Effect of a four-week course of interleukin-10 on cytokine production in a placebo-controlled study of HIV-1-infected subjects

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ABSTRACT. Interleukin (IL)-10 suppresses synthesis of the pro-inflammatory cytokines tumor necrosis factor (TNF)α, IL-1β, and interferon (IFN)γ. Since pro-inflammatory cytokines have been implicated in the production of human immunodeficiency virus type 1 (HIV-1), cytokine synthesis in whole blood cultures were determined during a 4-week course of subcutaneous IL-10 injections in 33 HIV-1-infected patients. Patients were randomized into four groups: placebo (nine), IL-10 at 1 µg/kg/day (nine), IL-10 at 4 µg/kg/day (six) and IL-10 at 8 µg/kg three times per week (nine). Whole blood was obtained at the beginning and conclusion of the study and was stimulated for 24 hours with the combination of IL-18 plus lipopolysaccharide. TNFα production in stimulated whole blood was reduced three and six hours after the first injection of IL-10 compared to subjects injected with the placebo. After four weeks of treatment, production of IFNγ was suppressed in a greater number of patients in the IL-10 treatment groups compared to subjects in the placebo group. Similarly, IL-1β production was lower in the IL-10 treatment groups compared to subjects receiving placebo. In contrast, after four weeks of IL-10, circulating levels of the anti-inflammatory TNF soluble receptor p55 increased dose-dependently compared to placebo subjects. Patient heterogeneity and small sample size presented difficulties in establishing statistical significance. Although the cytokine changes in our study did not demonstrate statistically significant changes, the data nevertheless reveal that four weeks of IL-10 therapy in HIV-1 infected subjects produced the anticipated suppression of pro-inflammatory cytokines.

Keywords: HIV-1, interleukin-10, pro-inflammatory cytokines, whole blood, *ex vivo*

The morbidity and mortality of human immunodeficiency virus type 1 (HIV-1)-infected patients have decreased significantly following the introduction of highly active antiretroviral therapy (HAART) regimens [1]. However, HIV-1 infected patients on HAART may harbor viruses containing resistance mutations [2], and the presence of these mutated viruses is associated with clinical failure of antiretroviral therapies [3, 4]. Novel treatment approaches include administration of substances that enhance natural defenses against HIV-1 rather than targeting virus-specific components. Several cytokines or cytokine antagonists can suppress HIV-1, including interferon (IFN)γ [5, 6], interleukin (IL)-1 receptor antagonist (IL-1Ra), tumor necrosis factor (TNF) soluble receptors [7, 8], IL-18 [9], and the β-chemokines regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-1β [10]. Clinical applications of cytokine-based therapy in conjunction with HAART regimens could prove more effective than HAART alone.

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IL-10 is an 18 kDa protein that is active as a homodimer and produced by several cell types including monocytes, macrophages, T-cells, and B-cells [11]. IL-10 has anti-inflammatory properties that include suppression of gene expression and synthesis of pro-inflammatory cytokines such as TNFα and IL-1β [12]. Consistent with these *in vitro* effects, IL-10-deficient mice develop spontaneous, fatal enterocolitis with growth retardation and anemia [13]. These findings are thought to result from excessive inflammation initiated by the endogenous intestinal flora in the absence of IL-10 anti-inflammatory activity [13, 14]. This observation underscores the role of endogenous IL-10 in protecting the host from inflammation.

The pro-inflammatory cytokines TNFα and IL-1β, as well as IL-6 stimulate HIV-1 production *in vitro*, and elevated levels of circulating pro-inflammatory cytokines have been documented in patients with HIV-1 infection [8, 15-19]. Since pro-inflammatory cytokines increase HIV-1 expression *in vitro*, and IL-10 inhibits production of pro-inflammatory cytokines *in vitro* [20], administration of IL-10 to HIV-1-infected patients may reverse the virus-inducing effect of pro-inflammatory cytokines. In fact, a single intravenous infusion of IL-10 administered to HIV-1-infected persons in an uncontrolled study inhibited pro-inflammatory cytokine production and decreased viral RNA levels in some subjects [21]. IL-10 also possesses antiretroviral function *in vitro* that may be independent of cytokine modulation [22-24].

In the present clinical study, a four-week course of subcutaneous (sc) IL-10 was administered to HIV-1-infected subjects in a double-blind, placebo-controlled phase II/III trial evaluating the effect of IL-10 on HIV-1 viral load [25]. We describe a sub-study of this trial where the effect of IL-10 on cytokine production was examined. To enhance clinical relevance, we evaluated *ex vivo* cytokine production in whole blood obtained from patients before and after IL-10 injection. In this design, manipulation of IL-10 blood concentrations occurred by administering IL-10 to subjects *in vivo*, and experiments were performed *in vitro* using samples of blood. Therefore, IL-10 in the blood samples reflects biologically relevant levels, and the observed effects likely reflect *in vivo* IL-10 activity. Our investigations showed that IL-10 suppressed the induction of pro-inflammatory cytokines in whole blood cultures, and IL-10-injected subjects possessed elevated levels of circulating anti-inflammatory TNFsRp55.

METHODS

Subject selection

Thirty-nine HIV-1-infected subjects between 22 and 54 years of age were enrolled in a phase II/III study of IL-10 as an antiretroviral therapy as described previously [25]. After informed consent was obtained, patients were enrolled in six centers following Institutional Review Board approval. Eligibility requirements included HIV-1 RNA quantification by PCR of at least 1500 RNA copies/mL, $CD4^+$ lymphocyte count of at least 200/mm^3, a Karnofsky performance score greater than 50, life expectancy of at least 12 weeks, and absence of systemic opportunistic infection. Patients were either antiretroviral therapy naive or were receiving a stable antiretroviral regimen for at least one month prior to entry. Additional requirements included a normal screening chest x-ray, no history of systemic opportunistic infection or immunization within the preceding three months, and weight of at least 104.4 pounds (47.5 kg). Required laboratory values included a platelet concentration of at least 100 000/mm^3, hemoglobin at least 9.0 gm/L, absolute neutrophil count 750/mm^3 or greater, serum creatinine less than 1.5 times the upper limit of normal, SGOT and SGPT each less than 5.0 times the upper limit of normal, and direct bilirubin less than 3.0 mg/dL. Women of childbearing potential required a negative serum β-HCG pregnancy test within 24 hours of the initial study injection.

Exclusion criteria included clinically significant disorders not related to HIV-1 disease, participation in a clinical trial of a new medication within one month of the first dose of IL-10, allergy to *E. coli* proteins or IL-10, malignancy including Kaposi's sarcoma, cytotoxic therapy for cancer within the past year, blood transfusion within 60 days, receipt of HIV-1 vaccination within the preceding three months, or inability to understand informed consent. Additional exclusion criteria included immunomodulatory therapies within eight weeks of study entry, systemic corticosteroids (excluding anabolic agents), intravenous immune globulin, interferons, interleukins, pentoxifylline, cimetidine, thalidomide, granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), dinitrochlorobenzene, thymosin alpha-1, thymopentin, isoprinosine, polyribonucleoside, ditiocarb (diethyldithiocarbamate), or N-acetylcysteine.

