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Peripheral Blood Cell-Specific Cytokines in Persons with Untreated HIV Infection in Malawi, Africa

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ABSTRACT

Human immunodeficiency virus (HIV) infection is the primary cause of morbidity and mortality in Malawi, Africa, because of its many effects on the immune system. Immune cells communicate through cytokines; therefore, we examined the relationships between HIV serostatus and cell-specific cytokine production for 40 asymptomatic, employed adults and 312 acutely ill, hospitalized patients in Malawi. We also measured the plasma HIV-1 RNA levels of 13 asymptomatic persons and 83 patients found to be HIV^+. We incubated peripheral whole blood with brefeldin-A ± phorbol 12-myristate 13-acetate and ionomycin and then permeabilized, fixed, fluorescently stained, and examined the mononuclear cells with four-color, six-parameter flow cytometry. The percentage of lymphocytes expressing CD4 did not differ significantly between the HIV^+ and HIV^- healthy adults (medians, 35.2 vs. 40.8%, respectively), but a wide array of cytokine parameters were lower in the HIV^+ than in the HIV^- asymptomatic persons, for example, median percentages of T cells producing induced interleukin 2 (IL-2) (8.7 vs. 16.5%, respectively) and spontaneously producing IL-6 (0.7 vs. 11.0%, respectively). Also, four T cell parameters reflecting type 2-to-type 1 cytokine balances (T2/T1) were higher in the HIV^+, versus HIV^-, asymptomatic persons. Unlike the healthy adults, for patients with mycobacteremia/fungemia or malaria, the HIV^+ patients had higher median percentages of T cells and $CD8^+$ T cells producing induced interferon γ than did the HIV^- patients. For both asymptomatic and acutely ill persons, HIV-1 plasma levels were positively correlated with T2/T1 parameters. Cell-specific cytokine effects of HIV infection may precede measurable effects on CD4 expression. Cytokine therapies, even beyond periodic administration of IL-2, may improve the responses of HIV-infected persons to both HIV and coinfections.

INTRODUCTION

In the United States, most persons known to be infected with human immunodeficiency virus (HIV) are able to receive highly active antiretroviral therapy (HAART), if such therapy is indicated. The economic situation in most African countries, however, does not permit similar treatment of HIV-infected individuals. Thus, although in the United States HIV infection is now in many ways a chronic disorder, in Africa HIV is the primary cause of premature mortality, through its many effects on the immune system and the resulting opportunistic infections occurring secondary to these effects. We studied immune determinants of bloodstream infections in hospitalized Malawians, many of whom were infected with HIV.[1,2] Herein, we sought to determine the specific contribution of HIV infection to cell-specific cytokine production by comparing peripheral blood cells of asymptomatic HIV-infected persons, acutely ill HIV-infected patients, and non-HIV-infected persons.

Cytokines represent a major means of communication between immune cells. Cytokines and chemokines interact with HIV in complex ways.[3,4] On the most basic level, certain

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chemokine receptors act as cofactors for viral entry into $CD4^+$ cells. In addition, HIV affects the balance between pro- and antiinflammatory cytokines and between type 1 and type 2 cytokines. Cytokines, in turn, affect both HIV viral replication and the ability of the immune system to control the virus. They have been used therapeutically to treat HIV infection, with some success reported with interleukin 2 (IL-2) therapy.[5,6] Much of what is known or postulated about the roles of cytokines in persons with untreated HIV infection is based on *in vitro* studies or non-cell-specific assays (e.g., those measuring serum or supernatant cytokines or cellular cytokine-related RNA of peripheral blood or bronchial lavage cells).

Cell-specific cytokine assessment using flow cytometry became practically feasible only after the implementation of HAART in the United States. Thus, although research concerning cell-specific cytokines is proceeding rapidly, most studies focus on the relationship between cell-specific cytokine profiles and the response to HAART.[7,8] We have adapted intracellular cytokine assessment technology for use in developing countries and assessed cell-specific cytokines in relation to bloodstream infections, malaria parasitemia, iron deficiency, and vitamin A deficiency.[1,2,9–11] In a study of U.S. HIV-infected persons, we found that in the first weeks of HAART, cytokine changes preceded changes in $CD4^+$ T cells.[7] We hypothesized that, similarly, in healthy HIV-infected persons, cytokine alterations might exist before declines in $CD4^+$ T cell percentages. We also wanted to assess the effects of intercurrent acute opportunistic infections on HIV-infected persons cytokine profiles. In particular, we postulated that asymptomatic HIV infection would be associated with a relative type 2 cytokine dominance and/or changes in the production of the $CD4^+$ T cell cytokine IL-2 even when $CD4^+$ cell changes were not noted. Further, we postulated that the occurrence of acute opportunistic infections in patients with more advanced HIV infection might cause the immune profile to revert to more of a type 1 profile. We therefore evaluated the relationships between the cell-specific cytokines and (1) the presence or absence of HIV infection, (2) plasma HIV-1 RNA levels in infected persons, and (3) the presence or absence of coexistent acute coinfections. Our goal was to clarify the interaction between the immune cell-specific cytokine network and untreated HIV infection. It has been suggested that cytokine-modulatory therapies may prove useful in controlling HIV infection.[3,12] If so, our findings may be valuable in determining new and, perhaps, less expensive, less toxic, and more effective additions to HIV therapy.

