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HIV-1 is not a major driver of increased plasma IL-6 levels in chronic HIV-1 disease

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Abstract

Objective—Increased plasma IL-6 levels have been associated with HIV-1 disease progression risk, yet the drivers of IL-6 production in HIV-1 infection are not known. This study was designed to explore the relationship between HIV-1 replication and IL-6 induction.

Design—Correlations between plasma levels of IL-6 and HIV-1 RNA were examined in two clinical studies. To more directly assess the induction of IL-6 by HIV-1, several cell and tissue types that support HIV-1 replication *in vivo* were infected with HIV-1 and expression of IL-6 was measured.

Methods—Spearman's rank correlations were used to examine the relationship between plasma levels of IL-6 and HIV-1 RNA. Macrophages, and colonic and lymph node histocultures were infected with HIV-1 or stimulated with bacterial products, LPS or flagellin, and IL-6 levels in supernatant were measured by ELISA or multiplex bead assay.

Results—In the clinical studies there was weak or no correlation between plasma levels of IL-6 and HIV-1 RNA but IL-6 levels were correlated with plasma levels of the LPS coreceptor CD14. Macrophages stimulated with LPS or flagellin showed robust production of IL-6, but there was no increase in IL-6 production after HIV-1 infection. IL-6 expression was not increased in lymph node histocultures obtained from HIV-1 infected subjects nor after productive HIV-1 infection of colonic or lymph node histocultures *ex vivo*.

Conclusions—We find no evidence that HIV-1 replication is an important driver of IL-6 expression *in vivo* or in *in vitro* systems.

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Conflicts of Interest: For the remaining authors none were declared.

Keywords

IL-6; HIV-1 RNA; histocultures; macrophages; LPS; flagellin

Introduction

Chronic immune activation is a major predictor of HIV-1 disease progression. Increased expression of CD38 and HLA-DR on T cells is an indicator of this immune activation¹⁻³ as are plasma levels of cytokines such as IL-6 and TNF α ⁴. The advent of highly active antiretroviral therapy (HAART) has greatly extended the life expectancy of persons infected with HIV-1, however, even with controlled viral replication, HIV-1 infected patients are at increased risks for morbidities including cancer, liver disease, and cardiovascular disease⁵. Though viral replication has been linked to many of these indices of immune activation, many remain elevated even when viral replication is “fully” suppressed by HAART^{1, 6-7}. In the SMART trial of sustained versus intermittent antiretroviral therapy, three soluble factors were independent predictors of overall and cardiovascular morbidities: IL-6, C-reactive protein (CRP) and D-dimers⁸. In a nested case-control sub-study of the SMART trial, plasma levels of soluble CD14 (sCD14) a coreceptor for bacterial lipopolysaccharide (LPS) were also an independent predictor of mortality⁹. In other studies, increased plasma IL-6 levels have been associated with new AIDS-defining illnesses or death¹⁰, the progression of clinical disease¹¹⁻¹³, and the development of opportunistic infections¹⁴. While it is clear that increased plasma IL-6 is associated with HIV-1 disease progression; it remains unclear what is driving the increased levels of IL-6 in HIV-1 disease. This study was designed to explore the relationship between HIV-1 replication and increased plasma levels of IL-6 in persons with HIV-1 infection. We found little indication that HIV-1 replication plays an important role in the elevated levels of IL-6 found in the plasma of HIV-1 infected subjects, as plasma levels of IL-6 correlated weakly or not at all with plasma levels of HIV-1 RNA, and we could not find increased levels of IL-6 in supernatants of macrophages, lymph nodes or colonic histocultures after *in vitro* infection with HIV-1.

Methods

Patients

These studies were approved by the institutional review board (IRB) at University Hospitals/Case Medical Center and for the MERIT study by the Institutional Review Boards or Independent Ethics Committees (IEC) of all participating sites. All patients provided written informed consent in accordance with the Declaration of Helsinki.