Study design

This was a prospective, randomized, double-blind, placebo-controlled study of four weeks treatment with subcutaneous (sc) vehicle placebo (a lyophilized formulation without IL-10) once daily or three times weekly, or sc recombinant human IL-10 (Schering-Plough, Kenilworth, NJ, USA) at 1.0 μg/kg once daily, 4.0 μg/kg once daily, or 8.0 μg/kg three times each week (TIW). Randomization of subjects was in a 1:1:1:1 ratio. Venous blood was drawn immediately before dose of the first administration of placebo or IL-10 injection and three, six and 24 hours following the initial injection. Blood samples were also taken before the final dose of placebo or IL-10 (day 28 for once daily dosing groups or day 26 for the TIW dosing groups), and three, six, and 24 hours following the final dose of placebo or IL-10. Blood was assessed for white blood cell count and differential, platelet count, and concentration of $CD4^+$ lymphocytes [26]. Blood was also added to a Cytochek tube (National Genetics Institute, Culver City, CA, USA) to quantify viral load.

Twenty four-hour whole blood cytokine stimulation cultures

Whole blood drawn from an antecubital vein was aspirated into sterile, heparin-containing vacuum tubes or into polypropylene syringes containing heparin (final heparin concentration 10 units/mL). One milliliter of heparinized blood was added to sterile polypropylene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) that con-

tained 1.0 mL of RPMI (Mediatech, Herndon, VA, USA) with 20 ng lipopolysaccharide (LPS, *E. coli* O55:B5, Sigma, St. Louis, MO, USA) and IL-18 (20 ng PeproTech, Rocky Hill, NJ, USA). Following addition of heparinized blood, the tubes were inverted three to five times and incubated upright for 24 hours at 37°C, 5% CO_2. Following incubation, each 2.0 mL sample was mixed by inversion and transferred into a 5.0 mL cryogenic tube (Corning) containing 200 µL of 10% (vol/vol) Triton-X-100 (approximately 1% vol/vol final concentration, Fisher Scientific, Fair Lawn, NJ, USA). Tubes were inverted to lyse the blood and samples were frozen at -70°C until assayed at the University of Colorado at Denver and Health Sciences Center.

Circulating TNF soluble receptor p55 (TNFsRp55)

One milliliter of heparinized blood drawn at the beginning and conclusion of the study was transferred into 5.0 mL polypropylene cryogenic tubes (Corning, Cambridge, MA, USA) containing 100 µL of 10% (vol/vol) Triton-X-100 (approximately 1% vol/vol final concentration, Fisher Scientific, Fair Lawn, NJ, USA). Tubes were inverted to lyse the blood and the samples were immediately frozen at -70°C.

Cytokine assays

Blood samples were warmed to room temperature and analyzed by ELISA for mature IL-1β (Cistron) and MIP-1α as described previously [27]. Lysed whole blood specimens were diluted prior to assay. The samples and cytokine standards in each assay were diluted to the same degree using as diluent a pooled whole blood lysate from 20 healthy volunteers. Under these conditions, the lower limits of detection were 200 pg/mL for IL-1β and 100 pg/mL for MIP-1α.TNFα and IFNγ were quantified using electrochemiluminescence (ECL) assays as described previously [28]. Each ECL cytokine assay was unaffected by 1% Triton-X-100 and the cytokine standards in each assay were diluted using a pooled, whole blood lysate from 20 healthy volunteers. The lower limit of detection for TNFα was 40 pg/mL, and for IFNγ 60 pg/mL. TNF soluble receptor p55 (TNFsRp55) was measured using a radioimmunoassay as described previously [29]. The TNFsRp55 radioimmunoassay was unaffected by 1% Triton-X-100.

Data analysis

Production of cytokines in whole blood after 24 hours (hrs) of incubation was calculated as concentration per million cells derived from the concurrent white blood cell count and differential. Since T-cells are thought to be the primary source of IFNγ, whole blood IFNγ levels were calculated per million $CD4^+$ T-cells. For IL-1β, TNFα, and MIP-1α, the cytokine concentrations were calculated per million monocytes to reflect the likely cell of origin for these molecules. Since the source of circulating TNFsRp55 is not known, there was no normalization of TNFsRp55 levels for any type of white blood cell.

Differences in cytokine production were tested for statistical significance within each study group using paired t-tests *(figures 1-4)*, or between groups using a one-way ANOVA with Kruskal-Wallace analysis *(figure 5)*. Statistical significance was defined as $p < 0,05$.

RESULTS

Study group characteristics

Enrollment in this study assessing IL-10 as an antiretroviral therapy ceased after an interim analysis revealed that IL-10 treatment had no significant effect on HIV-1 RNA viral load [25]. Complete cytokine data were available for 33 of the 39 subjects and this subset of 33 comprises the group described in this report. *Table 1* shows characteristics of the 33 subjects. Caucasian volunteers predominated in the 1 µg/kg and 8 µg/kg IL-10 groups, whereas African Americans predominated in the 4 µg/kg group. The 8 µg/kg group had the highest mean weight (77.0 kg) and the lowest mean $CD4^+$ count (317.6 cells/mm^3). The mean HIV-1 RNA viral load measurements (in RNA copies/mL plasma) were within 0.15 $\log_{10}$ units of 4.0 in the study groups.

Effect of a four-week course of IL-10 on IL-18/LPS-induced IFNγ production

To analyze the effect of four weeks of IL-10 administration on *ex vivo* cytokine production in the HIV-1-infected individuals in this study, pre-injection cytokine production was measured in 24-hour whole blood cultures stimulated with a combination of IL-18 and LPS. This combination of stimuli induces IFNγ as well as other cytokines. Whole blood cytokine production was measured before the first dose of placebo or IL-10 and compared to measurements before the final dose of placebo or IL-10. Since IFNγ possesses anti-HIV-1 activity *in vitro* [5, 6], we measured the effect of IL-10 on whole blood IFNγ induction. For each subject, IFNγ production in blood cultured before the first study dose was set at 100% and the IFNγ obtained in cultures conducted before the final dose were expressed as a percentage of the pre-study level. As shown in *figure 1A*, five of nine patients randomized to the placebo group produced less IFNγ after the four-week course (range of percentage production compared to first dose 18-75%), whereas four patients produced greater amounts of IFNγ. In contrast, most subjects receiving any IL-10 dose exhibited decreased IFNγ production after the four-week course of treatment (18 of 24, *figures 1B-D*). Decreased production in subjects receiving 1, 4 or 8 µg/kg was observed in six out of nine, five out of six and seven out of nine subjects, respectively. In all subjects combined, pre-study IFNγ concentrations ranged from 29 to 6,083 pg/mL/10^6 T-cells, and after four weeks of study the ranges in the placebo and in the three combined IL-10 groups were 27-12,889 pg/mL/10^6 T-cells and 27-4,966 pg/mL/10^6 T-cells, respectively.

