

# The effect of HIV infection and HAART on inflammatory biomarkers in a population-based cohort of US women

Sheila M. Keating<sup>a,b</sup>, Elizabeth T. Golub<sup>e</sup>, Marek Nowicki<sup>f</sup>,  
Mary Young<sup>g</sup>, Kathryn Anastos<sup>h</sup>, Howard Crystal<sup>i</sup>, Mardge H. Cohen<sup>j,k</sup>,  
Jinbing Zhang<sup>e</sup>, Ruth M. Greenblatt<sup>c</sup>, Seema Desai<sup>k</sup>, Shiquan Wu<sup>a</sup>,  
Alan L. Landay<sup>k</sup>, Stephen J. Gange<sup>e</sup>, and Philip J. Norris<sup>a,b,d</sup> and the  
Women's Interagency HIV Study

**Objective:** HIV causes inflammation that can be at least partially corrected by HAART. To determine the qualitative and quantitative nature of cytokine perturbation, we compared cytokine patterns in three HIV clinical groups including HAART responders (HAART), untreated HIV non-controllers (NC), and HIV-uninfected (NEG).

**Methods:** Multiplex assays were used to measure 32 cytokines in a cross-sectional study of participants in the Women's Interagency HIV Study (WIHS). Participants from 3 groups were included: HAART (n = 17), NC (n = 14), and HIV NEG (n = 17).

**Results:** Several cytokines and chemokines showed significant differences between NC and NEG participants, including elevated IP-10 and TNF- $\alpha$  and decreased IL-12(p40), IL-15, and FGF-2 in NC participants. Biomarker levels among HAART women more closely resembled the NEG, with the exception of TNF- $\alpha$  and FGF-2. Secondary analyses of the combined HAART and NC groups revealed that IP-10 showed a strong, positive correlation with viral load and negative correlation with CD4+ T cell counts. The growth factors VEGF, EGF, and FGF-2 all showed a positive correlation with increased CD4+ T cell counts.

**Conclusion:** Untreated, progress HIV infection was associated with decreased serum levels of cytokines important in T cell homeostasis (IL-15) and T cell phenotype determination (IL-12), and increased levels of innate inflammatory mediators such as IP-10 and TNF- $\alpha$ . HAART was associated with cytokine profiles that more closely resembled those of HIV uninfected women. The distinctive pattern of cytokine levels in the 3 study groups may provide insights into HIV pathogenesis, and responses to therapy.

© 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

*AIDS* 2011, **25**:000–000

**Keywords:** CD4+ T cells, chemokines, cytokines, HAART, HIV

---

<sup>a</sup>Blood Systems Research Institute, San Francisco, California, USA, <sup>b</sup>Departments of Laboratory Medicine, <sup>c</sup>Pharmacy, <sup>d</sup>Medicine, University of California, San Francisco, San Francisco, California, USA, <sup>e</sup>Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA, <sup>f</sup>Keck School of Medicine of the University of Southern California, Los Angeles, California, USA, <sup>g</sup>Georgetown University Medical Center, Washington, DC, USA, <sup>h</sup>Albert Einstein College of Medicine, Bronx, New York, USA, <sup>i</sup>SUNY Downstate Medical Center, Brooklyn, New York, USA, <sup>j</sup>Department of Medicine, Stroger Hospital, and <sup>k</sup>Rush University Medical Center, Chicago, Illinois, USA.

Correspondence to Mailing address: Blood Systems Research Institute, 270 Masonic Avenue, San Francisco, CA 94118.

Tel: +415 923 5769; fax: +415 567 5899; e-mail: pnorris@bloodsystems.org.

Received: 28 January 2011; revised: 14 April 2011; accepted: 9 May 2011.

DOI:10.1097/QAD.0b013e3283489d1f

## Introduction

Infection with HIV leads to immune dysfunction and progression to AIDS within 10–11 years in the absence of antiretroviral therapy in the majority of infected persons [1,2]. There is considerable variability in the rate of disease progression, and the factors underlying the pathogenesis of HIV are not entirely understood. Persons with progressive HIV infection on average have higher viral loads [3] and elevated levels of activated T cells [3,4], including HIV-specific T cells [5]. Elevated cytokine levels in HIV infection could have positive or negative effects on viral control or CD4+ T cell homeostasis. *In vitro* studies have revealed that a number of factors can contribute to enhanced HIV replication, including TNF- $\alpha$  [6–8] and IL-2 [9,10]. In contrast, some cytokines appear to decrease HIV replication in tissue culture, including IFN- $\alpha$  [11,12], IFN- $\gamma$  [6,12,13], and GM-CSF [13].

Prior studies have assessed the associations of soluble markers of inflammation with disease outcome during chronic SIV or HIV infection. An SIV model of infection revealed no differences in cytokine perturbations between animals that progressed rapidly versus slowly to AIDS when studied at the terminal stage of AIDS, though animals with encephalitis had higher IL-2 and IL-6 levels compared to animals without encephalopathy [14]. Recent studies of pathogenic vs. non-pathogenic infection of rhesus macaques and African green monkeys have revealed a host of factors differentially regulated in these disease models [15–18]. In humans, prior work has demonstrated that patients with chronic HIV infection typically show elevations in serum or plasma TNF- $\alpha$  levels [19–21]. In a large cohort of HIV-infected men, the plasma activation markers soluble TNF receptor II (sTNF-RII), neopterin, and sIL-2R correlated well with each other and had some ability to predict progression to AIDS independent of CD4+ T cell count or plasma HIV viral load [22]. More recent data from the SMART trial showed that elevated levels of C-reactive protein, IL-6, and D-dimer were associated with increased risk of death in a cohort in which most participants received HAART [23].

The above studies suggest that soluble mediators of inflammation are associated with HIV disease progression. Our group and others have identified soluble markers of inflammation that are dysregulated in acute HIV infection [24,25], more recently using newly available multiplex cytokine testing reagents [26,27]. We applied these multiplex cytokine detection techniques to study HIV seronegative women and women with chronic HIV infection. Thirty-two soluble immune markers were quantified and correlated with clinical group, viral load, and CD4+ T cell count. This study furnishes a more complete picture of the degree and

importantly the breadth of immune dysfunction associated with uncontrolled viral replication, and reveals the ability and limitations of HAART to correct the systemic inflammation associated with HIV.

