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Resumption of HIV replication is associated with monocyte/macrophage derived cytokine and chemokine changes: results from a large international clinical trial

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Abstract

Background—There is increasing interest in the role of immune activation and inflammation in HIV disease, but data on direct effects of HIV replication on immune cell activation are limited.

Methods—High sensitivity multiplex bead array assays (MBAAs) were used to measure changes in plasma cytokines and chemokines [interleukin (IL)-1β, IL-2, IL-6, IL-7, IL-8, IL-12p70, IL-17, tumor necrosis factor-α (TNFα), interferon-γ, granulocyte macrophage colony-stimulating factor, IL-4, IL-5, IL-10, IL-13, CXCL10] from randomization (month 0) to month 2 in a random sample of 200 patients from both the drug conservation (DC) and viral suppression (VS) arms of the Strategies for Management of Antiretroviral Therapy (SMART) trial. IL-6 was also measured by ELISA. Data were evaluated using nonparametric correlation and censored parametric analysis of covariance and associations were declared as statistically significant when the Bonferroni-adjusted *P*-value was less than 0.003.

Results—Compared with the VS arm, significant increases were seen in the DC arm for TNFα (+0.34 $\log_e$ pg/ml, $P = 0.0001$), IL-10 (+0.33 $\log_e$ pg/ml, $P = 0.00001$) and CXCL10 (+0.66 $\log_e$ pg/ml, $P = 0.00001$). IL-6 ELISA poorly correlated with IL-6 MBAA (Spearman's rho = 0.29, $P = 0.0001$).

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Conclusion—Resumption of HIV replication after ceasing antiretroviral therapy is associated predominantly with an increase of monocyte/macrophage-derived cytokines. Measurement of IL-6 levels may be affected by assay method and this should be considered in future studies of biomarkers.

Keywords

antiretroviral therapy; chemokines; clinical trial; cytokines; interruption

Introduction

The role of immune activation and inflammation in HIV disease has become increasingly of interest to the field of HIV pathogenesis and clinical care. A number of studies have shown that cellular markers of immune activation, including CD38 expression on $CD8^+$ T cells, are stronger predictors of HIV disease progression than plasma viral load [1–8]. Soluble markers of immune activation, including neopterin and β2-microglobulin have been shown to predict HIV infection progression to AIDS or death [9–12], although these studies were carried out for the most part in the HAART era. More recent studies focused on individuals starting HAART have shown that baseline immune activation/inflammation markers are predictive of subsequent $CD4^+$ T-cell responses [13–15]. In addition, for some who experience viral suppression while on HAART, there is a persistence of immune activation/inflammation that may correlate with the development of non-AIDS morbidity and mortality, including cardiovascular disease (CVD), metabolic disease, neurocognitive decline, bone disease and cancer [16]. Recently, there has been speculation that measurement of biomarkers can even improve risk prediction of AIDS over and above what can be done using $CD4^+$ T-cell counts and plasma viral load levels and, if so, whether they should be used in routine HIV monitoring [17].

The Strategies for Management of Antiretroviral Therapy (SMART) trial randomized patients to a drug conservation (DC) arm, in which they stopped therapy, or to a viral suppression (VS) arm, in which they maintained therapy [18]. The findings of this trial have focused the field's attention on patients who developed non-AIDS-defining comorbidities that were significantly associated with inflammatory markers including interleukin (IL)-6, high sensitivity C-reactive protein and D-dimer [19]. A number of additional analyses have evaluated the association between microbial translocation markers and clinical outcomes [20,21], cystatin C and its role in renal disease [22] and hyaluronic acid and the risk of non-AIDS mortality in HIV/hepatitis co-infected patients [23].

Activation of various immune cells can be examined by assaying cytokines and chemokines produced by these cells in plasma. Examples of such cytokines include interferon-γ (IFNγ) and IL-2 produced by type 1 $CD4^+$ helper T (Th1) cells, IL-4 and IL-5 produced by Th2 cells and IL-17 produced Th17 cells [24]. In addition, monocytes/macrophages are a source of cytokines and chemokines when activated including pro-inflammatory mediators such as IL-1, IL-6, tumor necrosis factor-α (TNFα) and CXCL10 [25]. In contrast, the regulatory cytokine IL-10 is produced by multiple cell types including monocytes/macrophages and regulatory T cells.

The current analysis was undertaken to evaluate the effects of HIV replication after ceasing antiretroviral therapy (ART) on changes in plasma cytokines and chemokines produced by peripheral blood lymphocytes or monocytes/macrophages. The specific cytokines and chemokines were selected in order to determine whether we could identify pathways that may be involved in driving immune activation/inflammation and $CD4^+$ T cell decline in the DC vs. VS arms of the SMART Study.