Effect of a four-week course of IL-10 on IL-18/LPS-induced IL-1β production

In vitro studies in chronically infected U1 cells showed that IL-1β induced HIV-1 replication, suggesting that

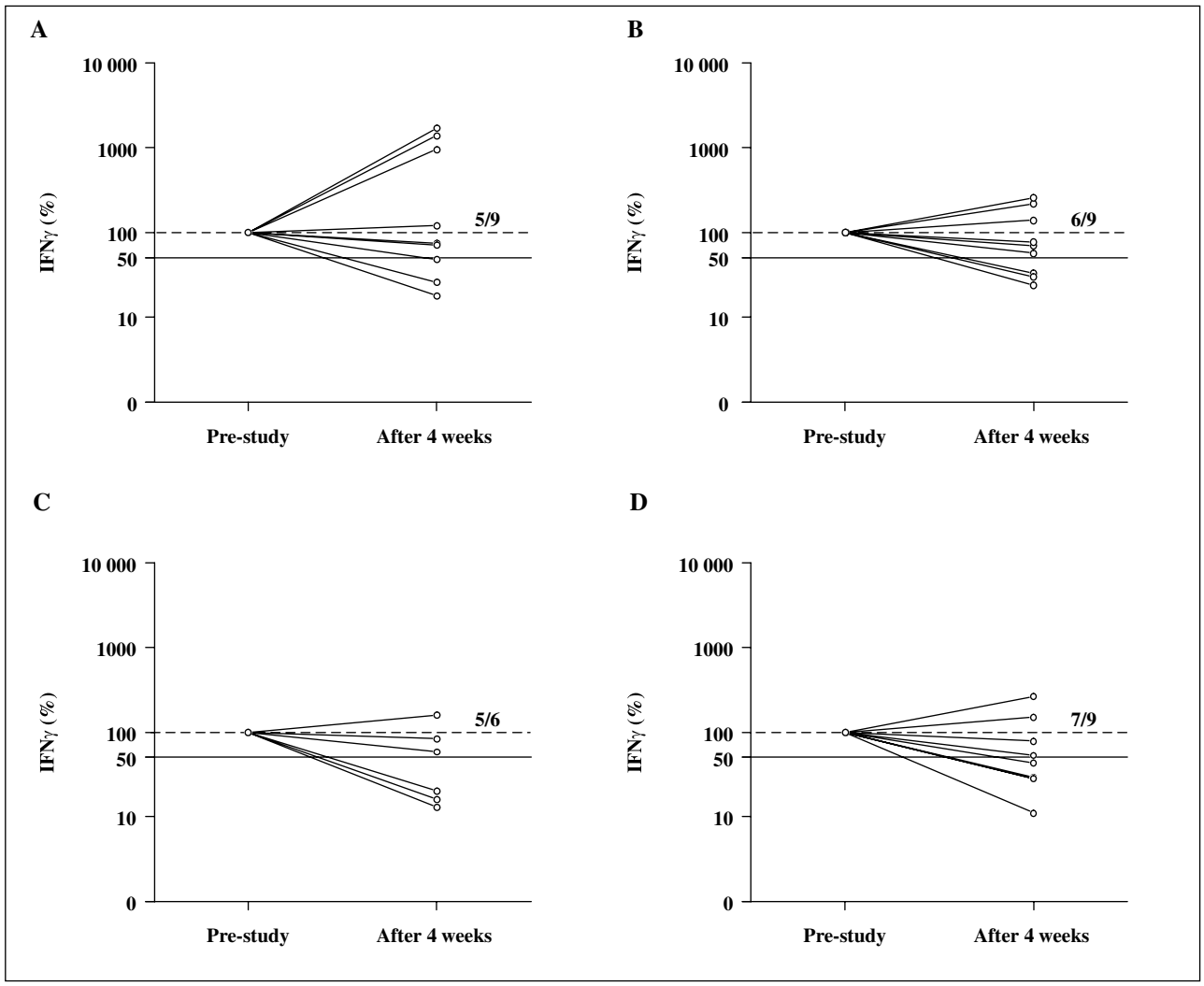

Figure 1

Stimulated whole blood IFNγ production after four weeks of placebo or IL-10. IL-18/LPS-induced IFNγ values were calculated per million $CD4^+$ T-cells and expressed as a percentage compared to the pre-study level (100%) for each subject. Log_{10} percentage IFNγ levels are shown on the vertical axes. The horizontal dashed lines indicate 100% and the solid lines identify 50% reductions in 24-hour IFNγ production. The fractions shown indicate the number of subjects in each group exhibiting reduced IFNγ production after four weeks of study (numerator) compared to the total number of subjects in each group (denominator). Data are shown for the four study groups: (**A**) placebo, (**B**) IL-10 at 1 µg/kg/day, (**C**) IL-10 at 4 µg/kg/day, or (**D**) IL-10 at 8 µg/kg TIW.

IL-1β induces HIV-1 *in vivo* [7, 8]. Since IL-10 can inhibit pro-inflammatory cytokines and HIV-1 production *in vitro*, we assessed the effect of four weeks of placebo or IL-10 administration on IL-1β production. In whole blood cultures stimulated with IL-18 and LPS, five out of nine placebo subjects exhibited decreased IL-1β production after four weeks of study, with a 54-91% range of production compared to pre-study levels *(figure 2A)*. For patients receiving IL-10 *(figure 2B-D)*, there was a direct relationship between the IL-10 dose and the fraction of patients with decreased IL-1β at study conclusion. For IL-10 at 1, 4, or 8 µg/kg, the number of patients exhibiting reduced IL-1β synthesis after four weeks were three out of nine (33%), three out of six (50%) and seven out of nine (78%), respectively. A dose-dependent inhibition of stimulated IL-1β is suggested since the suppression was greatest in the group receiving IL-10 at 8 µg/kg TIW (range of percentage production 45-94%). In all subjects combined, pre-study IL-1β concentrations were 2,041-28,731 pg/mL/10^6 monocytes, and after four weeks of study the ranges in the placebo and the in the three combined IL-10 groups were 2,946-24,867 pg/mL/10^6 monocytes and 2,860-20,123 pg/mL/10^6 monocytes, respectively.