## Methods

### Study participants

Subjects were participants in the Women's Interagency HIV Study (WIHS), an ongoing multi-site cohort study of HIV among US women, which enrolled participants in 1994–95 and 2001–02 [28,29]. Semiannual visits include interview, clinical exam, and collection of biologic specimens. HIV non-controllers (NC,  $n = 14$ ) were antiretroviral therapy naive and had a viral load  $>10\,000$  RNA copies/ml for at least one of two time points separated by 6 months. HAART responders ( $n = 17$ ) had undetectable viral load ( $<80$  RNA copies/ml) for at least 12 months while on a potent combination antiretroviral regimen. HIV uninfected women (NEG,  $n = 17$ ) in WIHS undergo the same follow-up procedures as the HIV infected women, and have HIV serology performed every 6 months. HCV serology was performed at study entry, and HCV plasma RNA quantitation was performed on seropositive women to determine if infection was ongoing versus resolved. Participants for the current study were chosen from the total WIHS cohort of 3,766 women to match within the three study groups (NEG, HAART, and NC) based on ethnicity (African-American versus other), age, body mass index, HCV antibody status at study entry, and time of follow-up in the cohort (within one year).

### Sample selection

Two serum samples for each subject were tested, with the samples chosen near the beginning and end of the period of clinical interest (i.e. during the period of undetectable viremia for the HAART group and during a period of the highest level viremia for the NC group). The average time span between the paired samples from each subject was 2.7, 3.3, and 2.9 years for the NEG, HAART, and NC groups, respectively. For three HAART participants one of the two samples tested was plasma, and these samples were excluded from the analysis due to potential differences in cytokine levels between these two sample types (data not shown).

### Multiplex cytokine and chemokine analysis

Serum samples were assayed using the high-sensitivity LincoPlex kit (Millipore, Billerica, MA) for IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p70, IL-13, IFN- $\gamma$ , GM-CSF and TNF- $\alpha$ , and the standard-sensitivity Milliplex Map kit (Millipore) for epidermal growth factor (EGF), Eotaxin, fibroblast growth factor

(FGF)-2, fractalkine, IL-1 $\alpha$ , IL-1R $\alpha$ , IL-9, IL-12(p40), IL-15, IL-17, IP-10, monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , sIL-2R $\alpha$ , TNF- $\beta$ , and vascular endothelial growth factor (VEGF) following the manufacturer's protocols. Standard curves were run in duplicate, and samples were tested in duplicate. Samples were acquired on a Labsan 100 analyzer (Luminex) using Bio-Plex manager 4.1 software (Bio-Rad). FGF-2 levels were also assayed in secondary testing by high-sensitivity ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

### Statistical analysis

For participants with data available from two time points (all but the three HAART subjects noted above), cytokine levels were averaged over the two time points for analysis of the association of cytokine levels with clinical group. For analyses of cytokine level correlations with viral load or CD4+ T cell count the observations from each subject were not averaged. Cytokine levels were compared between clinical groups using one-way ANOVA and Tukey's HSD (Honestly Significant Differences) tests. Differences in subject characteristics between groups were evaluated by ANOVA for continuous variables and by Fisher's exact test for ongoing HCV infection. Associations of cytokine levels with HIV viral load and CD4+ T cell count were assessed via linear regression. For analyses that included viral load values, these were set at 40 RNA copies/ml for participants with an undetectable viral load (half the lower limit of detection of 80 RNA copies/ml). Cytokine and viral load values were log-transformed prior to analysis due to non-normal distribution of the data. P-values were adjusted into FDR (False Discovery Rates) by the Benjamini and Hochberg controlling procedure, a commonly used method for analysis of large sets of biological data [30]. Statistical significance was defined as  $p < 0.05$  and  $FDR < 0.1$ . R/Bioconductor software was utilized for analyses.

## Results

### Participant characteristics

Consistent with the overall WIHS cohort, each of the study groups (NEG, HAART, and NC) was predominantly African American (Table 1). The median age for each group at the time of sample collection was 35 to 40 years and did not differ significantly between groups ( $p = 0.3$ , ANOVA). Median CD4+ T cell counts were significantly lower in the NC group compared to NEG and HAART women ( $p < 0.001$ , ANOVA). The difference in CD4+ T cell counts between the NEG and HAART was not significant ( $p > 0.05$ , Tukey's post-test).

### Cytokine perturbations are most pronounced in viremic women

Multi-analyte bead-based assays were used to assess 32 soluble markers covering pro- and anti-inflammatory mediators, chemotaxis signaling, and growth factor secretion. The vast majority of analytes tested (27 of 32) did not show significant differences between the study groups (Table 2). However, five analytes were significantly different in one or both groups of HIV infected women. Compared to HIV negative women, NC participants had significantly higher serum levels of the inflammatory mediators TNF- $\alpha$  and IP-10 and significantly lower serum levels of IL-12(p40), IL-15, and FGF-2 (Fig. 1). Levels of the analytes also differed at the level of statistical significance after application of a false discovery rate step-down procedure to the dataset to account for the multiple analytes examined [30].

One possible confounder of the results would be ongoing HCV replication, which could drive soluble markers of inflammation. We first examined cytokine levels in HCV RNA positive vs. negative women within the HIV NEG and HAART groups. Levels of IP-10 were significantly higher in both groups of HCV viremic women, and IL-10 was higher only in the HIV NEG, HCV RNA positive women ( $p < 0.05$ ,  $FDR < 0.1$ ). To control for this the

**Table 1. Demographic and clinical characteristics in 48 included women.**

	HIV negative	HAART	Non-controllers	p-value
Age (years)	35 (33–45)	40 (36–46)	38 (34–44)	0.3
Race (% Black)	82%	82%	79%	1
BMI	28.8 (24.2–36.3)	29 (26.7–32.7)	31.9 (24.8–39.4)	0.2
IDU	3 (18%)	3 (18%)	1 (7%)	0.6
HCV antibody+	4 (24%)	6 (35%)	2 (14%)	0.4
HCV RNA+	3 (18%)	3 (18%)	0 (0%)	0.2
CD4 count (cells/ $\mu$ l)	908 (703–1,193)	841 (703–1042)	553 (381–756)	<0.0001
Viral load (RNA copies/ml)		<80	14,000 (6,200–35,000)	N/A

Median and interquartile range (IQR) values are shown, except race which is shown as percent; IDU = self reported intravenous drug use at study entry; HCV RNA+ = hepatitis C virus RNA positive at study entry.

Table 2. Cytokine levels by disease category.