Patients and methods

Patient characteristics

We evaluated stored plasma samples obtained from a subset of patients enrolled in the SMART trial. The methods and results of the SMART trial have been shown in detail elsewhere [18]. Specimens collected at randomization and 2 months after randomization were identified for a random sample of 200 participants from each treatment group – the DC and VS arms – who at time of randomization consented to storing blood for future research and reported no history of CVD at randomization. In addition, all patients had to be receiving ART at randomization with a viral load suppressed to 400 copies/ml or less. More in detail, of the 5472 patients enrolled in SMART, 1330 (24%, 666 in the DC and 664 in VS arm) satisfied the following criteria: consented to have a sample stored both at randomization and at month 2, did not have a history of CVD at randomization and had a suppressed viral load of 400 copies/ml or less at randomization. For 95 in the DC (14%) and 109 in the VS arm (16%) of these 1330 patients, samples had been already been analyzed using ELISA [19] and are included in this investigation so that results could be compared. The remaining 105 patients in the DC arm and 91 in the VS arm were randomly chosen from the remaining 571 and 555 patients in the two arms, respectively. Patients with a known history of CVD were excluded because they had already developed a morbidity that was shown to be associated with increased values of soluble factors. In contrast, at the time of developing the analysis plan, there was no knowledge of what the distribution of these factors would be in CVD-free patients with one or more risk factors for CVD. However, previous analyses indicated that, in order to increase the power to detect differences, the study population should be restricted to patients with suppressed viral load at baseline and the analysis focused on changes at month 2. The institutional review board at the University of Minnesota approved plans for analysis of stored specimens for consenting participants.

Biomarkers analysis

Plasma specimens were collected using EDTA (lavender top) blood collection tubes and were shipped frozen to the INSIGHT specimen repository multiplex bead array assays (MBAAs) were performed strictly according to the manufacturer's protocol for plasma samples. Prior to adding samples to an assay plate, aliquots were gently vortexed and then centrifuged at 13 200 revolutions/min for 10 min at 48°C. Dilutions of plasma samples and standards were prepared according to the manufacturers' instructions and assayed in duplicate wells on each plate. Each sample was tested in a plate of the same lot number of each manufacturer's kit. Two separate multiplex kits were used: a High Sensitivity Human Cytokine LINCOplex Immunoassay Kit (13-plex, Linco/Millipore, Billerica, Massachusetts, USA) and a Human Cytokine Milliplex Map Immunoassay Kit (2-plex, Linco/Millipore). The biomarkers that were measured included IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12p70, IL-17, TNFα, IFNγ, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-10, IL-13 and CXCL10. A fluorescent bead-based instrument (Luminex-100; Luminex Corporation, Austin, Texas, USA) was used to read each multiplex plate. Luminex bead-based data were analyzed using Milliplex Analyst software v3.4 (Millipore; VigeneTech Inc., Boston, Massachusetts, USA) and a five parameter logistic curve fit. For a subset of patients, IL-6 was also measured by the Laboratory for Clinical Biochemistry Research at the University of Vermont using a standard ELISA assay both at baseline and 1 month after randomization: these values were used in a previous analysis of the SMART trial data [19]. To avoid confusion, IL-6 measured using MBAA is indicated with 'IL6-M', whereas the ELISA measurement with 'IL6-E'. Classical ELISA-based cytokine assays are robust, easy to use and very well suited for measurement of single cytokines. The rationale for using MBAA was that multiple cytokines and chemokines could be assayed on a single serum sample. This made the approach much more cost-effective and also allowed for a much

more comprehensive interrogation of cytokine and chemokine changes [26]. A potential drawback is that there may not be concordance of results for the two methods, particularly if there is no uniformity of assay reagents [27,28].

Statistical analysis

Some patients had biomarker values that were below the level of quantification. We adopted two separate approaches to deal with censored values: setting levels below the limit of quantification equal to the limit of quantification or, alternatively, a censored analysis – using PROC LIFETEST and LIFEREG in SAS (SAS Institute, Cary, North Carolina, USA), the latter with the normality assumption in the natural logarithmic scale ($\log_e$) [29].

The correlation between IL6-E and IL6-M (in the natural logarithmic scale, in the subset of patients for whom they were available) was analyzed using linear correlation analysis and Spearman's rank-correlation coefficient (rho statistic). The median (range) levels of the biomarkers at randomization (raw scale) and at month 2 were described in both the DC and VS arm, and we also calculated the mean change in biomarkers (natural logarithmic scale) by month 2 after randomization within each arm. In contrast, the Kaplan–Meier method and log-rank test were employed to compare medians when using the alternative approach. The difference in biomarker changes after 2 months between the DC and VS arm were estimated using analysis of covariance (ANCOVA) with a separate model for each biomarker and after controlling for the value at randomization of the biomarker under analysis. We performed a sensitivity ANCOVA after excluding patients with a viral load of 51–400 copies/ml at randomization. For IL6-E, month 2 values were unavailable, so the change after 1 month was analyzed instead. A parametric censored ANCOVA was used as an alternative analysis (results available as supplemental online materials http://links.lww.com/QAD/A137). For biomarkers showing more than 10% censored values (Table 1), the proportion of values below the lower limit of quantification were also compared using a χ^2-test.

A number of general patients' characteristics were collected (sex, age, race, mode of HIV infection, hepatitis virus co-infection, prior AIDS, current and prior smoking status, diabetes and treatment with blood pressure or lipid lowering therapy) and we checked that patients in the two arms were balanced for these factors. Co-infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) were defined on the basis of serology tests alone. In addition, for a selected subset of biomarkers, we evaluated whether the differences in change between the DC and VS arm varied according to some of these demographic and treatment factors by stratification and formally performing an interaction test.

The association between biomarker changes (natural logarithmic scale), HIV-RNA ($\log_{10}$ scale) at month 2 and $CD4^+$ T-cell count change at 2 months from randomization was also evaluated by linear regression analysis using the data of the DC arm alone. The correlation between biomarkers was studied using linear regression and Spearman's rho statistic separately in the DC arm. This was done only for the subset of biomarkers showing an association with randomization arm $CD4^+$ T-cell count and viral load.