Effect of a four-week course of IL-10 on IL-18/LPS-induced TNFα production

TNFα stimulates HIV-1 replication in lymphocyte cell lines and substantially enhances viral production in primary blood monocyte-derived macrophages [15, 30]. *Figure 3* depicts the effect of four weeks of placebo or IL-10 administration on IL-18 and LPS-induced TNFα production *ex vivo*. Six out of nine (67%) patients in the placebo group had decreased TNFα production at conclusion of study (range of percentage production of 4-96%). None of the groups receiving IL-10 had a greater proportion of subjects with decreased TNFα production. In all subjects, pre-study TNFα concentrations were 59-32,654 pg/mL/10^6 monocytes, and after four weeks of

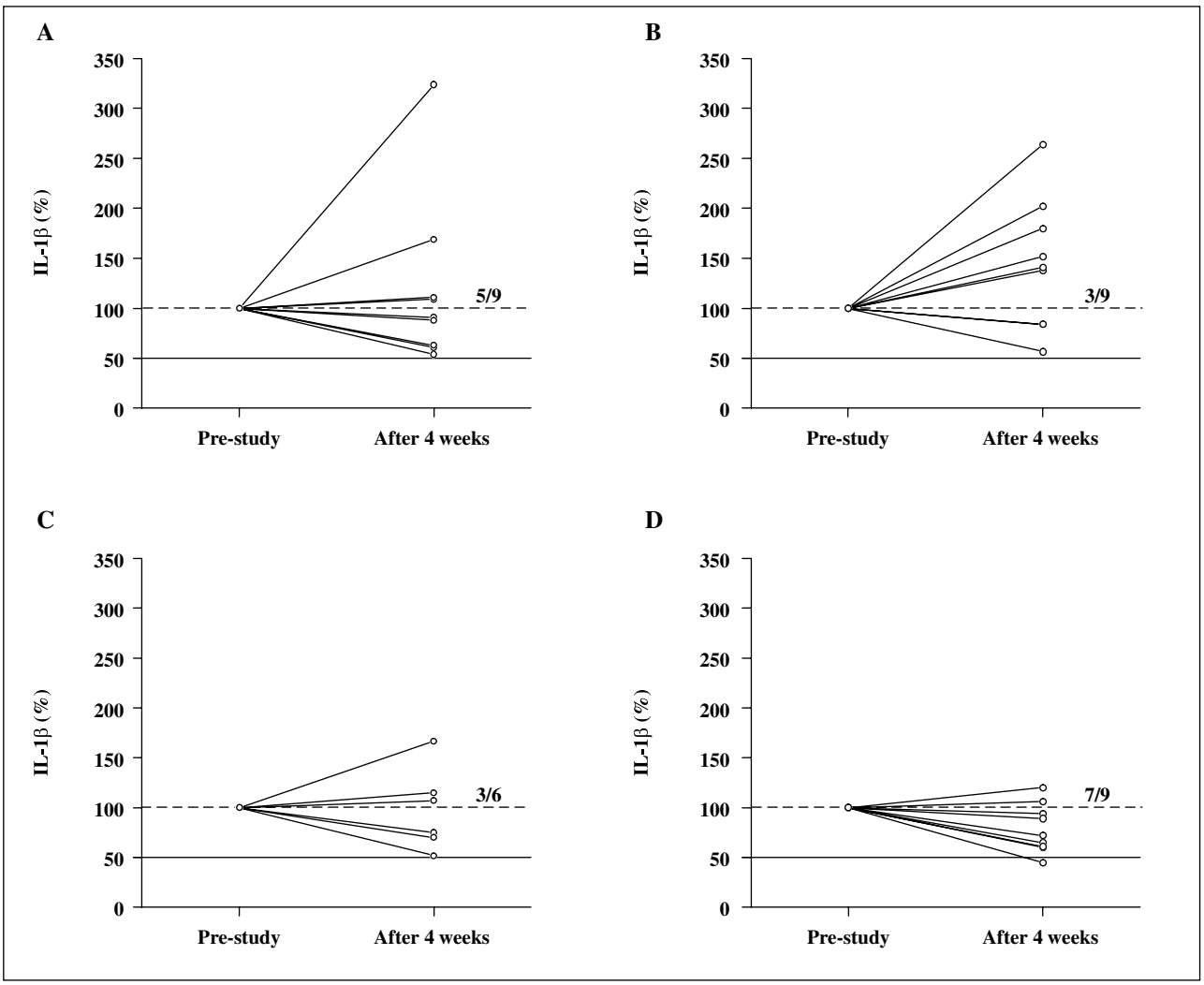

Figure 2

Stimulated whole blood IL-1β production after four weeks of placebo or IL-10. IL-1β was measured in the same IL-18/LPS-stimulated whole blood cultures described in *figure 1*. The concentrations of IL-1β were calculated per million monocytes and shown as $\log_{10}$ percentage productions on the vertical axes. The horizontal dashed lines indicate 100% and the solid lines identify 50% reductions in 24-hour, IL-1β production. The fractions indicate the number of subjects in each group exhibiting a decrease in IL-1β production (< 100%, numerator) compared to the total number of subjects in each group (denominator). Data are shown for (**A**) placebo, and IL-10 given at (**B**) 1 μg/kg/day, (**C**) 4 μg/kg/day, or (**D**) 8 μg/kg TIW.

study, the ranges in the placebo and in the three combined IL-10 groups were 120-4 083 pg/mL/10^6 monocytes and 95-14 473 pg/mL/10^6 monocytes, respectively.

Effect of a four-week course of IL-10 on IL-18/LPS-induced MIP-1α production

The β-chemokines are natural ligands for the CCR5 HIV-1 co-receptor and these molecules may have a role in moderating HIV-1 progression [10]. In *figure 4*, pre-injection IL-18 and LPS-stimulated MIP-1α was measured at the beginning and conclusion of the study. One out of nine (11%) placebo subjects exhibited decreased MIP-1α production after four weeks *(figure 4A)*. In contrast, decreased MIP-1α production was observed in four out of nine (44%), two out of six (33%) and three out of nine (33%) subjects receiving 1, 4 or 8 μg/kg IL-10, respectively *(figure 4B-D)*. Thus, 37.5% of all subjects receiving IL-10 produced less MIP-1α after four weeks, compared to 11% in the placebo group. In all subjects, pre-study MIP-1α concentrations ranged from 213 to 8,531 pg/mL/10^6 monocytes. After four weeks, the MIP-1α range in the placebo group was 772-6,039 pg/ mL/10^6 monocytes. The range in the three combined IL-10 groups was 207-29,779 pg/mL/10^6 monocytes.

Effect of a four-week course of IL-10 on TNFsRp55 levels

Figure 5 depicts TNFsRp55 levels measured at the end of the study in whole blood, expressed as mean percentage level compared to the pre-study concentrations (set at 100%). After four weeks, there was a dose-dependent increase in TNFsRp55 associated with IL-10 administration. Compared to pre-study values (100%), mean TNFsRp55 concentrations measured at the conclusion of study in the placebo, 1, 4 and 8 μg/kg IL-10 groups were 95 ± 12, 131 ± 17, 142 ± 19 and 146 ± 27 percent (mean ± standard error of the mean (SEM)), respectively. *Table 2* summarizes the results for IL-18 and LPS 24 hr whole blood cytokine production.