	NEG		HAART		NC	
	Median	IQR	Median	IQR	Median	IQR
Pro-inflammatory/ T cell						
IL-1 $\alpha$	38.6	1.6–134	1.6	1.6–75	1.6	1.6–1.6
IL-1 $\beta$	0.1	0.1–0.7	0.1	0.1–0.1	0.1	0.1–0.1
IL-2	2.8	0.3–8.6	0.3	0.1–0.5	0.3	0.1–1.3
IL-6	3.3	2.1–5.8	2.2	1–5.1	2.2	1.2–4.8
IL-7	7.2	5.0–9.3	8.8	5.7–9.7	7.6	5.4–13.9
IL-8	22.8	6.3–50.1	13.7	6.4–22.4	11.4	7.1–20.7
IL-9	6.8	1.6–80.6	1.6	1.6–5.9	2.1	1.6–19.4
<b>IL-12(p40)</b>	67.2	3.6–244	1.6	1.6–93.9	<b>1.6</b>	1.6–1.6
IL-12(p70)	0.2	0.1–2.7	0.5	0.1–1.2	0.1	0.1–0.2
<b>IL-15</b>	5.8	3.8–32.1	5.3	2.7–5.9	<b>1.6</b>	1.6–2.7
IL-17	6.4	2.0–58.7	8.2	1.6–45.1	1.6	1.6–2.5
IFN- $\gamma$	1.8	0.1–3.4	0.2	0.1–0.5	0.1	0.1–1.1
<b>TNF-<math>\alpha</math></b>	4.7	3.9–7.3	<b>10.4</b>	9.2–14.1	<b>12.7</b>	11.4–18.2
TNF- $\beta$	1.6	1.6–11.1	1.6	1.6–1.6	1.6	1.6–1.6
GM-CSF	1.6	0.6–3.3	0.2	0.1–1.3	0.2	0.1–1.3
Anti-inflammatory/Th2						
IL-1R $\alpha$	8.2	1.6–75.3	1.6	1.6–10.4	1.6	1.6–24.5
sIL-2R $\alpha$	23.5	1.6–53.1	77.8	55.8–189	46.8	4.3–120
IL-4	0.2	0.1–5.8	0.3	0.1–0.3	0.3	0.1–8.9
IL-5	0.1	0.1–0.5	0.1	0.1–0.3	0.2	0.1–0.5
IL-10	6.4	3.4–12	7.5	3.3–9.7	12.1	6–16
IL-13	2.7	1.3–10.2	0.3	0.2–5.3	1.1	0.1–8.1
Chemoattractants						
<b>IP-10</b>	143	110–224	204	146–322	<b>514</b>	412–773
MCP-1	411	307–560	552	393–675	541	445–633
MCP-3	29.4	2.5–58.5	1.6	1.6–19	1.6	1.6–32.6
MDC	2570	1990–3460	3460	2910–4140	2720	2020–3060
MIP-1 $\alpha$	125	69.1–242	71	29.2–128	68.3	35.8–144
MIP-1 $\beta$	74.0	52.3–195	82	60.3–140	56.1	48.9–78.1
Eotaxin	93.2	60.3–160	80.3	73.6–165	95.8	71.6–138
Fractalkine	35.6	1.6–278	64.7	1.6–400	1.6	1.6–50.1
Growth factors						
VEGF	222	122–404	360	116–544	194	156–272
EGF	167	120–245	199	134–291	208	121–247
<b>FGF-2</b>	32.8	18.6–73.2	<b>13.8</b>	1.6–23.4	<b>8.3</b>	1.6–17.8

Bold numbers denote values different from HIV NEG,  $p < 0.05$ ,  $FDR < 0.1$ .

results were re-analyzed, excluding the six women who had detectable HCV RNA at study entry. Each of the associations found in the initial comparison of HIV NC vs. NEG women remained significant with  $p < 0.05$ , though the FDR was  $> 0.1$  for the associations between IL-12(p40) and IL-15 and NC status. In summary, uncontrolled HIV replication was associated with perturbed cytokine and chemokine levels impacting multiple inflammatory and immune pathways.

### Cytokine levels of women on effective HAART are similar but not identical to those in HIV seronegative women

HAART has been shown to correct much of the inflammation induced by HIV at the level of CD4+ and CD8+ T cells [31]. Abnormalities in a number of serum markers have been shown to improve but not resolve on HAART [32,33], so it was of interest to see if these changes extended to a broader panel of soluble mediators of inflammation. In general, we found the distortion of

the pattern of soluble inflammatory mediators was less pronounced in participants with suppressed viral replication. The extent of increase in TNF- $\alpha$  and decrease of FGF-2 seen among NC compared with NEG women were mitigated in women in the HAART group. However, the levels of these cytokines did remain different from NEG women (Fig. 1 and Table 2). The significant differences compared to NEG women in levels of IP-10, IL-12(p40), and IL-15 that existed in NC participants were not seen in the HAART group. Limitation of analyses to HCV RNA negative women did not change the associations except that the HAART group had significantly lower IP-10 levels compared to NC women and the FDR was  $> 0.1$  for FGF-2 in HAART vs. NEG women, though the  $p$  value remained  $< 0.05$ . These findings demonstrate that while suppression of HIV viremia was associated with much less cytokine perturbation than in the NC women, some cytokines such as TNF- $\alpha$  and FGF-2 did not match levels seen in NEG women.

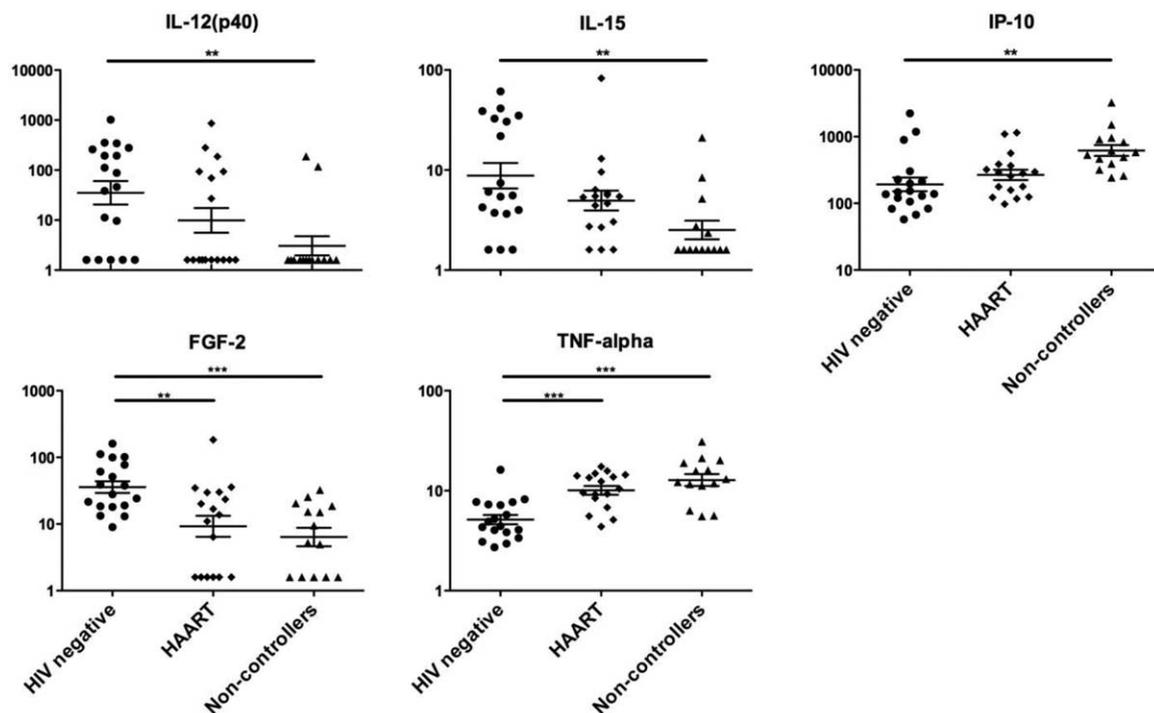


Fig. 1. Cytokine concentration by clinical group. Cytokine levels for each of the analytes showing significant differences between groups are shown. Horizontal bar represents mean level, with error bars denoting the standard error of the mean. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### Correlation between cytokine levels and plasma viral load