As 15 biomarkers were concomitantly evaluated, associations were declared to be statistically significant when showing a *P*-value below 0.05/15 = 0.003 (i.e. using a conservative Bonferroni-type adjustment for the inflation of type I error). Statistical analyses were performed using SAS 9.1.3 (SAS Institute, 2002–2003). All reported *P*-values are two-sided.

Results

Baseline cytokine/chemokine levels

Table 1 shows the comparison between the median plasma levels of cytokines and chemokines both at randomization and at the second time point. At randomization the two groups had similar levels for all biomarkers except IL-2 (3.19 vs. 4.27 pg/ml, $P = 0.02$) and IL-12p70 (2.15 vs. 3.46 pg/ml, $P = 0.01$), which were nominally significantly higher in the VS compared with the DC arm. The median and range of values of IL6-M (4.94 pg/ml, range 0.02–136.11) and IL6-E (2.47 pg/ml, range 0.16–88.63), both measured at randomization, differed substantially and were weakly correlated with each other (Fig. 1).

Patient's characteristics at randomization

Characteristics at randomization of participants assigned to the DC and VS arm in this substudy of the SMART trial were well balanced (Table 2). By inclusion criteria, at randomization, all patients had a HIV-RNA plasma viral load suppressed to a value of 400 copies/ml or less on ART and none of the patients had previously experienced CVD events. In four out of 10 (40%) HBV surface antigen-positive patients, HBV-DNA was detectable at more than 357 copies/ml, whereas HCV-RNA was available only for 62 HCV antibody-positive patients and 46 of these (74%) had a value of more than 615 copies/ml.

Change in biomarkers between randomization and month 2 within each arm and between arms

Within the VS arm, biomarkers levels remained fairly stable between randomization and month 2 with a tendency to slight decrease, with the exception of perhaps GM-CSF that instead showed a slight increase (Table 3a). In contrast in the DC arm, large increases were observed for IL6-E (+0.43 $\log_e$ pg/ml) and IL6-M (+0.21 $\log_e$ pg/ml), TNFα (+0.35 $\log_e$ pg/ml), IL-10 (+0.34 $\log_e$ pg/ml) and CXCL10 (+0.62 $\log_e$ pg/ml). ANCOVA demonstrated that by 2 months after randomization, there was a significantly greater increase in the DC vs. the VS arm in the following biomarkers: TNFa [mean difference +0.34 $\log_e$ pg/ml, 95% confidence interval (CI) 0.24–0.45, $P = 0.0001$], IL-10 (mean difference +0.33 $\log^e$ pg/ml, 95% CI 0.18–0.49, $P = 0.0001$) and CXCL10 (mean difference +0.66 $\log_e$ pg/ml, 95% CI 0.53–0.79, $P = 0.00001$). Consistent with previous observation in the SMART study, IL6-E levels showed a positive difference (+0.26 $\log_e$ pg/ml, 95% CI 0.06–0.46, $P = 0.01$), although the association did not reach significance at the Bonferroni-adjusted level of 0.003 for IL6-M (mean difference +0.20 $\log_e$ mg/dl, 95% CI 0.00–0.40, $P = 0.05$). Results were similar after restricting to patients who had a viral load of 50 copies/ml or less at randomization (+0.40 $\log_e$ mg/dl, 95% CI 0.24–0.56 for TNFα; +0.32 $\log_e$ mg/dl, 95% CI 0.09–0.55 for IL-10; +0.71 $\log_e$ mg/dl, 95% CI 0.52–0.90 for CXCL10; and +0.28 $\log_e$ mg/dl 95% CI 0.00–0.56 for IL6-E). In the alternative analysis replacing the censored values with the value of the limit of quantification, these results were also confirmed.

Of interest, serum levels of IL-12p70, IL-17 and GM-CSF were found to be significantly decreased in the DC vs. the VS arm in the main analysis (Table 3a), but not in the censored analysis (Table S2, http://links.lww.com/QAD/A137). However, for these biomarkers, the percentage of censored values was high (13, 53 and 17%, respectively) and, therefore, likely to have affected the results of the censored analysis. Indeed, for, IL-17 and GM-CSF, the proportion of patients with a value below the lower limit of detection at month 2 was also significantly higher in the DC arm compared with the VS arm (Table 1).

After stratifying for demographics and treatment factors, we found that the difference between the DC and VS arm in CXCL10 could be seen only in HIV mono-infected patients, but not in the hepatitis virus (either HCV or HBV) co-infected patients and this was

confirmed by a significant interaction test ($P = 0.002$). In the HIV mono-infected, the mean (95% CI) $\log_e$ change in CXCL10 within the DC arm was 0.75 (0.88) and -0.07 (0.64) within the VS arm, whereas the corresponding estimates in the hepatitis co-infected patients were 0.29 (0.72) in the DC arm vs. 0.11 (0.70) in the VS arm, again suggesting that the difference in the increase between arms was driven by a large increase in HIV mono-infected patients in the DC arm. Moreover, mean (SD) CXCL10 levels at randomization were significantly higher in co-infected patients 6.12 $\log_e$ pg/ml (0.74) compared with mono-infected 5.55 $\log_e$ pg/ml (0.74) (*t*-test $P = 0.0001$).

