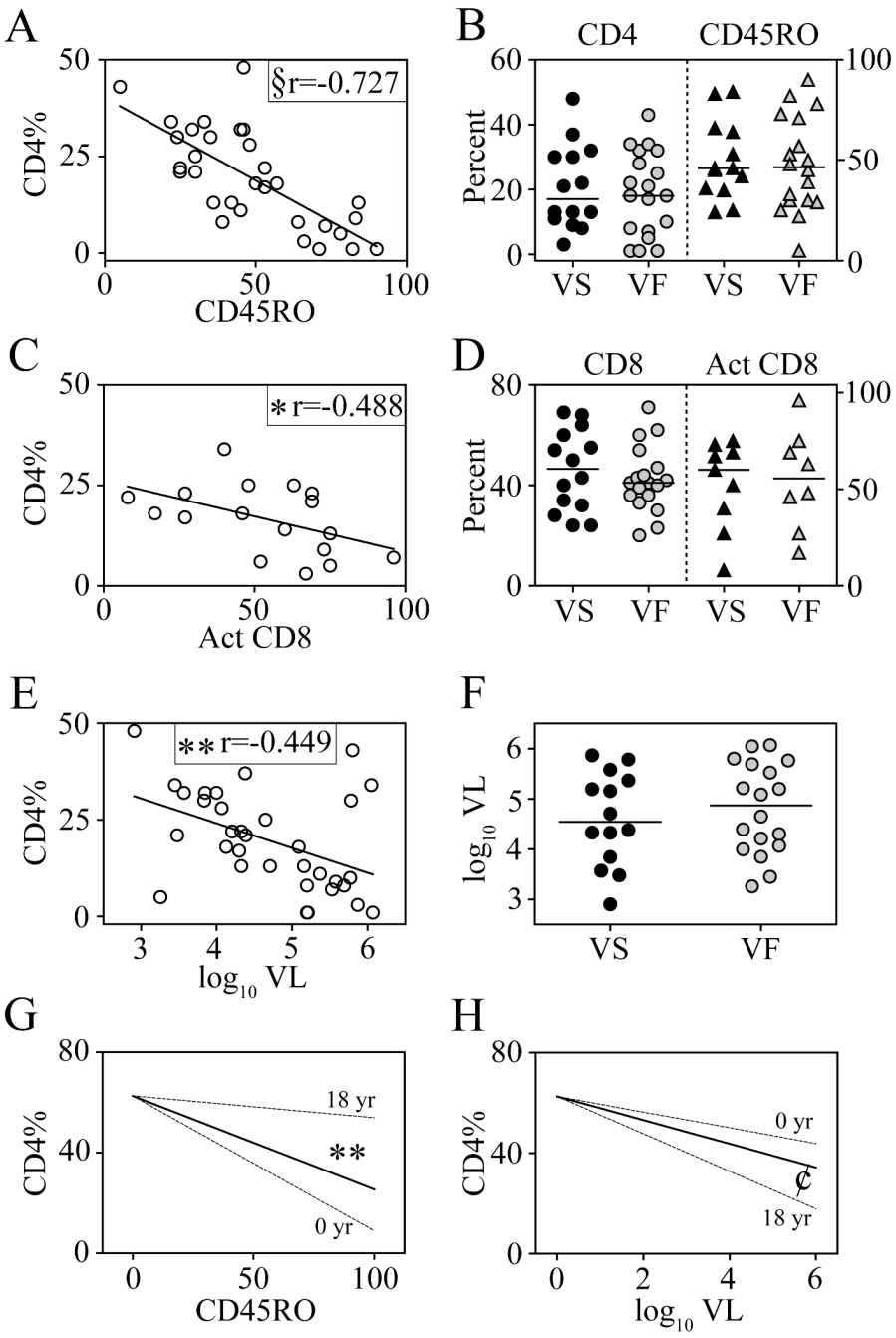

Figure 1. T cell activation and uncontrolled viremia mediate age-dependent pathogenesis in pre-therapy HIV-1 infection