To address the contribution of HIV replication to cytokine levels, we compared analyte levels to plasma HIV RNA copy numbers. Cytokine and plasma viral load levels were log-transformed and linear regression was performed for each analyte. Five analytes were found to be associated with viral load, and of those only IP-10 was also among the five analytes that differed between NEG and NC women ( $p < 0.001$ ). While IL-12(p40), IL-15, TNF- $\alpha$ , and FGF-2 were all elevated in NC participants, the levels of these cytokines were not associated with viral load (Table 3a). IP-10 was the only factor showing a positive correlation with viral load. By contrast, levels of IL-17, MDC, MIP-1 $\beta$ , and fractalkine were inversely related to viral load (Fig. 2a). When HCV RNA positive women were excluded from the analysis, each of the above correlations remained significant with the exception of MDC, where the FDR was  $>0.1$ . Additionally, IL-10 showed a significant positive correlation with viral load and FGF-2 showed a negative correlation after exclusion of HCV RNA positive women. Irrespective of HCV status, the inclusion of HAART women, whose viral load was set at half the limit of detection of the assay (40 RNA copies/ml), strongly influenced the associations. We therefore also performed analyses restricted to the NC women: the only analyte to show a weak positive correlation with the level of viremia was IFN- $\gamma$  ( $r^2 = 0.12$ ,  $p = 0.044$ ), which was not significant after

correction for multiple comparisons. With the exception of IP-10, the cytokine and chemokine levels that were significantly correlated with viral load were distinct from those that differed among the NEG, HAART, and NC groups. Coupled with the finding that levels of TNF- $\alpha$  were elevated and FGF-2 were decreased in the HAART group compared to NEG women (Fig. 1), these findings imply that HIV viremia is not the only determinant factor driving differences in most of the analytes that had distinctive patterns among the NEG, HAART, and NC groups.

### Correlation between cytokine levels and CD4+ T cell count

The level of viral replication is an important determinant of HIV disease outcome, but even more relevant is the level of CD4+ T cells in the periphery, which is a marker of the degree of immunological impairment. We measured whether cytokine levels correlated with CD4+ T cell count in HIV infected women. Two of the five analytes found to be disrupted in NC women also showed a significant correlation with peripheral CD4+ T cell count, IP-10, and FGF-2 (Table 3b, Fig. 2b). In addition, all of the analytes associated with viral load were also significantly inversely associated with CD4+ T cell count, likely reflecting the predictive value of higher viral load for lower CD4+ T cell counts. The markers significantly correlated with both lower viral load and higher CD4+ T cell count included IL-17, MDC,

**Table 3a. Correlation between cytokine level and HIV viral load.**

	Slope	r <sup>2</sup>	p value <sup>a</sup>	FDR
<b>Pro-inflammatory/ T cell</b>				
IL-1 $\alpha$	-0.18	0.07	0.05	0.2
IL-1 $\beta$	-0.02	0.003	0.7	0.9
IL-2	0.01	0.001	0.9	1
IL-6	0.005	0	0.9	1
IL-7	0.01	0.005	0.6	0.9
IL-8	-0.03	0.01	0.4	0.7
IL-9	-0.01	0.001	0.8	1
IL-12(p40)	-0.15	0.05	0.08	0.2
IL-12(p70)	-0.12	0.07	0.04	0.2
IL-15	-0.09	0.09	0.02	0.1
<b>IL-17</b>	<b>-0.17</b>	<b>0.13</b>	<b>0.005</b>	<b>0.04</b>
IFN- $\gamma$	0.06	0.01	0.4	0.8
TNF- $\alpha$	0.03	0.04	0.1	0.3
TNF- $\beta$	-0.10	0.08	0.06	0.2
GM-CSF	-0.05	0.009	0.5	0.7
<b>Anti-inflammatory/Th2</b>				
IL-1R $\alpha$	0.02	0.001	0.8	1
sIL-2R $\alpha$	-0.13	0.03	0.2	0.4
IL-4	-0.05	0.003	0.7	0.9
IL-5	0.02	0.002	0.7	0.9
IL-10	0.08	0.08	0.03	0.1
IL-13	-0.007	0	1	1
<b>Chemoattractants</b>				
<b>IP-10</b>	<b>0.14</b>	<b>0.26</b>	<b>&lt;0.001</b>	<b>0.002</b>
MCP-1	0.02	0.03	0.2	0.4
MCP-3	0.001	0	1	1
<b>MDC</b>	<b>-0.04</b>	<b>0.1</b>	<b>0.01</b>	<b>0.07</b>
MIP-1 $\alpha$	-0.09	0.009	0.5	0.7
<b>MIP-1<math>\beta</math></b>	<b>-0.09</b>	<b>0.18</b>	<b>0.001</b>	<b>0.01</b>
Eotaxin	-0.03	0.03	0.2	0.4
<b>Fractalkine</b>	<b>-0.35</b>	<b>0.18</b>	<b>0.001</b>	<b>0.01</b>
<b>Growth factors</b>				
VEGF	-0.02	0.002	0.7	0.9
EGF	-0.14	0.09	0.02	0.1
FGF-2	-0.1	0.06	0.07	0.2

<sup>a</sup>Bold p values significant after false discovery rate step-down.

MIP-1 $\beta$ , and fractalkine, while IP-10 was associated with higher viral load and lower CD4 count. Interestingly, CD4+ T cell count was the only clinical parameter studied that positively correlated with all three growth factors measured in the panel (VEGF, EGF, and FGF-2). In an attempt to measure whether some factors were associated with CD4+ T cell count independently of viral load, we performed a second analysis limited to the HAART participants (all with viral load <80 copies/ml). MIP-1 $\beta$  and VEGF correlated with CD4+ T cell count in the HAART group ( $p < 0.05$ ), though significance was lost after correction for multiple comparisons (data not shown). Finally, none of the correlations between CD4+ T cell count and soluble markers of inflammation were changed when HCV RNA positive women were excluded from the analysis.