Association between change in biomarkers and change in CD4 cell count and viral load

Median viral load at month 2 in the DC arm was 4.35 $\log_{10}$ copies/ml (interquartile range 3.52–4.86), and 172 patients (86%) of patients in the VS arm remained with viral load suppression of 400 copies/ml or less at month 2. The estimates for the change in $CD4^+$ T-cell count were -186 cells/μl (186) and -10 cells/μl in the DC and VS arms, respectively. Table 4a/b shows the association between change in biomarkers over 0–2 months from randomization and their association with the concomitant change in $CD4^+$ T-cell count and month 2 viral load from fitting a linear regression analysis using only patients allocated to the DC arm. In univariable analyses, each $\log_e$ picogram per milliliter (pg/ml) increase in IL-17 was associated with a 0.11 $\log_{10}$ copies/ml lower month 2 viral load (95% CI -0.21 to -0.01, $P = 0.03$). In contrast, increases in $\log_e$ pg/ml of TNFα, IL-10 and CXCL10 showed a large and positive correlation with month 2 viral load (+0.26 $\log_{10}$ copies/ml, $P = 0.02$; +0.17 $\log_{10}$ copies/ml, $P = 0.01$; and +0.35 $\log_{10}$ copies/, $P = 0.001$, respectively, Table 4a) and $CD4^+$ T-cell count decreases (-40 cells/μl, $P = 0.04$; -20 cells/μl, $P = 0.10$; and -36 cells/μl, $P = 0.02$, respectively, Table 4b). However, although the association between IL-10 and CXCL10 change and $CD4^+$ T-cell count decrease appeared to be entirely mediated by month 2 viral load, this was less so for TNFα (adjusted mean change -23 cells/μl per 1 $\log_e$ increase in TNFα, $P = 0.20$, Table 4b).

Correlation between biomarker changes in the DC arm

The Spearman's correlation analysis identified a single strong correlation between changes in TNFα and IL-10 (rho = 0.57, $P = 0.00001$, Fig. S1, http://links.lww.com/QAD/A137). The only other correlation showing a significant *P*-value was the association between changes in IL-10 and IL-17, although the degree of correlation was weak (rho = -0.35, $P = 0.018$).

Discussion

This analysis, designed to identify changes in plasma levels of cytokines and chemokines in patients randomly assigned to the DC and VS arms of the SMART study, might provide information about activation of immune cells associated with resumption of HIV replication in the DC arm. We found that significant increases occurred in the DC arm in IL6-E (by ELISA), TNFa, IL-10 and CXCL10 levels. These changes were significantly larger than the changes observed in the VS arm. In addition, there was correlation between the changes in TNFα, IL-10 and CXCL10 (strong in the case of TNFα and IL-10), suggesting that there was a common driver for the increased production. We hypothesize that this common cause is monocyte/macrophage activation, which typically determines increased production of these particular cytokines and chemokines.

We also examined the association of increased cytokine and chemokine production with changes in $CD4^+$ T-cell numbers in patients allocated to the DC arm only in whom any differences are likely to be due to the major stimulus induced by stopping ART. After adjustment for the effect of month 2 viral load level on $CD4^+$ T-cell count, increased plasma

levels of IL-10 and CXCL10 were no longer associated with decreased $CD4^+$ T-cell count. TNFα also was no longer associated, although it retained the smaller *P*-value ($P = 0.20$), suggesting that TNFα production might be associated with depletion of $CD4^+$ T cells by mechanisms other than viral load increase (e.g. inducing apoptosis). Indeed, TNFα is known to be an important mediator of apoptosis through engagement of TNFα receptors I and II [30–33]. Furthermore, it has been previously shown that genetic polymorphism in TNFα can impact $CD4^+$ T-cell recovery on HAART and may also be an important mediator of $CD4^+$ T-cell loss as seen in the current analysis [34,35]. In addition, serum TNFα has been associated with HIV disease progression in a number of studies [36–40]. A recent analysis has demonstrated a significant association of soluble TNF receptor 1 and HIV disease progression, including development of AIDS associated malignancies [41]. Another potential role for TNF in HIV pathogenesis is its ability to drive $CD8^+$ T cells to a senescent phenotype [42]. This suggests that high levels of TNF may be linked to the non-AIDS comorbidities seen in the SMART study, as cells with a senescent phenotype have been linked to CVD, neurocognitive decline and osteoporosis [43].

The planned treatment interruption in the DC arm also provided an opportunity to determine changes in cytokine/chemokine levels during a period of a significant increase in HIV replication. This period of intense viral replication is similar to what is seen during acute HIV infection. A study by Norris *et al*. [44] demonstrated significant increases in TNFα, IL-10 and IFNγ during acute HIV. Similar results were seen in a more recent study by Stacey *et al*. [45] who compared cytokine changes in acute HIV, HCV and HBV infection. These investigators found early changes in IL-15 and IFNα with a greater proportion of individuals showing an increase in CXCL10. They also noted that over 80% of individuals had significant increases in IL-10 and TNFα. These same levels of cytokine changes were not seen in samples from either HCV or HBV acutely infected individuals. Rychert et al. [46] have recently evaluated the correlation of plasma gp120 with cytokine changes in early HIV infection and found that gp120 levels correlated with an increase in TNFα, IL-10 and IL-6. These changes are consistent with our data. We did see a significant increase in CXCL10, which is an IFN-inducible protein in which production is known to be predominantly stimulated by IFN [47]. All of these data on changes in cytokines and chemokines early in HIV seroconversion point to a critical role for modulation of the immune response against HIV and impact on immune function.