The frequency of **(A)** CD4 CD45RO T cells (n = 31) or **(C)** activated CD8 CD45RA $CD27^{-}$ $CD11a^{++}$ T cells (n = 17) were compared to the overall frequency of CD4 T cells by linear regression analysis. **(B)** Comparison of CD4% and frequency of CD4 CD45RO T cells between viral success [VS] versus viral failure [VF] subjects. **(D)** Comparison of CD8% and frequency of activated CD8 T cells between VS and VF subjects. **(E)** The relationship between plasma viral load [VL] and CD4% was determined by linear regression analysis (n=33). **(F)** Comparison of viral load between VS and VF subjects. (For panels A, C and E, *P<0.05, **P<0.01, and §P<0.0001; Pearson correlation.) A multiple regression model was used to

determine age/LOI effects on correlations between CD4% and CD45RO frequency **(G)** or viral load **(H)**. Solid line represents correlations at the median age of the cohort (6.6 yrs) and dashed lines represent correlations at birth (0 yrs) or maturity (18 yrs). (*P<0.05 and ¢P=0.097 represent interaction between age/LOI and either CD45RO or viral load by a multiple regression model.) The specific values for interactions between age/LOI and either CD4 CD45RO T cell frequency or viral load used to generate Figures 1G and 1H are presented in Table S1 (See Supplemental Digital Content).

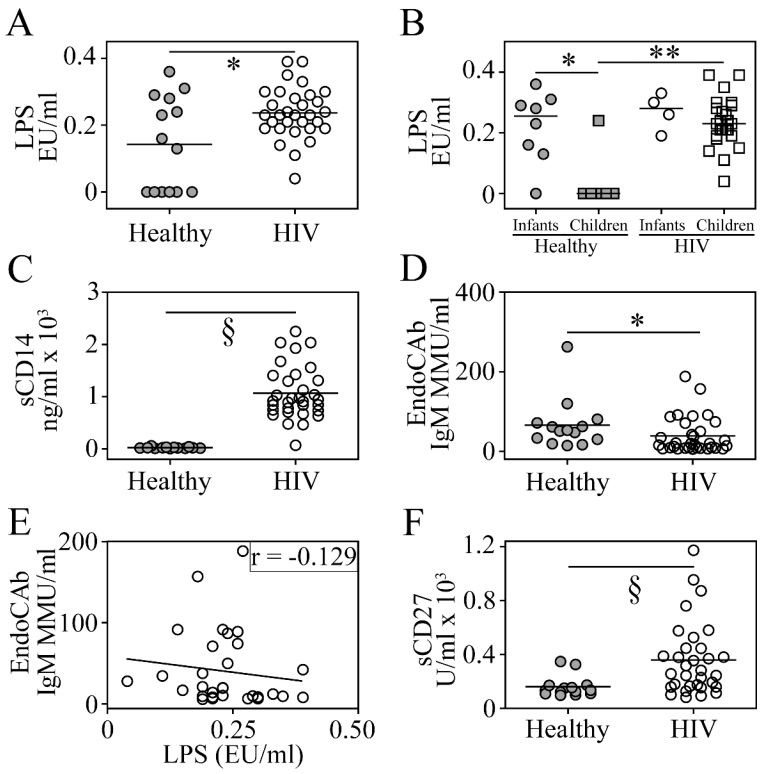

Figure 2. Microbial translocation and multi-lineage leukocyte activation develop simultaneously in HIV-1 infected subjects

(A) Plasma levels of LPS were determined by LAL assay in healthy controls and HIV-1-infected subjects and **(B)** LPS data were divided into groups based upon age (Infants, 0-2 yrs; Children, >2-18 yrs). Plasma levels of **(C)** sCD14 and **(D)** EndoCAb IgM levels were determined by ELISA and **(E)** linear regression analysis was performed to compare EndoCAb to LPS in HIV-1-infected subjects. **(F)** Levels of sCD27 were determined by ELISA in healthy controls and HIV-1-infected subjects. (*P<0.05, **P<0.01 and §P<0.0001; Mann-Whitney U test).