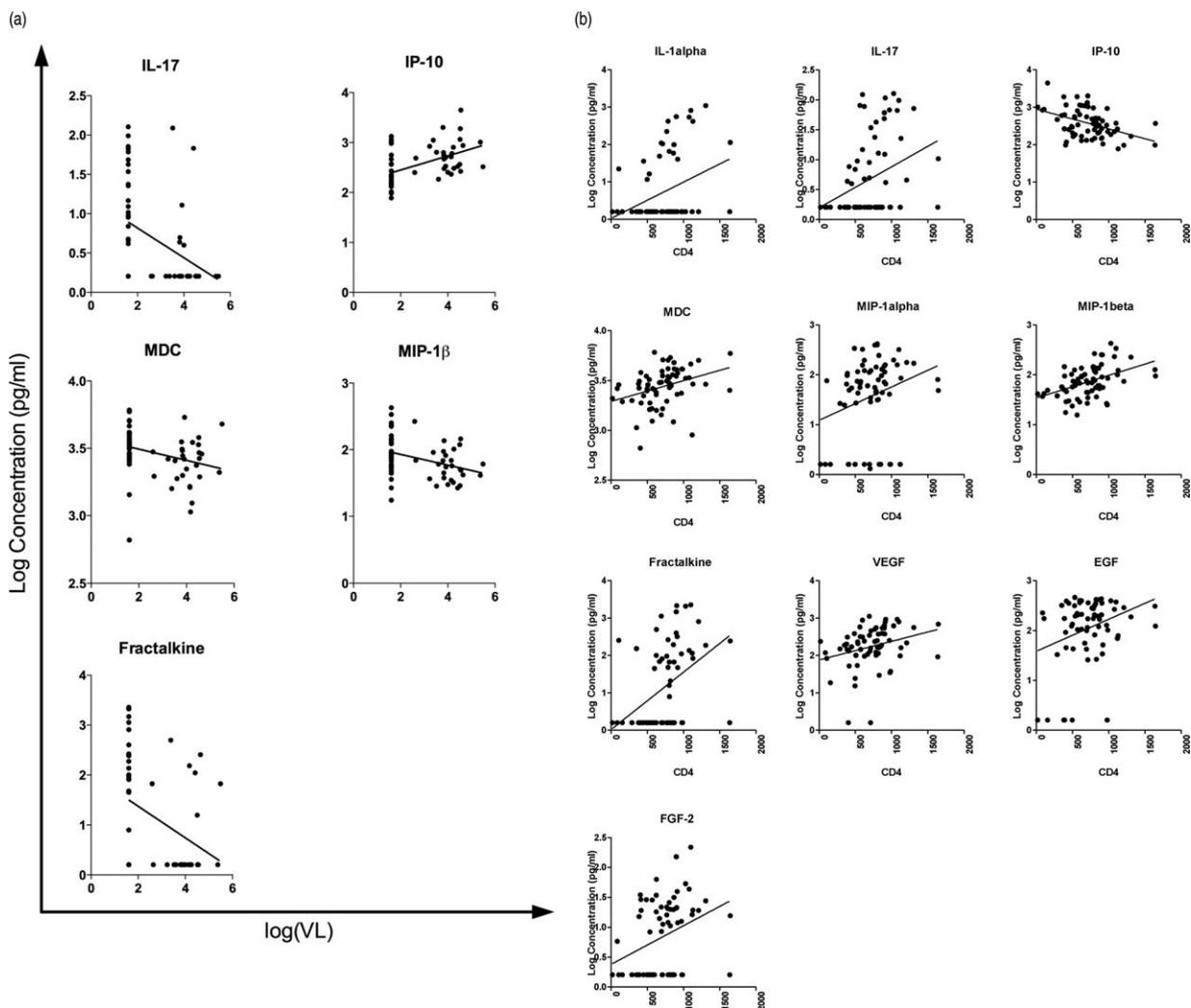
## Discussion

This study showed that serum levels of the innate immune system pro-inflammatory modulators TNF- $\alpha$  and IP-10

were elevated, increased among untreated HIV patients, compared with uninfected comparisons, a finding that is consistent with a broad inflammatory response. Additionally, serum IL-12(p40) and IL-15, important for T cell homeostasis and function, were decreased in chronic, untreated HIV infection compared to otherwise similar HIV-uninfected women. When compared with untreated women the HAART recipients showed fewer detectable differences in cytokines from HIV uninfected persons (2 vs. 5 analytes), though TNF- $\alpha$  and FGF-2 were still different in the HAART compared to NEG group. Finally, while as expected many factors correlated with both plasma viral burden and CD4+ T cell counts, some factors correlated with one or the other. Interestingly, the growth factors VEGF, EGF, and FGF-2 showed a positive correlation with higher CD4+ T cell counts.

Analytes found to be elevated in chronic infection in this study have been shown to be elevated using separate test methodologies for TNF- $\alpha$  [19–21] and IP-10 [34]. During primary infection IP-10 and TNF- $\alpha$  correlated positively with quantitative viral load [35]. We found that the only factor to show a significant positive correlation with viremia in untreated women during chronic HIV infection was IP-10, underlining the importance of this chemokine in the response to HIV and consistent with *in vitro* experiments demonstrating its ability to stimulate HIV replication [36]. Elevated IP-10 also has been detected in multiple viral infections, including acute West Nile virus [37], severe influenza infection [38,39] acute HCV [26] and chronic, persistent HCV in this study, suggesting a general role for this chemokine in the immune response to viral infections. Elevated IP-10 levels in chronic HIV infection could be deleterious and contribute to ongoing immune activation and T cell depletion, supported by the strong negative correlation between IP-10 levels and CD4+ T cell count we found in this study (Fig. 2b). Finally, the suppression of plasma IL-12 levels in untreated HIV-infected participants is consistent with published work demonstrating cellular defects in production of these cytokines in chronic HIV infection [40,41].

While most of the cytokine changes previously described to be elevated or reduced during chronic HIV infection were confirmed in our cohort of women with uncontrolled HIV replication, we did not find significant elevations reported by others in predominantly male populations using ELISA tests for or IL-6 [42,43], IL-10 [44,45], or FGF-2 [46]. Although median IL-10 levels in our untreated HIV-infected group were nearly two-fold higher compared to the HIV-negative participants, this difference was not statistically significant. In contrast, median IL-6 and FGF-2 values were lower in the NC than NEG group. The IL-6 data are consistent with our recent study of acute HIV infection, where only a subset of participants showed elevations in IL-6 levels [26], though the different assay format (ELISA vs. Luminex)



**Fig. 2.** Cytokine correlation with HIV plasma viral load and CD4+ T cell count. (a) Plots show log(cytokine level) vs. log(viral load) or (b) log(cytokine level) vs. CD4+ T cell count for each of the analytes showing a significant correlation between the two parameters. A linear regression line is included on each plot. P values and  $r^2$  values are shown in Tables 3a and 3b.

may have been responsible for the discrepant results. We re-tested the samples in the current study using an FGF-2 ELISA kit from the same manufacturer used in the prior study and the significant differences in FGF-2 levels between the clinical groups seen by Luminex testing was not seen using the ELISA kit (data not shown). The discrepancy between the ELISA test and Luminex assay for FGF-2 is potentially due to the different dynamic ranges of the two assays or to the smaller sample size of the current study.