Our analyses also showed a weak but negative correlation between the change in IL-10 levels and change in IL-17 levels over a period of 2 months after randomization when restricting the analysis to the DC arm alone. IL-10 has been shown to suppress Th17 cells in several, though not all, studies [48–51]. The role of Th17 cells in HIV infection has not been fully established, but Th17 cell depletion may contribute to the defect of gut mucosal immune responses induced by HIV infection [52]. Furthermore, IL-10 reduces Th17-mediated immune responses in an animal model of influenza virus infection [53] and, therefore, could affect other antiviral responses.

Of note, we observed that CXCL10 levels at 2 months only increased in patients who did not have HCV co-infection, possibly because serum CXCL10 levels are increased by HCV co-infection itself [54]. Indeed, our data show that CXCL10 levels at randomization were elevated in co-infected patients compared with mono-infected patients and that in the co-infected, there was a similar increase by month 2 in CXCL10 levels regardless of whether they interrupted therapy or not. We cannot, however, rule out that this is a chance finding because of the large number of subgroup analysis performed. In addition, hepatitis co-infection was defined on the basis of serology tests alone and 26% of our HCV-positive population at serology had cleared the infection.

There are other limitations to our analysis. We utilized a MBAA to evaluate plasma levels of cytokines and chemokines at baseline and following 2 months of the intervention because this assay method has the advantage of being able to measure a large number of analytes on the same plasma sample with reduction in time of execution and costs. However, it is recognized that there can be poor concordance between MBAA and ELISA in the assay of some cytokines, which to a large degree reflects differences in the activity of capture and reporter antibodies [27]. This is the probable explanation for our finding of a weak correlation between IL-6 levels assayed by MBAA or ELISA (Fig. 1). For IL-6, differences in the ability these assays to detect the effects of 'regulatory' molecules (e.g. sIL-6R or gp130 or both) might also be a factor [55]. It is, therefore, important to take these factors into consideration when comparing the results of studies that have used different assay methods. Despite these caveats, our data have clearly shown changes in plasma cytokine and chemokine levels within and between patient groups using the same assay method (MBAA).

Despite the high sensitivity of the MBAAs employed in this analysis, IL-12p70, IL-13, IL-17 and GM-CSF were not detectable in a large percentage of samples (Table 1). This produced a discrepancy in results between the main analysis and the alternative analysis. It is known that when the censoring is heavy, the censored ANCOVA can produce unreliable results [56]. Nevertheless, only cytokine/chemokine changes that were significantly different by randomization arm in both the main and the alternative analysis were considered to be unlikely to have occurred by chance alone. Finally, because monocyte/macrophage activation was not assessed directly, we cannot rule out the possibility that the observed changes in serum cytokine and chemokine levels were an effect of HIV replication on another source of these molecules.

In conclusion, the results of our analysis demonstrate a highly significant correlation of viral load increase following ART interruption with both $CD4^+$ T-cell decline and increased production of IL6-E (by ELISA), TNFα, CXCL10 and IL-10, which are all known to be monocyte/macrophage derived. These results suggest that the effect of HIV replication on monocyte/ macrophages may constitute a critical pathway in the induction of HIV-mediated immune activation/inflammation, as well as immune deficiency. Similar studies should be performed to confirm the hypothesis generated by these data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Specific contribution of single authors:

Conception and design (A.L., J.N.).

Acquisition of data (M.A.F., J.B., P.O., M.P.).

Analysis and interpretation of the data (A.C.-L., A.L., M.A.F.).

Drafting of the manuscript (A.C.-L., A.L., M.A.F.).

Critical revision of the manuscript (A.C.-L., M.A.F., J.B., P.O., M.P., J.N., A.L., INSIGHT SSC).

Statistical analysis (A.C.-L.).

The INSIGHT Scientific Steering Committee (SSC) participated in the conception and design of the study. Full access to all data and responsibility for integrity and accuracy of data/analysis (A.L., J.N.).

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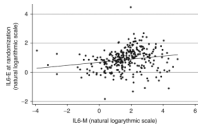

Fig. 1.

Log_e IL6-E (by ELISA) vs. log_e IL6-M (by multiplex bead array assay) at randomization in a subset of patients with both baseline measurement available (n = 296 patients). Adjusted R^2 = 0.03 (P = 0.001). Spearman's rank statistic rho = 0.29 (P = 0.0001).

Table 1

Median (interquartile range) cytokine and chemokine values and percentages of patients with a value below the limit of detection at randomization and the second time point.