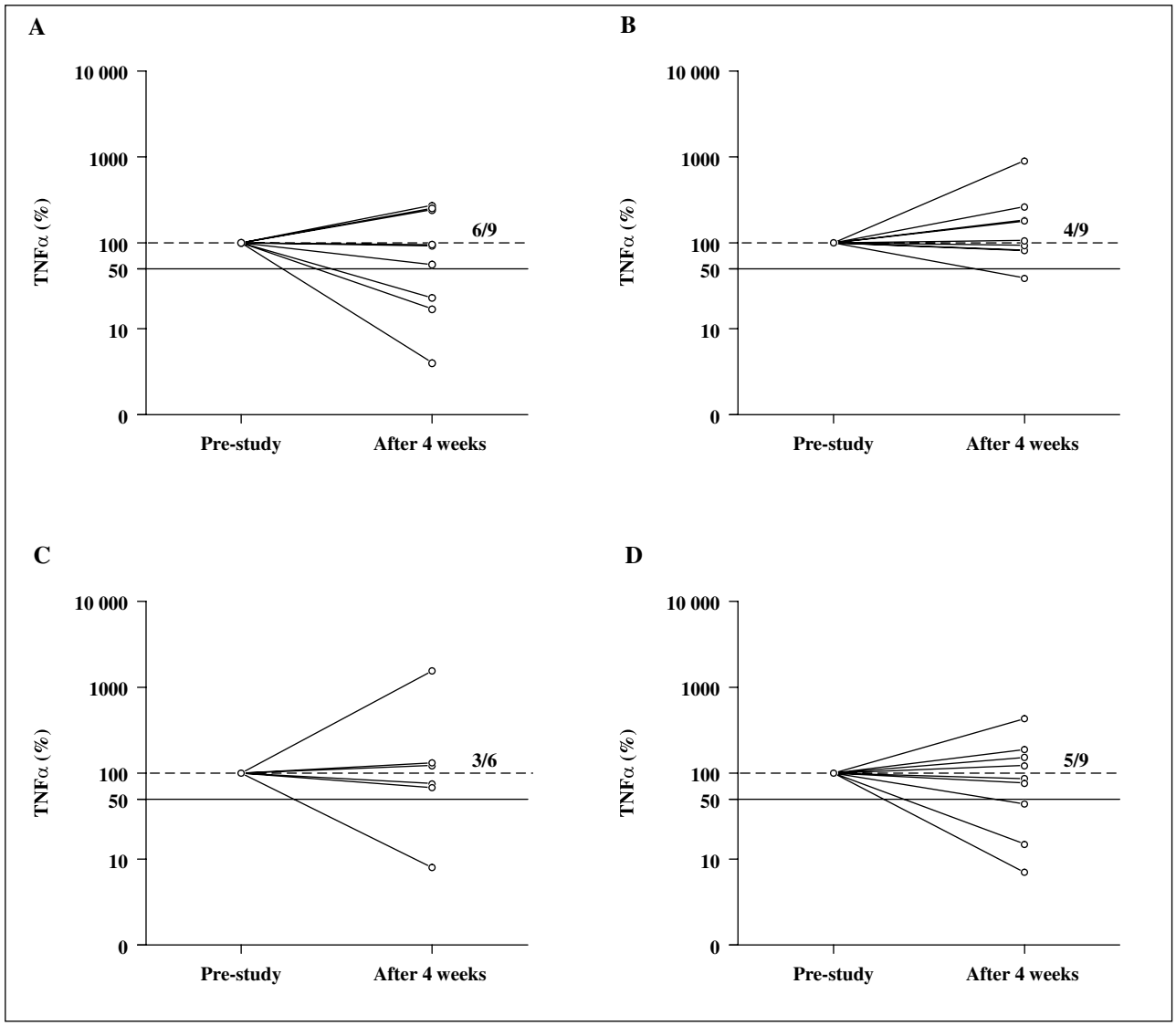

Figure 3

Stimulated whole blood TNFα production after four weeks of placebo or IL-10. TNFα was measured in the IL-18/LPS-stimulated whole blood samples described in *figure 1*. TNFα concentrations were calculated per million monocytes and the results presented as described in *figure 1*. The fractions indicate the number of subjects in each group exhibiting a decrease in TNFα production (< 100%, numerator) compared to the total number of subjects in each group (denominator). Data are shown for (**A**) placebo, (**B**) IL-10 at 1 µg/kg/day, (**C**) IL-10 at 4 µg/kg/day, or (**D**) IL-10 at 8 µg/kg TIW.

DISCUSSION

In vitro studies have shown that IL-10 reduces HIV-1 production in chronically or acutely infected cells [22-24, 31]. The likely mechanisms of IL-10 antiretroviral activity include effects on signal transduction, inhibition of cellular differentiation, decreased HIV-1 RNA half-life, and suppression of cytokines with HIV-1-inducing activity. However, other *in vitro* studies have revealed that IL-10 stimulated HIV-1 production in monocytic cell lines [32-35]. The complex effects of IL-10 on HIV-1 production *in vitro* stress the need for clinical data to establish the role of IL-10 in infected persons. IL-10 administered as a single intravenous injection to healthy volunteers significantly decreased *ex vivo* whole blood IL-1β and TNFα production [26]. Since IL-1β and TNFα increase HIV-1 production in some *in vitro* models [7, 8, 15, 30], IL-10-dependent inhibition of these cytokines provided additional rationale for using IL-10 as an antiretroviral agent. In a previous phase I trial in HIV-1-infected individuals with $CD4^+$ T-cell counts between 200 and 500/µL, a single intravenous IL-10 dose induced a transient but substantial reduction in circulating HIV-1 RNA in a subset of enrollees [21].

In the present double-blind, placebo-controlled phase II/III trial in 39 HIV-1-infected volunteers, HIV-1 production after four weeks of recombinant human IL-10 was the primary outcome. Analysis of clinical endpoints showed no effect on viral load or disease progression during the four weeks of IL-10 therapy as reported previously [25]. In the subset of subjects (33 out of 39) in this study, we assessed the effect of IL-10 on cytokine production. The combination of IL-18 and LPS was used to initiate a cytokine response and reveal an IL-10 effect in whole blood cultures [36]. Cytokine production was adjusted per million cells to ensure that levels reflected cell activity and not cell concentration. An *ex vivo* design was used since blood removed from a subject during therapeutic intervention and then studied *in vitro* is more likely to reflect *in vivo* conditions.