The current study has a number of limitations, including a relatively small sample size, which made detection of only relatively large differences in cytokine concentrations between clinical groups possible. Another potential limitation of the study is the fact that our participants were female and primarily African American, therefore the results may or may not be generalizable to other HIV-

infected populations. Data are conflicting regarding the extent to which race affects cytokine responses, with African Americans showing higher baseline levels of inflammatory cytokines [47], but race having no effect on reactivity of immune cells to IFN- $\alpha$  [48] or on the association between heart failure and inflammatory markers [49]. Finally, we did not formally demonstrate that women initiating HAART showed normalization of perturbed cytokine levels. We also did not test cytokine levels in individuals with high viral load who were taking HAART. However, there are indications from the literature that HAART initiation can normalize or partially normalize inflammatory and coagulation markers [32,33,50]. This, along with the fact that our participants were matched on potential confounders, makes it likely that the differences that we observed between NC and HAART groups were in fact due to HAART.

**Table 3b. Correlation between cytokine level and CD4 counts in HIV+ women.**

	Slope	r <sup>2</sup>	p value <sup>a</sup>	FDR
<b>Pro-inflammatory/ T cell</b>				
<b>IL-1<math>\alpha</math></b>	<b>0.001</b>	<b>0.14</b>	<b>0.004</b>	<b>0.02</b>
IL-1 $\beta$	-0.00007	0.004	0.6	0.8
IL-2	-0.00005	0.001	0.8	0.9
IL-6	-0.0001	0.004	0.7	0.8
IL-7	-0.0002	0.04	0.2	0.3
IL-8	0.0002	0.01	0.4	0.6
IL-9	0.0003	0.02	0.4	0.6
IL-12(p40)	0.0006	0.04	0.1	0.3
IL-12(p70)	0.0004	0.03	0.2	0.3
IL-15	0.0003	0.05	0.1	0.3
<b>IL-17</b>	<b>0.0008</b>	<b>0.17</b>	<b>0.001</b>	<b>0.008</b>
IFN- $\gamma$	-0.0004	0.04	0.1	0.3
TNF- $\alpha$	-0.0001	0.04	0.1	0.3
TNF- $\beta$	0.0004	0.09	0.05	0.1
GM-CSF	-0.00001	0	1	1
<b>Anti-inflammatory/Th2</b>				
IL-1R $\alpha$	0.0001	0.002	0.8	0.9
sIL-2R $\alpha$	0.0002	0.007	0.6	0.8
IL-4	0.0002	0.002	0.7	0.9
IL-5	0.00001	0	0.9	1
IL-10	-0.00007	0.004	0.6	0.8
IL-13	0.0002	0.002	0.7	0.9
<b>Chemoattractants</b>				
<b>IP-10</b>	<b>-0.0006</b>	<b>0.27</b>	<b>&lt;0.001</b>	<b>0.001</b>
MCP-1	-0.00007	0.01	0.4	0.6
MCP-3	0.00004	0	0.9	1
<b>MDC</b>	<b>0.0002</b>	<b>0.18</b>	<b>0.001</b>	<b>0.008</b>
<b>MIP-1<math>\alpha</math></b>	<b>0.0008</b>	<b>0.1</b>	<b>0.009</b>	<b>0.03</b>
<b>MIP-1<math>\beta</math></b>	<b>0.0004</b>	<b>0.23</b>	<b>&lt;0.001</b>	<b>0.002</b>
Eotaxin	0.00009	0.01	0.4	0.6
<b>Fractalkine</b>	<b>0.001</b>	<b>0.18</b>	<b>0.001</b>	<b>0.008</b>
<b>Growth factors</b>				
<b>VEGF</b>	<b>0.0005</b>	<b>0.09</b>	<b>0.02</b>	<b>0.07</b>
<b>EGF</b>	<b>0.0008</b>	<b>0.17</b>	<b>0.001</b>	<b>0.08</b>
<b>FGF-2</b>	<b>0.0006</b>	<b>0.12</b>	<b>0.009</b>	<b>0.03</b>

<sup>a</sup>Bold p values significant after false discovery rate step-down.

Recent work by Roberts et al. identified a number of factors that were associated with viral load set point and the rate of CD4+ T cell decline during primary HIV infection [35]. IL-12, IFN- $\gamma$ , and GM-CSF were associated with lower viral load set point at 12 months and/or slower CD4+ T cell decline, while IL-1 $\alpha$ , IL-7, IL-15 and eotaxin showed the opposite effect. In our study none of these factors except IL-1 $\alpha$  showed a correlation with viral load or CD4+ T cell count, and the association between IL-1 $\alpha$  and CD4+ T cell count in chronic HIV infection was opposite to what was seen in primary infection. These differences highlight the fact that the interaction between HIV and the immune system is likely very different in primary and chronic HIV. For example, biasing the immune system toward a Th1 response with higher IL-12 levels in primary HIV infection may be of benefit to establish a lower viral load set point, whereas after the set point has been reached many individuals have lost detectable IL-12 secretion (Fig. 1). Similarly, high IP-10 levels during primary HIV infection did not appear to increase subsequent viral load set point or the rate of CD4+ T cell decline [35], while

they were associated with lower CD4+ T cell counts during chronic HIV infection.

In summary, we found that compared to HIV-negative women, untreated chronic HIV infection was associated with defects in T cell signaling pathways, coupled with evidence of activation of the innate immune system, and these differences were less apparent or absent in HAART-treated women with undetectable viral load. By examining a broad array of soluble immune mediators we were able to identify specific analytes that behaved differently from the majority of immune markers. For example, TNF- $\alpha$  and FGF-2 were different in the HAART compared to HIV-uninfected group, as opposed to the majority of other analytes measured. This finding suggests that the pathways driving the dysregulation of these two factors may require very low levels of virus for stimulation (or suppression), are influenced by antiretroviral drugs themselves, or are dependent on immune activation induced by HIV in an indirect fashion. Finally, while T cell homeostasis factors such as IL-2, IL-7, and IL-15 did not appear to be associated with higher CD4+ T cell counts, the growth factors VEGF, EGF, and FGF-2 were associated with higher CD4+ T cell counts. Given that the causal role of the relationship is unknown, it is unclear if these growth factors would have therapeutic potential to ameliorate the immune deficiency associated with HIV infection or would serve as useful biomarkers of a more preserved or restored immune system.