	Randomization			Month 2 (Month 1 for IL6-E)		
Cytokines, pg/ml	**DC arm**	**VS arm**	***P*-value[a]**	**DC arm**	**VS arm**	***P*-value[a]**
IL-1β						
Median (range)	0.64 (0.02, 39.22)	0.77 (0.02, 19.30)		0.60 (0.02, 13.32)	0.67 (0.02, 10.98)	
n (%) with value below limit	22 (11.0%)	24 (12.0%)	0.50	18 (9.0%)	21 (10.6%)	0.65
IL-2						
Median (range)	3.19 (0.02, 219.9)	4.27 (0.01, 145.1)		2.47 (0.01, 65.32)	3.56 (0.08, 81.04)	
n (%) with value below limit	9 (4.5%)	9 (4.5%)	1.00	9 (4.5%)	11 (5.5%)	0.64
IL6-M						
Median (range)	4.72 (0.02, 136.1)	5.54 (0.06, 100.2)		5.43 (0.05, 139.1)	5.74 (0.09, 147.1)	
n (%) with value below limit	2 (1.0%)	1 (0.5%)	0.56	1 (0.5%)	2 (1.0%)	0.56
IL6-E						
Median (range)	2.37 (0.16, 14.79)	2.54 (0.27, 88.63)		3.52 (0.45, 15.50)	2.61 (0.59, 163.4)	
IL-7						
Median (range)	10.47 (0.02, 77.63)	11.71 (0.90, 111.9)		9.05 (0.17, 33.53)	11.37 (0.82, 51.89)	
n (%) with value below limit	3 (1.5%)	1 (0.5%)	0.04	0 (0.0%)	0 (0.0%)	0.24
IL-8						
Median (range)	5.40 (0.02, 53.55)	5.63 (0.13, 71.61)		5.85 (1.03, 54.14)	5.50 (0.06, 47.08)	
n (%) with value below limit	1 (0.5%)	0 (0.0%)		1 (0.5%)	1 (0.5%)	
IL-12 p70						
Median (range)	2.15 (0.03, 798.4)	3.46 (0.04, 476.6)		1.50 (0.03, 99.70)	2.30 (0.03, 302.7)	
n (%) with value below limit	32 (16.0%)	32 (16.0%)	0.19	23 (11.5%)	21 (10.6%)	0.11
IL-17						
Median (range)	3.00 (0.07, 302.2)	3.04 (0.16, 631.6)		2.98 (0.07, 162.1)	3.04 (0.17, 762.0)	
n (%) with value below limit	108 (54.0%)	137 (68.5%)	0.92	107 (53.5%)	101 (50.8%)	0.001
TNFα						
Median (range)	10.92 (0.10, 101.2)	11.48 (1.37, 120.9)		15.61 (2.28, 94.94)	11.08 (1.12, 77.28)	
n (%) with value below limit	1 (0.5%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	

Cytokines, pg/ml	Randomization			Month 2 (Month 1 for IL6-E)		
	DC arm	VS arm	*P*-value[a]	DC arm	VS arm	*P*-value[a]
IFNγ						
Median (range)	5.47 (0.09, 696.7)	7.17 (0.04, 293.0)		4.30 (0.08, 142.2)	5.43 (0.03, 301.2)	
n (%) with value below limit	18 (9.0%)	16 (8.0%)	0.86	17 (8.5%)	19 (9.5%)	0.58
GM-CSF						
Median (range)	1.23 (0.05, 367.3)	1.42 (0.05, 169.1)		0.86 (0.01, 71.86)	1.46 (0.01, 110.7)	
n (%) with value below limit	34 (17.0%)	52 (26.0%)	1.00	34 (17.0%)	31 (15.6%)	0.01
IL-4						
Median (range)	22.99 (0.01, 1567)	30.12 (0.01, 1743)		19.13 (0.01, 1361)	25.73 (0.01, 1541)	
n (%) with value below limit	15 (7.5%)	21 (10.5%)	0.85	14 (7.0%)	14 (7.0%)	0.22
IL-5						
Median (range)	0.49 (0.01, 38.41)	0.67 (0.02, 30.62)		0.42 (0.01, 65.74)	0.59 (0.01, 16.40)	
n (%) with value below limit	28 (14.0%)	29 (14.5%)	0.22	20 (10.0%)	19 (9.5%)	0.13
IL-10						
Median (range)	23.63 (0.08, 478.2)	25.85 (1.54, 651.1)		28.16 (4.47, 438.9)	24.69 (0.18, 402.8)	
n (%) with value below limit	3 (1.5%)	0 (0.0%)	0.04	0 (0.0%)	1 (0.5%)	0.24
IL-13						
Median (range)	4.60 (0.01, 676.3)	5.67 (0.01, 601.9)		2.69 (0.01, 685.5)	5.89 (0.01, 611.0)	
n (%) with value below limit	62 (31.0%)	77 (38.5%)	0.38	54 (27.0%)	58 (29.1%)	0.05
CXCL1						
Median (range)	269.4 (50.48, 2251)	271.8 (31.09, 3193)		522.3 (95.70, 8728)	273.7 (51.75, 2903)	
n (%) with value below limit	0 (0.0%)	0 (0.0%)		0 (0.0%)	0 (0.0%)	

For the calculation of the medians, values below detectable levels were set equal to the lower limit. DC, drug conservation; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; VS, viral suppression.

[a] χ^2-test.

Table 2

Main characteristics of random sample of discontinuation and viral suppression participants at randomization.

	DC (*n* = 200)	VS (*n* = 200)	*P*-value[a]
Characteristics age (years)			
Median (IQR)	45 (39, 51)	46 (40, 50)	0.408
≥45 years, *n* (%)	96 (48.0)	110 (55.0)	0.161
Sex, *n* (%)			0.820
Female	51 (25.5)	53 (26.5)	
Race, *n* (%)			0.766
Black	93 (46.5)	86 (43.0)	
White	81 (40.5)	85 (42.5)	
Other	26 (13.0)	29 (14.5)	
Co-infected, *n* (%)			0.114
Yes	41 (20.5)	29 (14.5)	
HCV alone	35 (17.5)	25 (12.5)	
HBV alone	5 (2.5)	3 (1.5)	
HCV/HBV	1 (0.5)	1 (0.5)	
Smoker, *n* (%)			0.604
Yes	71 (35.5)	76 (38.0)	
Diabetes, *n* (%)			0.749
Yes	21 (10.5)	23 (11.5)	
Lipid-lowering drugs, *n* (%)			0.908
Yes	51 (25.5)	50 (25.0)	
Treatment received			0.890
PI without NNRTI	86 (43.0)	82 (41.0)	
NNRTI without PI	78 (39.0)	88 (44.0)	
Neither PI or NNRTI	36 (18.0)	30 (15.0)	

DC, drug conservation; HBV, hepatitis B virus; HCV, hepatitis C virus; IQR, interquartile range; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; VS, viral suppression.