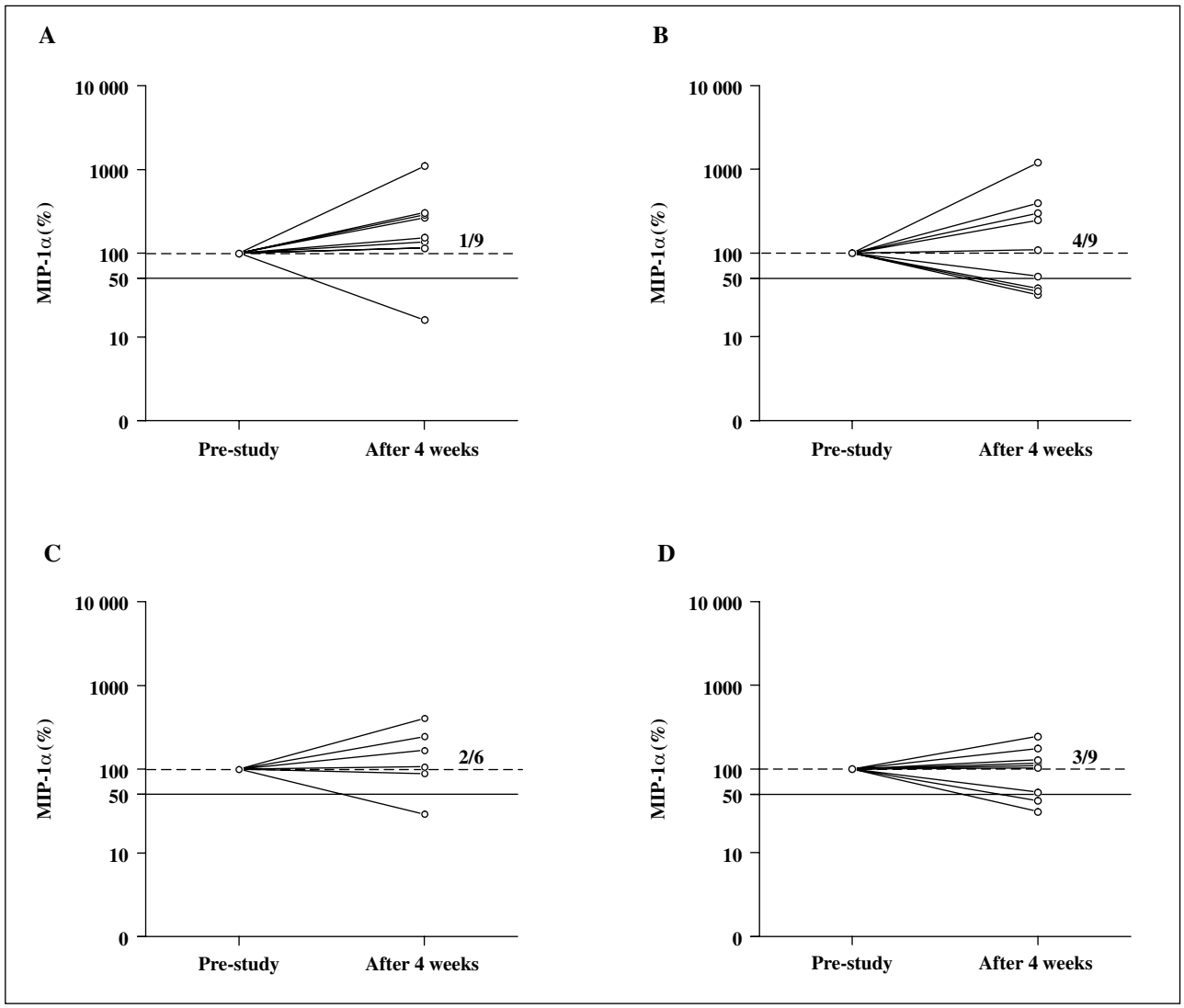

Figure 4

IL-18/LPS-stimulated whole blood MIP-1α production after four weeks of placebo or IL-10. MIP-1α was measured in the IL-18 and LPS-stimulated samples described in *figure 1*. The fractions indicate the number of subjects in each group exhibiting a decrease in IL-1β production (< 100%, numerator) compared to the total number of subjects in each group (denominator). MIP-1α concentrations were calculated per million monocytes and the data calculated for each study group, including (**A**) placebo, or IL-10 given at (**B**) 1 µg/kg/day, (**C**) 4 µg/kg/day, or (**D**) 8 µg/kg TIW.

Since IL-10 possesses anti-inflammatory effects *in vitro* and *in vivo* [12, 21, 26, 37], we anticipated IL-10 inhibition of pro-inflammatory cytokines. Consistent with previous experiments, IL-10 suppressed production of the pro-inflammatory cytokines IFNγ and IL-1β in stimulated whole blood cultures. Compared to blood obtained prior to the start of study drug, blood obtained after four weeks of therapy in the 8 µg/kg TIW IL-10 group revealed non-significant reductions in IL-18/LPS-induced IFNγ and IL-1β production. In the placebo group, five out of nine (66%) subjects exhibited decreased IFNγ production after four weeks of study compared to IFNγ measured just prior to study. In contrast, six out of nine (67%), five out of six (83%) and seven out of nine (78%) patients receiving 1, 4 or 8 µg/kg IL-10, respectively, showed decreased IFNγ after four weeks of study *(figure 1)*. For IL-1β, 78% of patients in the group receiving the highest IL-10 dose demonstrated reduced cytokine production at the conclusion of the study compared to five out of nine patients (56%) in the placebo group *(figure 2)*. No notable differences in TNFα concentrations before the first and last treatments were observed in the placebo or any IL-10 dose groups *(figure 3)*. However, circulating levels of the naturally occurring, soluble TNF receptor p55 dose-dependently increased after four weeks of IL-10 injections compared to levels before IL-10 treatment *(figure 5)*. In patients receiving placebo, there was no increase.

Since β-chemokines are natural ligands for the CCR5 HIV-1 co-receptor, modulation of β-chemokines in HIV-1-infected persons receiving IL-10 is of interest [10]. While 11% of patients receiving placebo exhibited decreased IL-18/LPS-induced MIP-1α after four weeks *(figure 4)*, 37.5% of all subjects receiving IL-10 experienced decreases. Although subjects receiving IL-10 had greater overall reduction in MIP-1α compared to patients in the placebo group, inhibition was not IL-10 dose-dependent and the proportion of IL-10 subjects with decreased MIP-1α production (37.5%) was relatively small, suggesting a lack of effect of IL-10 on β-chemokine production.

During prolonged exogenous administration of a biological response modifier, decreased responsiveness may