## Acknowledgments

Data in this manuscript were collected by the Women's Interagency HIV Study (WIHS) Collaborative Study Group with centers (Principal Investigators) at New York City/Bronx Consortium (Kathryn Anastos); Brooklyn, NY (Howard Minkoff); Washington DC, Metropolitan Consortium (Mary Young); The Connie Wofsy Study Consortium of Northern California (Ruth Greenblatt); Los Angeles County/Southern California Consortium (Alexandra Levine); Chicago Consortium (Mardge Cohen); Data Coordinating Center (Stephen Gange). The WIHS is funded by the National Institute of Allergy and Infectious Diseases (UO1-AI-35004, UO1-AI-31834, UO1-AI-34994, UO1-AI-34989, UO1-AI-34993, and UO1-AI-42590) and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (UO1-HD-32632). The study is co-funded by the National Cancer Institute, the National Institute on Drug Abuse, and the National Institute on Deafness and Other Communication Disorders. Funding is also provided by the National Center for Research Resources (UCSF-CTSI Grant Number UL1 RR024131). The contents of this publication are solely the responsibility of the authors and do not necessarily

represent the official views of the National Institutes of Health.

#### Author Contributions

SMK Performed experiments and analyses, SMK, ETG, RMG, SD, ALL, SJG, and PJN designed the study, JZ and SW performed statistical analyses, MN, MY, KA, HC, MHC, and RMG collected data and provided clinical samples, SMK and PJN wrote the manuscript.

## References

- Dou Z, Chen RY, Wang Z, Ji G, Peng G, Qiao X, *et al.* **HIV-infected former plasma donors in rural Central China: from infection to survival outcomes, 1985-2008.** *PLoS One* 2010; **5**:e13737.
- Brette RP, McNeil AJ, Burns S, Gore SM, Bird AG, Yap PL, *et al.* **Progression of HIV: follow-up of Edinburgh injecting drug users with narrow seroconversion intervals in 1983-1985.** *Aids* 1996; **10**:419-430.
- Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA. **Prognosis in HIV-1 infection predicted by the quantity of virus in plasma [see comments] [published erratum appears in Science 1997 Jan 3;275(5296):14].** *Science* 1996; **272**:1167-1170.
- Liu Z, Cumberland WG, Hultin LE, Prince HE, Detels R, Giorgi JV. **Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression.** *J Acquir Immune Defic Syndr Hum Retrovirol* 1997; **16**:83-92.
- Owen RE, Heitman JW, Hirschhorn DF, Lanteri MC, Biswas HH, Martin JN, *et al.* **HIV+ elite controllers have low HIV-specific T-cell activation yet maintain strong, polyfunctional T-cell responses.** *Aids* 2010; **24**:1095-1105.
- Wong GH, Krowka JF, Stites DP, Goeddel DV. **In vitro anti-human immunodeficiency virus activities of tumor necrosis factor-alpha and interferon-gamma.** *J Immunol* 1988; **140**:120-124.
- Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, Fauci AS. **Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone.** *Proc Natl Acad Sci U S A* 1989; **86**:2365-2368.
- Israel N, Hazan U, Alcamí J, Munier A, Arenzana-Seisdedos F, Bachelier F, *et al.* **Tumor necrosis factor stimulates transcription of HIV-1 in human T lymphocytes, independently and synergistically with mitogens.** *J Immunol* 1989; **143**:3956-3960.
- Foli A, Saville MW, Baseler MW, Yarchoan R. **Effects of the Th1 and Th2 stimulatory cytokines interleukin-12 and interleukin-4 on human immunodeficiency virus replication.** *Blood* 1995; **85**:2114-2123.
- Kinter AL, Poli G, Fox L, Hardy E, Fauci AS. **HIV replication in IL-2-stimulated peripheral blood mononuclear cells is driven in an autocrine/paracrine manner by endogenous cytokines.** *J Immunol* 1995; **154**:2448-2459.
- Shirazi Y, Pitha PM. **Alpha interferon inhibits early stages of the human immunodeficiency virus type 1 replication cycle.** *J Virol* 1992; **66**:1321-1328.
- Fan SX, Turpin JA, Aronovitz JR, Meltzer MS. **Interferon-gamma protects primary monocytes against infection with human immunodeficiency virus type 1.** *J Leukoc Biol* 1994; **56**:362-368.
- Hammer SM, Gillis JM, Groopman JE, Rose RM. **In vitro modification of human immunodeficiency virus infection by granulocyte-macrophage colony-stimulating factor and gamma interferon.** *Proc Natl Acad Sci U S A* 1986; **83**:8734-8738.
- Orandle MS, Williams KC, MacLean AG, Westmoreland SV, Lackner AA. **Macaques with rapid disease progression and simian immunodeficiency virus encephalitis have a unique cytokine profile in peripheral lymphoid tissues.** *J Virol* 2001; **75**:4448-4452.
- Meythaler M, Martinot A, Wang Z, Pryputniewicz S, Kasheta M, Ling B, *et al.* **Differential CD4+ T-lymphocyte apoptosis and bystander T-cell activation in rhesus macaques and sooty mangabeys during acute simian immunodeficiency virus infection.** *J Virol* 2009; **83**:572-583.
- Lederer S, Favre D, Walters KA, Proll S, Kanwar B, Kasakow Z, *et al.* **Transcriptional profiling in pathogenic and non-pathogenic SIV infections reveals significant distinctions in kinetics and tissue compartmentalization.** *PLoS Pathog* 2009; **5**:e1000296.
- Jacquelin B, Mayau V, Targat B, Liovat AS, Kunkel D, Petitjean G, *et al.* **Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response.** *J Clin Invest* 2009; **119**:3544-3555.
- Bosinger SE, Li Q, Gordon SN, Klatt NR, Duan L, Xu L, *et al.* **Global genomic analysis reveals rapid control of a robust innate response in SIV-infected sooty mangabeys.** *J Clin Invest* 2009; **119**:3556-3572.
- Lahdevirta J, Maury CP, Teppo AM, Repo H. **Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome.** *Am J Med* 1988; **85**:289-291.
- von Sydow M, Sonnerborg A, Gaines H, Strannegard O. **Interferon-alpha and tumor necrosis factor-alpha in serum of patients in various stages of HIV-1 infection.** *AIDS Res Hum Retroviruses* 1991; **7**:375-380.
- Zangerle R, Gallati H, Sarcelletti M, Wachter H, Fuchs D. **Tumor necrosis factor alpha and soluble tumor necrosis factor receptors in individuals with human immunodeficiency virus infection.** *Immunol Lett* 1994; **41**:229-234.
- Fahey JL, Taylor JM, Manna B, Nishanian P, Aziz N, Giorgi JV, Detels R. **Prognostic significance of plasma markers of immune activation, HIV viral load and CD4 T-cell measurements.** *Aids* 1998; **12**:1581-1590.
- Kuller LH, Tracy R, Bellosso W, De Wit S, Drummond F, Lane HC, *et al.* **Inflammatory and coagulation biomarkers and mortality in patients with HIV infection.** *PLoS Med* 2008; **5**:e203.
- Norris PJ, Pappalardo BL, Custer B, Spotts G, Hecht FM, Busch MP. **Elevations in IL-10, TNF-alpha, and IFN-gamma from the earliest point of HIV Type 1 infection.** *AIDS Res Hum Retroviruses* 2006; **22**:757-762.
- Barcellini W, Rizzardi GP, Poli G, Tambussi G, Velati C, Meroni PL, *et al.* **Cytokines and soluble receptor changes in the transition from primary to early chronic HIV type 1 infection.** *AIDS Res Hum Retroviruses* 1996; **12**:325-331.
- Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J, *et al.* **Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections.** *J Virol* 2009; **83**:3719-3733.
- Bebell LM, Passmore JA, Williamson C, Mlisana K, Iriogbe I, van Loggerenberg F, *et al.* **Relationship between levels of inflammatory cytokines in the genital tract and CD4+ cell counts in women with acute HIV-1 infection.** *J Infect Dis* 2008; **198**:710-714.
- Barkan SE, Melnick SL, Preston-Martin S, Weber K, Kalish LA, Miotti P, *et al.* **The Women's Interagency HIV Study. WIHS Collaborative Study Group.** *Epidemiology* 1998; **9**:117-125.
- Bacon MC, von Wyl V, Alden C, Sharp G, Robison E, Hessel N, *et al.* **The Women's Interagency HIV Study: an observational cohort brings clinical sciences to the bench.** *Clin Diagn Lab Immunol* 2005; **12**:1013-1019.
- Benjamini Y, Hochberg Y. **Controlling the false discovery rate: a practical and powerful approach to multiple testing.** *Journal of the Royal Statistical Society* 1995; **B57**:289-300.
- Almeida M, Cordero M, Almeida J, Orfao A. **Relationship between CD38 expression on peripheral blood T-cells and monocytes, and response to antiretroviral therapy: a one-year longitudinal study of a cohort of chronically infected ART-naive HIV-1+ patients.** *Cytometry B Clin Cytom* 2007; **72**:22-33.