The MERIT Study compared the effects of Maraviroc or Efavirenz added to a treatment regimen containing Zidovudine and Lamivudine among treatment-naïve HIV-1 infected persons¹⁵. Stored samples obtained from 60 trial participants (30 receiving MVC and 30 treated with Efavirenz) who controlled viremia on these regimens were analyzed for markers of immune activation (CD38) and for markers of inflammation and coagulation (IL-6, D-dimers, TNF α I, TNF α II, and hs CRP) in plasma and comparisons between the study arms have been reported earlier¹⁶. In these subjects, median HIV-1 RNA level was 4.9 log₁₀ copies/mL and the median CD4 T cell count was 274 cells/uL (Supplemental Table 1). The second clinical study was a cross sectional study of 154 HIV-1 infected patients who were receiving care at University Hospitals/Case Medical Center. The median CD4 T cell count was 350 cells/uL, and the HIV-1 RNA level in plasma ranged from <50 copies/mL to >750,000 copies/mL. Stored plasma samples also were analyzed for levels of IL-6 and sCD14.

Whole pelvic lymph nodes were obtained from 11 HIV-1 infected adults and 10 uninfected adults who were undergoing medically indicated surgery at University Hospitals of Cleveland (Cleveland, OH). Lymphocyte phenotypes and spontaneous expression of cytokines were reported earlier¹⁷.

Normal human colonic mucosal tissue was obtained through the Tissue Procurement, Histology, and Immunohistochemistry Core Facility of the Case Comprehensive Cancer Center. Colonic tissue was cleaned of muscle and fat and then mucosa was cut into 3 mm × 3 mm explants for histoculture as described previously¹⁸⁻¹⁹.

Virus preparation and storage

HIV-1_{Ba-L} was propagated in a PM1 cell line (kindly provided by Dr. Donald E. Mosier Scripps Research Institute, La Jolla, CA) which is permissive for growth of macrophage and T cell tropic HIV-1 viruses. PM1 cells were grown in RPMI 1640 (BioWhittaker Lonza) containing 10% Fetal Bovine Serum (Gemini Bio-Products), 2 mM L-Glutamine (Biowhittaker Lonza) and 100 Units/mL Penicillin/Streptomycin (BioWhittaker Lonza). Supernatants from uninfected PM1 cells were used as controls for infection experiments. Undiluted HIV-1_{Ba-L} viral stock propagated in PM1 cells contained <0.5pg/mL IL-6.

HIV-1 infection of macrophages

Monocytes were obtained from heparinized whole blood of healthy, HIV-1 uninfected subjects by mixing whole blood with RosetteSep monocyte enrichment antibody cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) and incubating for 20 minutes at room temperature following manufacturer's instructions. Blood was then separated over Ficoll; unbound monocytes separated at the Ficoll-plasma interface while unwanted cell types pelleted with the erythrocytes. Enriched monocytes were plated in complete medium consisting of RPMI (Lonza, Walkersville, MD), 10% Fetal Bovine serum (Gemini Bio-Products, West Sacramento, CA), 1% L-glutamine and 1% penicillin streptomycin (Lonza, Walkersville, MD) at 1 million cells/mL in 24-well Falcon tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ) for 7 days, with medium replacement on day 4. On day 7 supernatant was collected and frozen. Macrophages were then infected overnight with HIV-1_{Ba-L} at 100, 1,000, or 10,000 TCID₅₀ or stimulated with 50ng/mL LPS (Sigma-Aldrich, St. Louis, MO), or with 1µg/mL flagellin (Invivogen, San Diego, CA). Following overnight infection or stimulation, supernatant was collected and fresh complete medium was added to all wells. The wells stimulated with LPS or flagellin received new medium containing fresh LPS or flagellin. Supernatants were collected at days 1, 3, 7, 10, and 14 after infection or stimulation.

Cytokine production

IL-6 levels in supernatant from colonic and macrophage cultures were measured using a human IL-6 ELISA kit (Quantikine) or a high sensitivity IL-6 ELISA kit (Quantikine HS) both from R&D Systems (Minneapolis, MN). Supernatants harvested from lymph node histocultures were tested for IL-6 levels using a multiplex bead array assay as described previously¹⁷.