[a] Likelihood ratio χ^2-test or Wilcoxon test whichever was appropriate.

Table 3

Summary results for drug conservation vs. viral suppression at month 2 compared with baseline from fitting an analysis of covariance.

	DC arm (*N* = 200) Mean $\log_e$ change (SD)	VS arm (*N* = 200) Mean $\log_e$ change (SD)	Difference in mean $\log_e$ change DC/VS[a] (95% CI)	*P*-value
(a) Overall results				
Cytokines				
IL-1β	–0.02 (1.25)	–0.16 (1.16)	0.04 (–0.17, 0.25)	0.708
IL-2	–0.16 (1.59)	–0.16 (1.34)	–0.16 (–0.41, 0.09)	0.206
IL6-M	0.21 (1.26)	–0.05 (0.97)	0.20 (0.00, 0.41)	0.049
IL6-E (month 1)	0.43 (0.76)	0.06 (0.78)	0.26 (0.06, 0.46)	0.010
IL-7	–0.05 (1.02)	–0.10 (0.90)	–0.07 (–0.22, 0.09)	0.400
IL-8	0.08 (0.77)	–0.03 (0.69)	0.09 (–0.04, 0.21)	0.168
IL-12 p70	–0.29 (1.82)	–0.16 (1.49)	–0.34 (–0.63, –0.05)	0.022
IL-17	–0.41 (1.43)	–0.02 (1.03)	–0.44 (–0.65, –0.23)	<0.001
TNFα	0.35 (0.70)	–0.08 (0.62)	0.34 (0.24, 0.45)	<0.001
IFNγ	–0.17 (1.63)	–0.25 (1.46)	–0.03 (–0.29, 0.24)	0.854
GM-CSF	–0.30 (1.76)	0.05 (1.43)	–0.40 (–0.68, –0.12)	0.006
IL-4	–0.20 (2.51)	–0.28 (1.89)	–0.06 (–0.45, 0.34)	0.785
IL-5	–0.13 (1.45)	–0.11 (1.16)	–0.13 (–0.36, 0.10)	0.270
IL-10	0.34 (1.08)	–0.13 (0.88)	0.33 (0.18, 0.49)	<0.001
IL-13	–0.26 (2.48)	–0.19 (2.08)	–0.11 (–0.54, 0.31)	0.599
CXCL10	0.62 (0.87)	–0.04 (0.65)	0.66 (0.53, 0.79)	<0.001

	DC vs. VS; adjusted differences[b] (95% CI)					
	Biomarker					
	IL6-M	IL-12	IL-17	TNFα	IL-10	CXCL10
(b) Biomarkers levels according to baseline subgroups						
Characteristics						
Sex						
Male	0.18 (–0.06, 0.42)	–0.35 (–0.68, –0.01)	–0.52 (–0.76, –0.27)	0.36 (0.24, 0.49)	0.31 (0.13, 0.49)	0.61 (0.46, 0.76)
Female	0.27 (–0.13, 0.67)	–0.31 (–0.88, 0.25)	–0.24 (–0.66, 0.17)	0.29 (0.08, 0.50)	0.40 (0.09, 0.70)	0.80 (0.54, 1.06)
Interaction test *P*-value	0.71	0.92	0.26	0.54	0.63	0.21

	DC vs. VS; adjusted differences[b] (95% CI)					
	Biomarker					
	IL6-M	**IL-12**	**IL-17**	**TNFα**	**IL-10**	**CXCL10**
Age, years						
<45	0.31 (0.01, 0.60)	–0.37 (–0.79, 0.05)	–0.43 (–0.73, –0.12)	0.40 (0.25, 0.55)	0.43 (0.20, 0.65)	0.71 (0.52, 0.90)
>45	0.12 (–0.17, 0.40)	–0.29 (–0.70, 0.11)	–0.47 (–0.77, –0.18)	0.32 (0.17, 0.46)	0.27 (0.05, 0.48)	0.63 (0.44, 0.81)
Interaction test *P*-value	0.36	0.79	0.83	0.43	0.31	0.56
Ethnicity						
Black	0.11 (–0.21, 0.42)	–0.33 (–0.78, 0.12)	–0.64 (–0.97, –0.31)	0.37 (0.21, 0.54)	0.31 (0.07, 0.55)	0.63 (0.42, 0.83)
White/other	0.27 (0.01, 0.54)	–0.35 (–0.73, 0.03)	–0.30 (–0.58, –0.02)	0.32 (0.19, 0.46)	0.35 (0.15, 0.55)	0.68 (0.51, 0.86)
Interaction test *P*-value	0.43	0.95	0.12	0.66	0.81	0.69
Class of third drug[c]						
PI based	0.12 (–0.18, 0.42)	–0.58 (–1.00, –0.17)	–0.38 (–0.69, –0.07)	0.29 (0.13, 0.45)	0.42 (0.18, 0.65)	0.69 (0.48, 0.89)
NNRTI based	0.32 (0.02, 0.62)	0.10 (–0.32, 0.52)	–0.49 (–0.80, –0.18)	0.38 (0.22, 0.54)	0.31 (0.07, 0.54)	0.67 (0.46, 0.88)
Interaction test *P*-value	0.35	0.02	0.64	0.43	0.52	0.90
Co-infected						
No	0.21 (–0.02, 0.43)	–0.27 (–0.59, 0.04)	–0.44 (–0.68, –0.21)	0.36 (0.24, 0.48)	0.38 (0.20, 0.55)	0.75 (0.60, 0.89)
Yes	0.14 (–0.35, 0.63)	–0.73 (–1.43, –0.03)	–0.39 (–0.90, 0.13)	0.24 (–0.02, 0.50)	0.08 (–0.29, 0.46)	0.20 (–0.12, 0.52)
Interaction test *P*-value	0.81	0.25	0.84	0.40	0.17	0.002