32. French MA, King MS, Tschampa JM, da Silva BA, Landay AL. **Serum Immune Activation Markers Are Persistently Increased in Patients with HIV Infection after 6 Years of Antiretroviral Therapy despite Suppression of Viral Replication and Reconstitution of CD4(+) T Cells.** *J Infect Dis* 2009; **200**:1212–1215.
33. Wolf K, Tsakiris DA, Weber R, Erb P, Battegay M. **Antiretroviral therapy reduces markers of endothelial and coagulation activation in patients infected with human immunodeficiency virus type 1.** *J Infect Dis* 2002; **185**:456–462.
34. Relucio KI, Beernink HT, Chen D, Israelski DM, Kim R, Holodniy M. **Proteomic analysis of serum cytokine levels in response to highly active antiretroviral therapy (HAART).** *J Proteome Res* 2005; **4**:227–231.
35. Roberts L, Passmore JA, Williamson C, Little F, Bebell LM, Mlisana K, et al. **Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression.** *Aids* 2010; **24**:819–831.
36. Lane BR, King SR, Bock PJ, Strieter RM, Coffey MJ, Markovitz DM. **The C-X-C chemokine IP-10 stimulates HIV-1 replication.** *Virology* 2003; **307**:122–134.
37. Tobler LH, Cameron MJ, Lanteri MC, Prince HE, Danesh A, Persad D, et al. **Interferon and interferon-induced chemokine expression is associated with control of acute viremia in West Nile virus-infected blood donors.** *J Infect Dis* 2008; **198**:979–983.
38. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, et al. **Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia.** *Nat Med* 2006.
39. Lee N, Wong CK, Chan PK, Lun SW, Lui G, Wong B, et al. **Hypercytokinemia and hyperactivation of phospho-p38 mitogen-activated protein kinase in severe human influenza A virus infection.** *Clin Infect Dis* 2007; **45**:723–731.
40. Chehimi J, Starr SE, Frank I, D'Andrea A, Ma X, MacGregor RR, et al. **Impaired interleukin 12 production in human immunodeficiency virus-infected patients.** *J Exp Med* 1994; **179**:1361–1366.
41. Marshall JD, Chehimi J, Gri G, Kostman JR, Montaner LJ, Trinchieri G. **The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals.** *Blood* 1999; **94**:1003–1011.
42. Birx DL, Redfield RR, Tencer K, Fowler A, Burke DS, Tosato G. **Induction of interleukin-6 during human immunodeficiency virus infection.** *Blood* 1990; **76**:2303–2310.
43. Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, Hirano T, et al. **Infection with HIV is associated with elevated IL-6 levels and production.** *J Immunol* 1990; **144**:480–484.
44. Brockman MA, Kwon DS, Tighe DP, Pavlik DF, Rosato PC, Sela J, et al. **IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells.** *Blood* 2009; **114**:346–356.
45. Stylianou E, Aukrust P, Kvale D, Muller F, Froland SS. **IL-10 in HIV infection: increasing serum IL-10 levels with disease progression—down-regulatory effect of potent anti-retroviral therapy.** *Clin Exp Immunol* 1999; **116**:115–120.
46. Ascherl G, Sgadari C, Bugarini R, Bogner J, Schatz O, Ensoli B, Sturzl M. **Serum concentrations of fibroblast growth factor 2 are increased in HIV type 1-infected patients and inversely related to survival probability.** *AIDS Res Hum Retroviruses* 2001; **17**:1035–1039.
47. Slopen N, Lewis TT, Gruenewald TL, Mujahid MS, Ryff CD, Albert MA, Williams DR. **Early life adversity and inflammation in African Americans and whites in the midlife in the United States survey.** *Psychosom Med* 2010; **72**:694–701.
48. Pos Z, Salleri S, Spivey TL, Wang JK, Liu H, Worschech A, et al. **Genomic scale analysis of racial impact on response to IFN-alpha.** *Proc Natl Acad Sci U S A* 2010; **107**:803–808.
49. Kalogeropoulos A, Georgiopoulou V, Psaty BM, Rodondi N, Smith AL, Harrison DG, et al. **Inflammatory markers and incident heart failure risk in older adults: the Health ABC (Health, Aging, and Body Composition) study.** *J Am Coll Cardiol* 2010; **55**:2129–2137.
50. Francisci D, Giannini S, Baldelli F, Leone M, Belfiori B, Guglielmini G, et al. **HIV type 1 infection, and not short-term HAART, induces endothelial dysfunction.** *Aids* 2009; **23**:589–596.