Colonic mucosal histoculture

Two 3×3mm colonic explants were placed in 100 µL of RPMI 1640 (BioWhittaker Lonza, Walkersville, MD) supplemented with 15% Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 0.1 mM MEM-nonessential amino acids (Gibco Invitrogen, Grand Island, NY), 2.5 µg/mL Fungizone (Gibco Invitrogen), 1 mM MEM-Sodium Pyruvate (Gibco Invitrogen), 100 Units/mL Penicillin/Streptomycin (BioWhittaker Lonza) and 50 µg/

mL Gentamicin (Gibco Invitrogen) in a 96-well flat-bottomed plate and incubated for 1 hour at 37°C in 5% CO₂ to equilibrate prior to infection. After 1 hour, 1×10⁴ TCID₅₀ HIV-1_{Ba-L} in 100uL or 100 uL of control supernatant from the uninfected PM-1 cell line was added to each well. Explants were incubated with virus or control supernatant for 2 hours at 37°C in 5% CO₂ then washed with PBS. Following the wash step, tissues were placed on Gelfoam absorbable gelatin sponges (Pfizer, New York, NY) that had been soaked in medium in 24 well plates for 1 hour at 37°C in 5% CO₂. Plates containing explants on Gelfoam at the air-liquid interface²⁰ were incubated at 37°C in 5% CO₂. Supernatants were collected at the indicated times and frozen at -80°C for later analysis.

HIV-1 P24 measurement

P24 ELISA assay plates/kits were kindly provided by Dr. John Sullivan and Dr. Katherine Luzuriaga, (University of Massachusetts Medical Center) and were used for measurement of HIV-1 p24 levels in supernatant as described previously²¹.

Lymph node histoculture

Lymph nodes were dissected, and tissue blocks were placed on collagen sponge gels and cultured at the air-liquid interface for 15 days as described previously^{17, 20}. Using HIV-1 p24 enzyme-linked immunosorbent assay (ELISA), we detected HIV-1 replication in tissues of 8 of 11 patients with HIV-1, with average levels of 1046 pg/mL (range, 645-2462 pg/mL)¹⁷. Ten lymph node sections prepared from the nodes of healthy controls were infected overnight with approximately 300 TCID₅₀ of HIV-1_{LAI.04} virus per block. Sections were then washed with PBS and cultured in fresh medium and medium was changed every 3 days thereafter. Supernatant was pooled from each subject's samples and was tested for IL-6 levels as noted above.

Statistics

Plasma levels of IL-6, HIV-1 RNA, and other biomarker levels were compared using Spearman's rank correlation method. Differences between groups were compared using Mann-Whitney U tests.

Results

Plasma IL-6 levels correlate weakly with plasma HIV-1 RNA levels before administration of antiretroviral therapies

In the MERIT study at baseline, before initiation of antiretroviral therapy, plasma IL-6 levels were correlated to plasma HIV-1 RNA levels ($r=0.2949$; $p=0.0261$, Fig.1a). By weeks 4 and 12 after the initiation of therapy, plasma levels of HIV-1 and IL-6 were no longer significantly correlated (Fig.1b, 1c). IL-6 levels remained elevated when compared to levels seen in uninfected controls⁶ and did not decrease significantly until week 48 (Mann-Whitney $p=0.0112$) irrespective of the dramatic and early decreases in plasma HIV-1 RNA levels (Fig.1d).

Next, we analyzed data from a cross sectional study performed among 154 HIV-1 infected patients who were receiving care at University Hospitals/Case Medical Center. Plasma levels of IL-6 and sCD14, and HIV-1 RNA were measured. In this patient cohort the median CD4 T cell count was 350 cells/uL and plasma HIV-1 RNA levels ranged from <50 copies/mL to > 750,000 copies/mL. There was no correlation between plasma levels of IL-6 and plasma levels of HIV-1 RNA (Fig 2a). On the other hand, soluble CD14 levels in plasma correlated significantly with both plasma HIV-1 RNA levels (Fig.2b $r = 0.4216$; $p<0.0001$) and with IL-6 levels (Fig.2c $r = 0.3016$; $p=0.0009$). Considering the weak relationship between IL-6 levels and HIV-1 RNA levels in plasma in these clinical studies, we next

asked if we could demonstrate the induction of IL-6 expression in primary cells and in histocultures by HIV-1 exposure and replication.