PARTICIPANTS AND METHODS

Patients

During three periods in 1997 and 1998, we determined the HIV serology of 165 febrile (oral temperature ≥38°C) adult (≥13 years old) and 147 pediatric (<13 years old) patients who had been admitted to the Lilongwe Central Hospital in Malawi, Africa, and had enrolled in a study of the immune determinants of infectious diseases[1,2] (Table 1). All children admitted to the hospital during the enrollment period were included in the study, irrespective of their temperature at admission, because infected children often do not present with fever. Selection for the study was random and participants were comparable to all hospitalized patients.[2] For each patient at admission, blood samples, epidemiologic data, and a medical history were obtained

TABLE 1. CHARACTERISTICS AND HIV-1 PLASMA TITERS OF PATIENT PARTICIPANTS,[a] BY HIV STATUS

Characteristic[b]	*HIV⁺* (n = 156)	*HIV⁻* (n = 156)	p *Value*[c]
Male (%)	43	55	0.041
Age (years)			
Mean	24.7	10.2	
Median	28.0	4.0	<0.001
Range	0.1–60.0	0.1–61.0	
Died (%)	18	5	0.001
Blood culture positive[d] (%)	29	13	0.001
Number mycobacteremic[d]	11	0	<0.001
Number fungemic[d]	4	1	NS[e]
Bacteria positive[d] (%)	20	13	NS
Malaria smear positive[d] (%)	11	14	NS
HIV-1 plasma RNA levels (copies/ml) (n = 82)[f]			
Median	483,388	NA[e]	
Range	ND[e]–11,037,736		

[a]Demographic information was not obtained for healthy adult participants; therefore they are not included.

[b]Sex was not recorded for four persons, and exact age was not recorded for six persons. Malaria smears were not done for four persons. Mortality status was not recorded for 45 persons and was unknown for those who were either still hospitalized at the end of the study (n = 18) or who left the hospital against medical advice (n = 12).

[c]Fisher exact or Wilcoxon rank sum test.

[d]Includes three persons with multiple organisms in their bloodstream.

[e]Not significant (NS), nonapplicable (NA), nondeductable (ND).

[f]For the 13 asymptomatic adult control persons, the median viral load was nondetectable (range, ND–1,016,178 copies/mm^3).

and a physical examination was performed by one of the investigators. Mortality status was based on the status of the patient at the close of the study period. It was not known for 18 patients who remained hospitalized at the conclusion of the study and for 12 patients who left the hospital against medical advice. Mortality status was not recorded for an additional 45 persons.

Healthy adult participants

We enrolled 40 actively employed adults, all healthy by self-report, 16 of whom were found to be HIV seropositive (HIV^-) at enrollment testing. These individuals were completely asymptomatic. Children were not included in this group for ethical reasons. Epidemiologic data were not obtained for these participants.

The study protocol was approved by the institutional review boards of the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and the Malawian Health Sciences Research Committee; informed consent was obtained from all participants or their guardians. As in most developing nations, HIV-infected persons in this study were neither receiving antiviral therapies nor being monitored for changes in $CD4^+$ cell count or HIV plasma RNA level.

Laboratory procedures

Viral/microbiologic. HIV antibody testing was done at study enrollment, using enzyme-linked immunosorbent assay (ELISA) test kits (Murex Diagnostics, Norcross, GA). HIV-2 has not been reported in Malawi. During the last two phases of the study, plasma HIV-1 RNA levels were determined for 48 HIV^+ adult patients, 35 HIV^+ children, and 13 HIV^-, asymptomatic, employed adult participants (Roche Monitor version 1.5; Roche Diagnostics, Indianapolis, IN). Of the 21 children <1.3 years old, 13 were HIV^+; all but 3 had detectable plasma HIV-1 RNA levels, indicating infection. Blood was cultured for bacteria, fungi, and mycobacteria as described previously.[13] Plates were incubated at 35°C and examined daily for 7 days. Of the patients, 97 adults (69 HIV^+ and 28 HIV^-) and 114 children (28 HIV^+ and 86 HIV^-) had negative blood cultures. The blood cultures of eight HIV^+ adults grew only mycobacteria and those of four adults (three HIV^+ and one HIV^-) grew only fungi. Only bacteria grew from the blood cultures of 31 adults (25 HIV^+ and 6 HIV^-) and 22 children (7 HIV^+ and 15 HIV^-). Thick and thin malaria smears were done at admission and read by a single, highly experienced individual not aware of the patient's clinical status or findings. A smear was considered positive if any *Plasmodium* (*flaciparum*) asexual parasites were seen on examination of peripheral blood smears (thick films and the tails of thin films).[2] Smears from 25 adults (14 HIV^+ and 11 HIV^-) and 13 children (2 HIV^+ and 11 HIV^-) were positive for *P. falciparum*. An additional three patients were positive for mycobacteria and fungi, malaria, or bacteria.