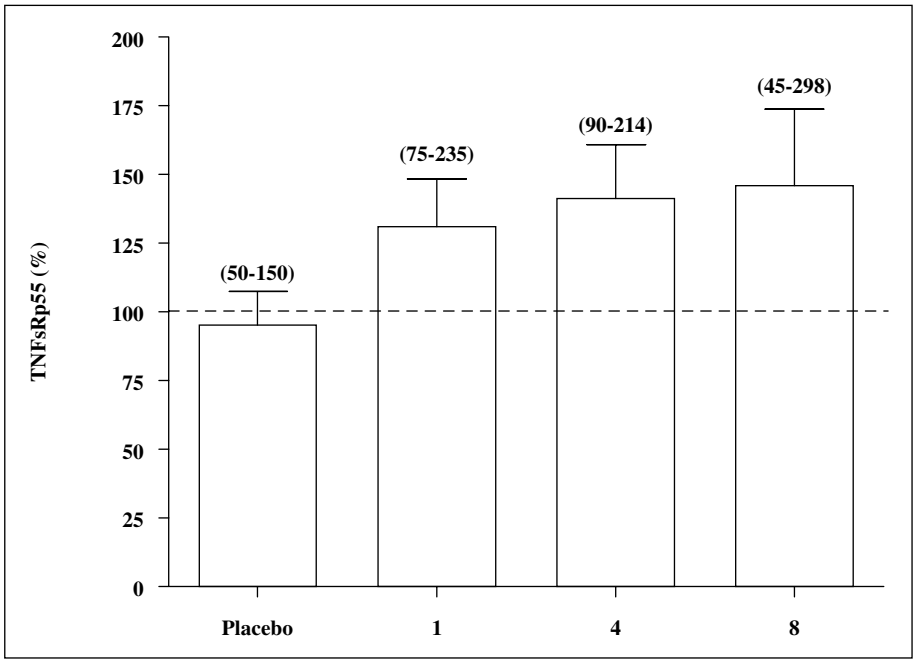

Figure 5
Effect of IL-10 on whole blood levels of TNFsRp55. TNFsRp55 levels measured in whole blood prior to initiation of study drug (pre-study) were set at 100%. The mean percentage production (± SEM) at four weeks compared to pre-study is shown for each study group. The vertical axis shows percentage TNFsRp55 levels for patient groups administered placebo (placebo) or IL-10 at 1 µg/kg/day, 4 µg/kg/day or 8 µg/kg TIW (depicted on the horizontal axis as placebo, 1, 4 and 8, respectively). The numbers in parentheses above each bar indicate the range of percentage TNFsRp55 levels obtained after four weeks of IL-10.

emerge (tachyphylaxis). Possible mechanisms include neutralization of the response modifier by antibody production, generation of soluble receptors, induction of specific antagonists, decreased expression of cell surface receptors, or reduced signal transduction following engagement of cell surface receptors.

The IL-10 effect has been examined over a one-day period in *ex vivo* studies in healthy, non-HIV-1-infected

Table 1
Patient characteristics

	Placebo (n = 9)	**IL-10 at 1 µg/kg/d (n = 9)**	**IL-10 at 4 µg/kg/d (n = 6)**	**IL-10 at 8 µg/kg TIW (n = 9)**
Age				
mean	42.7	36.0	33.5	42.4
(range)	(32-54)	(25-50)	(22-44)	(28-54)
SEM	2.09	2.75	3.16	2.46
Sex (M/F)	8/1	7/2	4/2	6/3
Race				
Caucasian	4	8	2	7
Black	4		4	2
Hispanic	1			
Native American		1		
Weight (kg)				
mean	70.9	74.8	68.7	77.0
(range)	(61.4-85.0)	(64.0-94.0)	(52.0-83.9)	(59.5-100.0)
SEM	4.3	3.6	5.1	4.6
CD4 (cells/mm^3)				
mean	377.0	410.8	386.4	317.6
(range)	(175-814)	(173-619)	(142-808)	(75-485)
SEM	57.1	50.4	78.5	40.2
HIV-1 RNA viral load ($\log_{10}$ RNA copies/mL plasma)	4.06	4.10	4.15	4.01

Table 2
Summary of cytokine data obtained in whole blood stimulated for 24 hr with combined IL-18 and LPS. The data are shown for each study group

	Placebo	IL-10 at 1 µg/kg/day	IL-10 at 4 µg/kg/day	IL-10 at 8 µg/kg TIW
IFNγ	5/9	6/9	5/6	7/9
IL-1β	5/9	3/9	3/6	7/9
TNFα	6/9	4/9	3/6	5/9
MIP-1α	1/9	4/9	2/6	3/9

(Number of subjects with reduced cytokine/number of subjects in each study group).

volunteers [38]. In this prior study, a single intravenous IL-10 injection suppressed *ex vivo* IFNγ induction in peripheral blood mononuclear cells three and six hours post-IL-10 injection, but not 24 hours post-IL-10 injection. In the present study, compared to the placebo group, reductions in stimulated (IL-18 and LPS) whole blood IFNγ, IL-1β and TNFα were noted three hours following the first as well as the last IL-10 administrations (data not shown). At the six-hour time point, corresponding reductions in cytokine synthesis after the first and last IL-10 administration were also observed (also not shown). The reductions in cytokines three and six hours post-IL-10 injected following the first dose of IL-10 are well-described, but this is the first report that after four weeks of IL-10, the three and six-hour reductions were again observed. These observations suggest a durable, suppressive effect of IL-10 on cytokine induction over four weeks of administration. The absence of tachyphylaxis to IL-10 administration supports the concept that the inability of IL-10 to reduce viral load during the study is not due to loss of IL-10 activity.

This study demonstrated that although multiple doses of recombinant human IL-10 did not affect HIV-1 viral load, the pro-inflammatory cytokines IFNγ and IL-1β were reduced, while circulating levels of TNFsRp55 were simultaneously increased. While four weeks of IL-10 administered to HIV-1-infected subjects resulted in trends suggesting a net reduction of pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, none of these results achieved statistical significance. The lack of statistical significance was likely due to patient heterogeneity and/or small sample size. Also, although *ex vivo* investigation is an attractive method with which to study the effects of a therapeutic intervention on cytokine production, *ex vivo* analysis may reflect *in vivo* cytokine production inaccurately.

The failure of IL-10 to reduce HIV-1 viral load in this study does not necessarily contradict the hypothesis that cytokine modulation may be antiretroviral. Since statistically significant IL-10 effects on HIV-1-modulating cytokines were not obtained in these patients, the data are insufficient to refute the hypothesis that cytokine modulation affects HIV-1 production *in vivo*. The considerable literature demonstrating the effects of cytokines on HIV-1 *in vitro* suggests that further study of cytokine modulation as a means of affecting HIV-1 production in patients should continue. Study designs should carefully consider study population, power analysis and the rationale for assessing particular cytokines.

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REFERENCES

1. Murphy EL, Collier AC, Kalish LA, Assmann SF, Para MF, Flanigan TP, Kumar PN, Mintz L, Wallach FR, Nemo GJ. Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. *Ann Intern Med* 2001; 135: 17.
2. Young B, Johnson S, Bahktiari M, Shugarts D, Young RK, Allen M. Ramey RRn, Kuritzkes DR. Resistance mutations in protease and reverse transcriptase genes of human immunodeficiency virus type 1 isolates from patients with combination antiretroviral therapy failure. *J Infect Dis* 1998; 178: 1497.
3. Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron Jr. JJ, Feinberg JE, Balfour Jr. HH, Deyton LR, Chodakewitz JA, Fischl MA. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* 1997; 337: 725.
4. Hirsch MS, Conway B, D'Aquila RT, Johnson VA, Brun-Vezinet F, Clotet B, Demeter LM, Hammer SM, Jacobsen DM, Kuritzkes DR, Loveday C, Mellors JW, Vella S, Richman DD. Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. International AIDS Society--USA Panel. *JAMA* 1998; 279: 1984.
5. 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 USA* 1986; 83: 8734.
6. Kornbluth RS, Oh PS, Munis JR, Cleveland PH, Richman DD. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. *J Exp Med* 1989; 169: 1137.
7. Granowitz EV, Saget BM, Wang MZ, Dinarello CA, Skolnik PR. Interleukin 1 induces HIV-1 expression in chronically infected U1 cells: blockade by interleukin 1 receptor antagonist and tumor necrosis factor binding protein type 1. *Mol Med* 1995; 1: 667.
8. Poli G, Kinter AL, Fauci AS. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc Natl Acad Sci USA* 1994; 91: 108.
9. Choi HJ, Dinarello CA, Shapiro L. Interleukin-18 inhibits human immunodeficiency virus type 1 production in peripheral blood mononuclear cells. *J Infect Dis* 2001; 184: 560.