HIV-1 infection of monocyte-derived macrophages does not induce IL-6 production

Monocytes were negatively selected from whole blood obtained from two HIV-1 uninfected subjects. Enriched monocytes were placed in plastic 24-well tissue culture plates in complete medium and allowed to mature for 7 days. After maturation, replicate wells containing monocyte-derived macrophages were incubated overnight with medium alone, or with LPS (50ng/mL), flagellin (1ug/mL), or with HIV-1_{Ba-L} 1,000 and 10,000 TCID₅₀. LPS or flagellin exposure induced substantial expression of IL-6 that ranged from 11.5 to 2288 pg/mL at day 3 (Table 1). In triplicate wells, macrophages became actively infected as reflected by increasing HIV-1 p24 levels in supernatants (Table 1). Despite productive HIV-1 infection, levels of IL-6 were not increased in the culture supernatants at any time in these infected wells from day 3 to day 14. In a third similar experiment (not shown), exposure of monocyte-derived macrophages to HIV-1_{Ba-L} did not result in productive HIV-1 infection in any well. In these wells too, IL-6 levels were not increased above levels in unexposed wells (not shown). This indicates that neither productive HIV-1 replication nor exposure to HIV-1 virions without infection could induce IL-6 expression. Unstimulated peripheral mononuclear cells (PBMC) isolated from whole blood were also incubated overnight with medium, LPS (50ng/mL), flagellin (1ug/mL), or with HIV-1_{Ba-L}. As was seen among monocyte-derived macrophages, no IL-6 was induced by HIV-1 infection of PBMC, yet exposure to LPS or flagellin induced substantial levels of IL-6 (data not shown).

HIV-1 infection of colonic histocultures does not induce IL-6 production

Since mucosal and secondary lymphoid tissues are the major sites of HIV-1 replication *in vivo*, we asked if HIV-1 infection of these tissues could induce production of IL-6. Colonic biopsies from three HIV-1 uninfected subjects (C3, C4, C5) were infected *ex vivo* with HIV-1_{Ba-L} for 2 hours then washed. Sections were cultured on absorbable gelatin sponges for 2 weeks at the air-liquid interface²⁰. Culture medium was collected and replaced with new medium on days 1, 3, 7, 10, and 14. Viral infection was confirmed by documenting increases in HIV-1 p24 levels in supernatant by ELISA (data not shown) and IL-6 levels were measured by ELISA. Although productive infection and IL-6 production were documented in the virus-exposed histocultures, no difference was noted between the levels of IL-6 generated by the infected histocultures when compared to the levels generated by the uninfected tissues at any time point (Fig.3).

Lymph node histocultures infected with HIV-1 *in vivo* or *ex vivo* do not increase IL-6 production

Lymph node samples obtained by surgical biopsy from 11 HIV-1+ patients and 10 uninfected subjects were cut into sections and cultured on collagen sponge gels at the air-liquid interface. Sections from the 10 uninfected subjects were cultured in medium or infected overnight with HIV-1_{LAI.04}. The following day the sections were washed and new medium was added. Culture supernatants were collected every three days and IL-6 was measured using a multiplex bead array assay¹⁷. Figure 4 shows the accumulated levels of IL-6 produced by lymph node cultures from 11 HIV-1+ patients (chronic), 10 uninfected subjects (control), and 10 lymph node sections from the same uninfected subjects that were infected by overnight exposure to HIV-1_{LAI.04} (acute). There was no difference in IL-6 levels in lymph node samples from controls that were left uninfected (control, median 17,528 pg/mL) or were infected with HIV-1 (acute, median 19,571pg/mL). Interestingly, levels of IL-6 produced spontaneously by lymph node histocultures from HIV-1 infected patients (chronic; median 2437pg/mL) were significantly lower than levels produced by the uninfected or *ex vivo* infected histocultures (Mann-Whitney U test, p=0.0288 and p=0.0232,

respectively). This may represent a depletion of IL-6 producing cells in the chronically infected node¹⁷ or a failure of spontaneous IL-6 expression in the chronically infected tissues.