CI, confidence interval; DC, drug conservation; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; TNF, tumor necrosis factor; VS, viral suppression.

[a] Model includes baseline level of marker (natural logarithmic scale).

[b] Adjusted for baseline using analysis of covariance.

[c] Thirty-three patients receiving both/neither NNRTI and PI were excluded.

Table 4

Estimated mean month 2 viral load [$\log_{10}$ copies/ml – part (a)] and CD4 cell count change by month 2 [cells/μl – part (b)] associated with 1 $\log_e$ higher values in biomarkers from fitting a linear regression model (drug conservation army only).

Per $\log_e$ higher	Unadjusted analysis Mean (95% CI)	*P*-value	Adjusted analysis[a] Mean (95% CI)	*P*-value
(a) Viral load at month 2 ($\log_{10}$ copies/ml)				
IL-1β	0.02 (–0.09, 0.14)	0.713	0.03 (–0.08, 0.13)	0.632
IL-2	0.05 (–0.04, 0.14)	0.301	0.05 (–0.03, 0.14)	0.190
IL6-M	–0.01 (–0.12, 0.11)	0.930	–0.01 (–0.12, 0.09)	0.812
IL-7	0.02 (–0.12, 0.16)	0.786	0.05 (–0.08, 0.17)	0.483
IL-8	0.02 (–0.16, 0.21)	0.796	0.02 (–0.15, 0.19)	0.800
IL-12	0.02 (–0.07, 0.10)	0.709	0.01 (–0.06, 0.08)	0.767
IL-17	–0.11 (–0.21, –0.01)	0.033	–0.10 (–0.19, –0.01)	0.038
TNFα	0.26 (0.05, 0.47)	0.015	0.16 (–0.03, 0.35)	0.104
IFNγ	0.03 (–0.06, 0.12)	0.455	0.03 (–0.05, 0.11)	0.466
GM-CSF	–0.01 (–0.10, 0.07)	0.739	–0.02 (–0.09, 0.06)	0.621
IL-4	0.01 (–0.04, 0.07)	0.612	0.02 (–0.04, 0.07)	0.560
IL-5	–0.02 (–0.12, 0.08)	0.696	–0.03 (–0.12, 0.06)	0.530
IL-10	0.17 (0.04, 0.30)	0.012	0.12 (0.00, 0.24)	0.047
IL-13	–0.00 (–0.06, 0.06)	0.935	–0.02 (–0.07, 0.04)	0.540
CXCL10	0.35 (0.18, 0.51)	<0.001	0.26 (0.11, 0.41)	<0.001
(b) Change in $CD4^+$ T-cell count (cells/μl)				
IL-1β	1.85 (–19.0, 22.68)	0.861	6.29 (–12.7, 25.26)	0.514
IL-2	2.74 (–13.7, 19.18)	0.743	7.21 (–7.77, 22.20)	0.344
IL6-M	–3.01 (–23.7, 17.72)	0.775	–2.04 (–20.9, 16.81)	0.831
IL-7	10.46 (–15.0, 35.89)	0.418	14.21 (–8.90, 37.31)	0.227
IL-8	–1.78 (–35.7, 32.09)	0.917	2.68 (–28.2, 33.51)	0.864
IL-12	–0.69 (–15.2, 13.84)	0.925	2.40 (–10.9, 15.65)	0.722
IL-17	7.15 (–11.1, 25.36)	0.439	–2.78 (–19.8, 14.19)	0.747
TNFα	–39.5 (–76.6, –2.41)	0.037	–22.5 (–57.2, 12.17)	0.202
IFNγ	–1.02 (–17.0, 14.99)	0.900	3.00 (–11.6, 17.64)	0.686
GM-CSF	–1.70 (–16.5, 13.14)	0.821	0.61 (–12.9, 14.14)	0.929
IL-4	0.54 (–9.85, 10.94)	0.918	1.99 (–7.48, 11.46)	0.679
IL-5	–3.04 (–21.0, 14.92)	0.739	–2.37 (–18.7, 14.00)	0.775
IL-10	–19.9 (–43.8, 4.01)	0.102	–5.39 (–27.7, 16.94)	0.634
IL-13	–5.49 (–16.0, 5.05)	0.306	–5.61 (–15.2, 3.97)	0.249
CXCL10	–35.8 (–65.7, –5.97)	0.019	–11.2 (–39.8, 17.49)	0.443

CI, confidence interval; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

[b]Adjusted for month 2 $\log_{10}$ viral load.

[a]Adjusted for change in $CD4^+$ T-cell count.