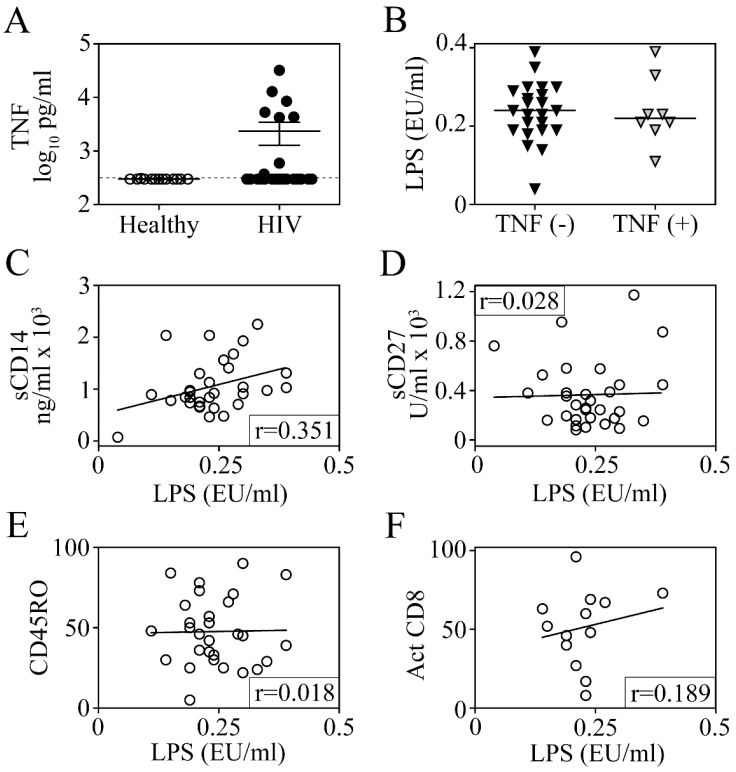

Figure 3. Microbial translocation is associated with monocyte/macrophage activation independent of lymphocyte activation

(A) Levels of TNF in plasma of healthy controls and HIV-1-infected subjects were compared (dotted line represents limit of detection 300 pg/ml). **(B)** HIV-1-infected subjects were delineated into TNF (-) and TNF (+) groups and the levels of plasma LPS in each group was compared. The relationships between levels of plasma LPS and **(C)** monocyte/macrophage activation or **(D)** systemic lymphocyte activation were determined by linear regression analyses. Association of plasma LPS with frequency of **(E)** effector/memory CD4 T cells or **(F)** activated CD8 T cells were determined by linear regression analyses.

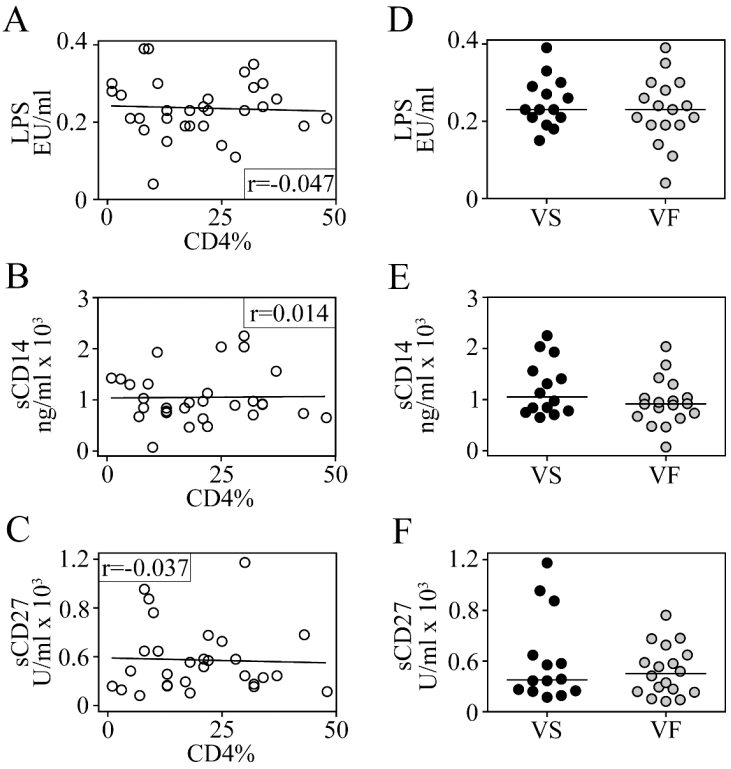

Figure 4. Microbial translocation and multilineage leukocyte activation develop independent of CD4 T cell attrition

The relationships between CD4 T cell frequency and plasma levels of **(A)** LPS, **(B)** sCD14 or **(C)** sCD27 were determined by linear regression analyses. The levels of **(D)** LPS, **(E)** sCD14 and **(F)** sCD27 were compared among viral success and viral failure subjects by Mann-Whitney U-test.