Discussion

Although increased plasma levels of IL-6 have been linked to risk of HIV-1 disease progression in some^{4, 8, 10-11, 13} but not all studies²², the determinants of IL-6 induction and immune activation in this setting are incompletely understood. Here we report the results of two clinical studies in which the relationship between plasma HIV-1 RNA levels and IL-6 are explored. In the first, an antiretroviral therapy trial among treatment-naïve subjects (MERIT), pre-treatment HIV-1 RNA levels in plasma correlated weakly with plasma IL-6 levels but this relationship disappeared rapidly as HIV-1 RNA levels fell with application of antiretroviral therapies. Plasma levels of IL-6 did not decrease significantly until 48 weeks of therapy (Mann-Whitney $p=0.0112$) but never reached levels seen in uninfected persons. Thus, the correlation between HIV-1 RNA levels and IL-6 levels in plasma was weak at baseline and disappeared with suppression of HIV-1 replication as the substantial decrease in HIV-1 replication in the first weeks of therapy were not reflected by a decrease in the plasma levels of IL-6. In a different study where plasma cytokines and HIV-1 RNA levels were monitored early after introduction of antiretroviral therapy, although HIV-1 RNA levels fell rapidly and significantly within the first few days of therapy, plasma levels of IL-6 did not decrease durably even after 24 weeks of therapy²³. In another large study that involved more than 1000 HIV-1 infected patients, examining the association of low level viremia with inflammation and mortality, HIV-1 RNA was only significantly associated with plasma IL-6 levels when virus levels exceeded 10,000 copies/mL²⁴. Interestingly, this association was strongly influenced by the CD4 T cell count²⁴.

In the second clinical study reported here, a cross sectional study of a heterogeneous population of HIV-1 infected adults, only a weak but statistically not significant relationship between plasma levels of IL-6 and HIV-1 RNA levels could be demonstrated. In a recently published study, we found that plasma levels of IL-6 remained elevated in patients who had therapy-controlled viremia (<400 copies/mL) for an average of more than 7 years. Importantly, IL-6 levels were significantly higher in subjects who failed to increase their CD4 T cell counts to levels >350/uL than in subjects whose CD4 T cell counts had increased to levels >500/uL²⁵. While other studies have also found a weak relationship between plasma levels of IL-6 and HIV-1 RNA^{9, 26}, the determinants of high level IL-6 expression in HIV-1 infection appear to be complex and there is dissociation between levels of HIV-1 and levels of IL-6 in the plasma of patients, especially during the dynamic changes in HIV-1 replication after the introduction of antiretroviral therapy.

As the immunologic and inflammatory interactions in HIV-1 infection are complex, we decided to utilize simpler systems of primary cell and histoculture models to ascertain if HIV-1 replication could induce IL-6 expression. Using unstimulated PBMC, macrophages, lymph nodes, and colonic mucosal histocultures, we could not find any evidence that *ex vivo* HIV-1 exposure or productive infection could drive IL-6 production in any of these systems. Although macrophages did not express IL-6 after HIV-1 exposure and infection, incubation with bacterial products lipopolysaccharide and flagellin, induced high level expression of IL-6. We also examined the expression of IL-6 in lymph node histocultures obtained from subjects who were HIV-1 infected and found that the spontaneous expression of IL-6 in these histocultures was actually less than in histocultures prepared from lymph nodes of uninfected controls.