10. Oravecz T, Pall M, Norcross MA. Beta-chemokine inhibition of monocytotropic HIV-1 infection. Interference with a postbinding fusion step. *J Immunol* 1996; 157: 1329.

11. Howard M, O'Garra A. Biological properties of interleukin 10. *Immunol Today* 1992; 13: 198.

12. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; 147: 3815.

13. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993; 75: 263.

14. Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, Thompson-Snipes L, Leach MW, Rennick D. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* 1996; 98: 1010.

15. Mellors JW, Griffith BP, Ortiz MA, Landry ML, Ryan JL. Tumor necrosis factor-alpha/cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages. *J Infect Dis* 1991; 163: 78.

16. Poli G, Kinter AL, Vicenzi E, Fauci AS. Cytokine regulation of acute and chronic HIV infection in vitro: from cell lines to primary mononuclear cells. *Res Immunol* 1994; 145: 578.

17. Rizzardi GP, Barcellini W, Tambussi G, Lillo F, Malnati M, Perrin L, Lazzarin A. Plasma levels of soluble CD30, tumour necrosis factor (TNF)-alpha and TNF receptors during primary HIV-1 infection: correlation with HIV-1 RNA and the clinical outcome. *AIDS* 1996; 10: F45.

18. Scott-Algara D, Vuillier F, Marasescu M, de Saint Martin J, Dighiero G. Serum levels of IL-2, IL-1 alpha, TNF-alpha, and soluble receptor of IL-2 in HIV-1-infected patients. *AIDS Res Hum Retroviruses* 1991; 7: 381.

19. 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.

20. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; 146: 3444.

21. Weissman D, Ostrowski M, Daucher JA, Gantt K, Blauvelt A, Altman D, Shen L, Ehler L, Hoxie J, Grint P, Katz SI, Fauci AS. Interleukin-10 Decreases HIV Plasma Viral Load: Results of a Phase I Clinical Trial. *4th Conference on Retroviruses and Opportunistic Infections* 1997.

22. Montaner LJ, Griffin P, Gordon S. Interleukin-10 inhibits initial reverse transcription of human immunodeficiency virus type 1 and mediates a virostatic latent state in primary blood-derived human macrophages in vitro. *J Gen Virol* 1994; 75(Pt 12): 3393.

23. Naif HM, Chang J, Ho-Shon M, Li S, Cunningham AL. Inhibition of human immunodeficiency virus replication in differentiating monocytes by interleukin 10 occurs in parallel with inhibition of cellular RNA expression. *AIDS Res Hum Retroviruses* 1996; 12: 1237.

24. Saville MW, Taga K, Foli A, Broder S, Tosato G, Yarchoan R. Interleukin-10 suppresses human immunodeficiency virus-1 replication in vitro in cells of the monocyte/macrophage lineage. *Blood* 1994; 83: 3591.

25. Angel JB, Jacobson MA, Skolnik PR, Giordano M, Shapiro L, LeBeaut A, Greaves W, Fuchs AC. A multicenter, randomized, double-blind, placebo-controlled trial of recombinant human interleukin-10 in HIV-infected subjects. *AIDS* 2000; 14: 2503.

26. Chernoff AE, Granowitz EV, Shapiro L, Vannier E, Lonnemann G, Angel JB, Kennedy JS, Rabson AR, Wolff SM, Dinarello CA. A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J Immunol* 1995; 154: 5492.

27. Muhl H, Dinarello CA. Macrophage inflammatory protein-1 alpha production in lipopolysaccharide-stimulated human adherent blood mononuclear cells is inhibited by the nitric oxide synthase inhibitor N(G)-monomethyl-L-arginine. *J Immunol* 1997; 159: 5063.

28. Puren AJ, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J Clin Invest* 1998; 101: 711.

29. Shapiro L, Clark BD, Orencole SF, Poutsiaka DD, Granowitz EV, Dinarello CA. Detection of tumor necrosis factor soluble receptor p55 in blood samples from healthy and endotoxemic humans. *J Infect Dis* 1993; 167: 1344.

30. Ito M, Baba M, Sato A, Hirabayashi K, Tanabe F, Shigeta S, De Clercq E. Tumor necrosis factor enhances replication of human immunodeficiency virus (HIV) in vitro. *Biochem Biophys Res Commun* 1989; 158: 307.

31. Weissman D, Poli G, Fauci AS. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Res Hum Retroviruses* 1994; 10: 1199.

32. Angel JB, Saget BM, Wang MZ, Wang A, Dinarello CA, Skolnik PR. Interleukin-10 enhances human immunodeficiency virus type 1 expression in a chronically infected promonocytic cell line (U1) by a tumor necrosis factor alpha-independent mechanism. *J Interferon Cytokine Res* 1995; 15: 575.

33. Barcellini W, Rizzardi GP, Marriott JB, Fain C, Shattock RJ, Meroni PL, Poli G, Dalgleish AG. Interleukin-10-induced HIV-1 expression is mediated by induction of both membrane-bound tumour necrosis factor (TNF)-alpha and TNF receptor type 1 in a promonocytic cell line. *AIDS* 1996; 10: 835.

34. Finnegan A, Roebuck KA, Nakai BE, Gu DS, Rabbi MF, Song S, Landay AL. IL-10 cooperates with TNF-alpha to activate HIV-1 from latently and acutely infected cells of monocyte/macrophage lineage. *J Immunol* 1996; 156: 841.

35. Weissman D, Poli G, Fauci AS. IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995; 9: 442.

36. Puren AJ, Razeghi P, Fantuzzi G, Dinarello CA. Interleukin-18 enhances lipopolysaccharide-induced interferon-gamma production in human whole blood cultures. *J Infect Dis* 1998; 178: 1830.

37. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; 174: 1209.

38. Fuchs AC, Granowitz EV, Shapiro L, Vannier E, Lonnemann G, Angel JB, Kennedy JS, Rabson AR, Radwanski E, Affrime MB, Cutler DL, Grint PC, Dinarello CA. Clinical, hematologic, and immunologic effects of interleukin-10 in humans. *J Clin Immunol* 1996; 16: 291.