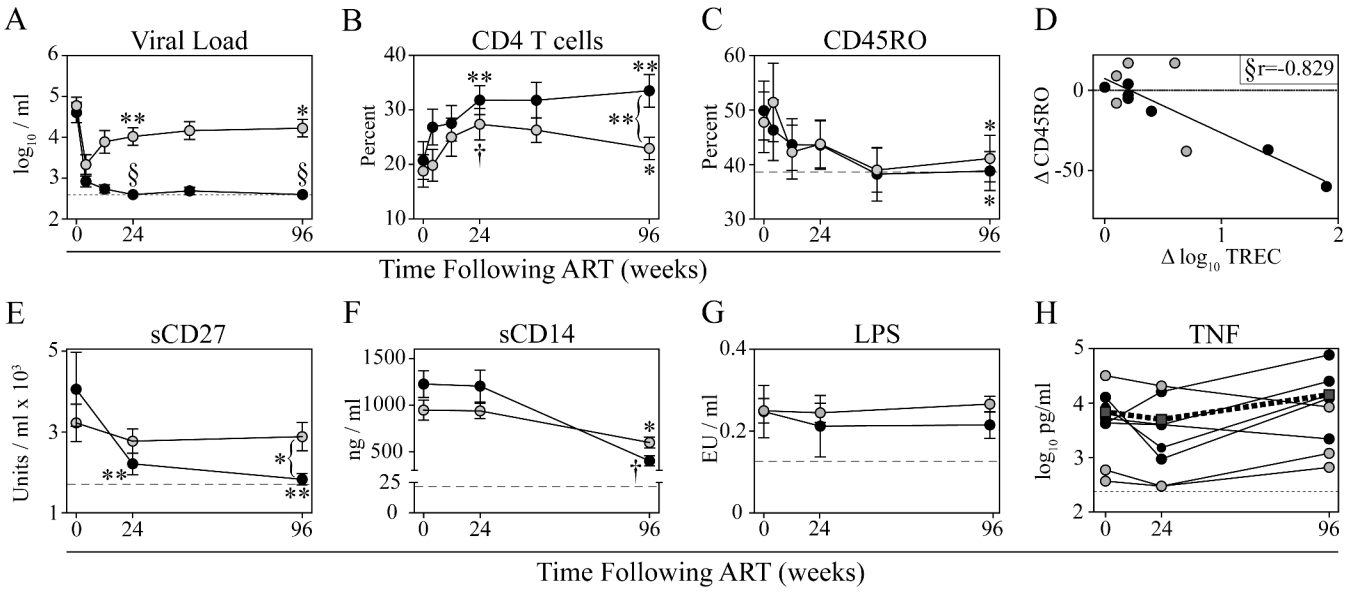

Figure 5. Longitudinal analyses of response to ART among viral success and viral failure subjects

(A) Plasma viral load **(B)** CD4% and **(C)** the frequency of effector/memory CD4 T cells were determined just prior to ART (0 weeks) and after 4, 12, 24, 48 and 96 weeks of ART in viral success (black circles) and viral failure (shaded circles) subjects (Panel B, dotted line represents limit of detection 2.6 $\log_{10}$ copies / ml). **(D)** The changes in CD4 CD45RO T cell frequency and $\log_{10}$ TREC following 24-48 weeks of ART were compared by linear regression. Plasma levels of **(E)** sCD27, **(F)** sCD14 and **(G)** LPS were determined prior to ART and after 24 and 96 weeks of ART in viral success and viral failure subjects. (Panels C, E, F and G, dashed lines represent mean values among healthy control subjects) **(H)** Longitudinal analysis of 8 subjects who had detectable plasma TNF. (Panel H, dotted line represents limit of detection 300 pg/ml TNF; Heavy dashed line with boxes represents mean value for all 8 subjects) (*P<0.05, **P<0.01, †P<0.001 and §P<0.0001, Wilcoxon matched pairs test for 0 wk vs. 24 wk or 0 wk vs. 96 wk, or Mann-Whitney U test for viral success versus viral failure).