Very high levels of IL-6 were produced by colonic histocultures whether HIV-1 infected or not and relatively high levels of IL-6 also accumulated in lymph node histocultures whether HIV-1 infected or not. The relatively high background levels of IL-6 seen in the colonic mucosal histocultures could be a consequence of exposure to colonic bacteria *in vivo* and this might have obscured induction of IL-6 by HIV-1 infection *ex vivo*. On the other hand, gut mucosal cells could be an important source of systemic IL-6 levels *in vivo*. Conceivably, greater exposure of mucosal immune cells to microbes and their products as a consequence of the sustained damage to gut mucosal integrity in HIV-1 infection²⁷⁻²⁸ may underlie the high levels of IL-6 typically found in untreated as well as treated HIV-1 infection. In this regard, a recent study examining a mouse model of colitis showed that microbiota of the gut could induce the production of IL-6 by dendritic cells and this could promote T cell activation and colitis²⁹. While we show here a good correlation between plasma levels of IL-6 and the LPS coreceptor sCD14, we did not have samples available for measurement of LPS. We had earlier reported elevated plasma levels of IL-6 in patients with controlled viremia but who experienced incomplete immune restoration⁶. On analysis of that data set we can now report that plasma IL-6 levels were correlated with plasma LPS levels (Spearman's rho; $r=0.287$, $p=0.009$, $n=81$)⁶. Marchetti et al. found that untreated patients with higher plasma levels of LPS also tended to have higher levels of IL-6 ($p=0.053$)²².

The lack of induction of IL-6 expression *in vitro* by HIV-1 replication is consistent with earlier reports that *in vitro* HIV-1 infection of PBMCs or macrophages alone does not induce IL-6 production³⁰⁻³¹. Molina et al. infected PBMC overnight with HIV-1 and then examined culture supernatant for IL-1 β , IL-6, and TNF α . They did not detect induction of any of these cytokines by HIV-1, however, PBMC exposed to LPS at concentrations as low as 0.5 ng/mL expressed significant amounts of IL-1 β , IL-6, and TNF α . On the other hand, Nakajima et al. showed that PBMC exposed to either live or inactivated HIV-1 could produce IL-6 as measured by the IL-6-dependent proliferation of the murine cell line MH60.BSF-2³². Gan et al. infected monocyte-derived-macrophages with HIV-1_{Ba-L} and saw no induction of IL-6. When HIV-1 infected cells were also incubated with LPS, levels of IL-6 mRNA were 7-fold higher than in uninfected cells that were exposed to LPS alone. HIV-1 proteins Tat³³⁻³⁴ and Vpr³⁵ have been reported to induce IL-6 gene expression however, these studies reported only the effects of recombinant viral proteins and not the induction of IL-6 by whole virus infection. We did not explore the effects of HIV-1 exposure on transcription of IL-6 RNA in any of the systems we employed. Thus, it is conceivable that HIV-1 exposure induces IL-6 transcription and cytokine synthesis but that the cytokine is degraded or absorbed by cells within these preparations. Should this be the case *in vivo*, we still cannot attribute the high systemic levels of IL-6 in HIV-1 infection to HIV-1 replication.

It is worth noting that IL-6 can reactivate provirus expression in HIV-1 infected macrophages³⁶ so it is possible that the weak correlations seen between plasma levels of IL-6 and HIV-1 RNA may be a consequence of IL-6 induction of latent virus expression rather than the other way around.

In summary, the correlations between HIV-1 RNA and IL-6 levels in plasma were weak at best, and we could find no evidence that HIV-1 infection could induce IL-6 expression *in vitro*. These findings suggest that HIV-1 replication does not directly drive the high systemic levels of IL-6 found in HIV-1 disease. The correlations we found between IL-6 and other indices of inflammation, such as sCD14, suggest that IL-6 levels are associated with the general immune activation and inflammation that are demonstrable even when HIV-1 replication is well-controlled by antiretroviral therapy. What is inducing this continued immune activation and inflammation remains a question. We suspect that this may be related to the apparent persistent defect in gut mucosal integrity seen even in treated HIV-1

infection³⁷ and the local and systemic exposure to gut-derived microbial products that can drive IL-6 expression *in vitro* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Plasma levels of IL-6 correlated weakly with HIV-1 RNA levels only before therapy and did not decrease commensurately with plasma HIV-1 RNA after antiretroviral therapy After initiation of HAART there was a rapid decline in plasma HIV-1-RNA levels, but plasma levels of IL-6 did not decrease significantly until week 48 (Mann-Whitney $p=0.0112$). a) HIV-1 RNA levels correlated with plasma IL-6 at baseline before HAART ($p=0.0261$) but not at 4wks (b) or 12wks (c). d) Mean plasma IL-6 and HIV-1 RNA levels at each time point, error bars represent standard deviation from mean ($n=60$). Spearman's rank correlation was used to evaluate associations. P values of <0.05 were considered significant.

Figure 2. Plasma levels of IL-6 did not correlate significantly with plasma HIV-1 RNA levels in a cross sectional study, but sCD14 levels correlated with both plasma HIV-1 RNA and with IL-6. Spearman's rank correlation was used to evaluate associations. P values of <0.05 were considered significant.

Figure 3. Colonic mucosa histocultures infected with HIV-1 did not produce more IL-6 than did uninfected histocultures

Supernatant from colonic mucosal histocultures from 3 subjects (C3, C4, C5) were collected on days 1, 3, 7, 10, and 14 and tested for IL-6 levels by ELISA. Bars represent the means and standard deviation of 4 replicate wells. Active infection was confirmed by detection of HIV-1 p24 antigen in supernatant by ELISA (data not shown).

Figure 4. IL-6 production was not increased in lymph nodes obtained from HIV-1 infected subjects or in histocultures infected *ex vivo* with HIV-1

IL-6 levels in supernatant of lymph node histocultures prepared from 11 HIV-1+ patients (chronic), 10 uninfected subjects (control), and 10 lymph node sections from uninfected subjects infected overnight *ex vivo* with HIV-1 (acute) are shown. While IL-6 levels were not significantly different between the uninfected controls and the acutely infected samples, the chronically infected samples produced less IL-6 than the control samples (Mann Whitney $p=0.0288$) and the acutely infected samples (Mann Whitney $p=0.0232$).

Table 1
HIV-1 infection of monocyte-derived macrophages did not induce production of IL-6, while stimulation with LPS or flagellin induced large amounts of IL-6

Monocyte-derived macrophages were incubated with HIV-1_{Ba-L}, bacterial LPS or flagellin as described in the methods. IL-6 was measured in culture supernatant by ELISA. Active infection was confirmed by detection of HIV-1 p24 antigen by ELISA. Macrophage infection and IL-6 production

Subject 1	well	Day 3		Day 7		Day 10		Day 14	
		HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL
Uninfected		BLD	8.75	BLD	0.35	BLD	0.32	108	<0.04
HIV-1 (1,000 _{TCID50})	a	36	1.77	77	0.06	1586	0.22	>2000*	0.32
	b	BLD	2.55	81	<0.04	>200*	<0.04	>200*	<0.04
	c	43	2.72	BLD	0.42	390	0.17	>2000*	<0.04
HIV-1 (10,000 _{TCID50})	a	101	0.34	68	0.17	200	<0.04	1053	0.32
	b	109	5.10	134	0.97	>200*	<0.04	727	0.05
	c	100	0.24	BLD	0.30	1203	0.06	828	<0.04
LPS		BLD	2288.0	BLD	ND	BLD	ND	BLD	ND
flagellin		BLD	258.5	BLD	ND	BLD	ND	BLD	ND
Subject 2	well	Day 3		Day 7		Day 10		Day 14	
		HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL	HIVp24 pg/mL	IL-6 pg/mL
Uninfected		BLD	0.05	BLD	<0.04	BLD	<0.04	BLD	<0.04
HIV-1 (1,000 _{TCID50})	a	BLD	0.18	>2000*	0.15	>2000*	<0.04	>2000*	<0.04
	b	BLD	0.59	5215	<0.04	BLD	<0.04	BLD	0.17
	c	BLD	0.76	8982	<0.04	43	<0.04	>200*	0.07
HIV-1 (10,000 _{TCID50})	a	75	1.15	770	<0.04	1051	<0.04	>2000*	0.66
	b	127	0.42	182	<0.04	175	<0.04	>200*	0.02
	c	133	0.93	>200*	<0.04	1167	<0.04	>2000*	0.32
LPS		BLD	51.5	BLD	ND	BLD	ND	BLD	ND
flagellin		BLD	11.5	BLD	ND	BLD	ND	BLD	ND

BLD; below the limits of detection (12.5pg/mL) ND; not done

* sample to concentrated, above the limit of detection

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