Stimulation of cytokine production. Blood was prepared for cytokine stimulation as described previously. Blood was either stimulated for 5 hr at 37°C with phorbol 12-myristate 13-acetate (PMA, 200 ng/ml; Sigma, St. Louis, MO) and ionomycin (4 μg/ml) (Sigma) in the presence of brefeldin-A (40 μg/ml; Sigma) and RPMI 1640 with L-glutamine (induced or stimulated cytokine expression) or retained in identical medium without PMA and ionomycin but with brefeldin-A (spontaneous or unstimulated cytokine expression). No serum was added to the cultures. After being washed, the red blood cells were lysed with ammonium chloride solution and lymphocytes were permeabilized and fixed with Ortho Permeafix (Ortho Diagnostic Systems, Raritan, NJ). After processing, samples were shipped at 4–8°C to the CDC for further analysis. In analyses, the percentage of lymphocytes expressing CD4 (% CD4) was based on data for unstimulated cells.

Flow cytometric reagents. The surface antigens assessed in this study were those shown in our laboratory to be stable with this permeabilization/fixation protocol, that is, using these techniques, we had comparable results for the surface-related antigens when staining was done either pre- or postpermeabilization. Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, peridinin chlorophyll protein (PerCP)-conjugated, or allphycocyanin (APC)-conjugated murine monoclonal antibodies were obtained from the following sources: (1) Becton Dickinson Immunocytometry Systems/PharMingen (BD/PMG; San Jose, CA) (CD8–FITC and –PE [clone SK1], CD3–PerCP and –APC [clone SK7], CD4–APC [clone SK3], CD45–FITC [clone 2D1], CD19–APC [clone SJ25C1], CD14–PE [clone MφP9], CD16–PE [clone B73.1], and CD56 [clone MY31], IL-4–PE [clone 8D4-8], IL8–PE [clone G265-8], and IL-10–PE [clone JES3-9D7]), (2) Research and Diagnostics (Minneapolis, MN) (IL-6–PE [clone 1927.311]), and (3) Immune Source (Reno, NV) (CD8–APC [clone KL-12], IL-2–APC [R-56.2], tumor necrosis factor α [TNF-α]–FITC [clone DTX.34], and interferon γ (IFN-γ)–APC [clone 13.TR]). Isotype controls were obtained from BD/PMG.

Flow cytometry. All staining was done at room temperature for 30 min in the dark after permeabilization, fixation, and shipment to the CDC. Staining was followed by a buffered saline wash. Four-color cytofluorometry was performed with a FACSort or FACSCalibur cytometer and CellQuest software (BD/PMG). Between 50,000 and 80,000 ungated events were collected from each tube in the panel.

Analytic techniques

For each participant, analyses were done for various combinations of all lymphocytes. $CD3^+$ (T) lymphocytes, $CD3^+CD8^+$ lymphocytes, $CD3^+CD8^-$ lymphocytes, $CD3^+CD16/56^+$ lymphocytes (natural T cells [NT]), $CD3^-CD16/56^+$ lymphocytes (natural killer cells [NK]), $CD19^+$ (B) lymphocytes, and monocytes, depending on the tube configuration.[14] The relative balances between type 2 and type 1 cytokines (T2/T1) were represented by the ratios of the percentage of $CD3^+$ lymphocytes producing IL-10 or IL-4 to the percentage producing each of the other cytokines. Note that this was not limited to $CD4^+$ T cells, because other T cells also produce most of the cytokines being assessed. We calculated similar ratios for monocyte production of IL-10 and IL-6, IL-8, and TNF-α.

IL-10 and IL-4 were both assessed and represent anti-inflammatory, regulatory, and type 2 (humoral) cytokines. They induce antibody maturation and production, as well as the pro-

duction of other type 2 cytokines (e.g., IL-3 or IL-6), and IL-10 inhibits the production of type 1 cytokines (e.g., IFN-γ). We refer to TNF-α, IL-6, and IL-8 as proinflammatory cytokines. IL-2 and IFN-γ represented type 1 cytokines, which induce and are involved in cellular immunity and are essential for immunity to intracellular organisms.

Statistical techniques

Comparisons of continuous variables between HIV^+ and HIV^- participants were made by Wilcoxon rank sum tests and logistic regression analyses. Categorical data were compared by Fisher exact or χ^2 tests. For all analyses of HIV-1 viral titers, nondetectable levels were set at half the detection limit. Comparisons of continuous variables to one another were made by determining Pearson correlation coefficients (r_p) and linear regression analyses. Univariate analyses were done on three participant subgroups: healthy adults, adult patients, and pediatric patients. Univariate analyses were also done for the following infection groups within each of the two patient age groups: those with bacterial infections, those with malaria parasitemia, and those with negative blood cultures and negative malaria smears. If a variable was statistically significant for any of these subgroups, multivariate analyses were done for each of the three subgroups and, when numbers would allow, to control for the effect of acute intercurrent infections, for the patient subgroups listed above. Numbers did not permit separate multivariate analyses of those with mycobacteremia/fungemia. For logistic and linear regression analyses, the statistics provided here are for the final, reduced models containing only the significant variables. In the initial models involving patients, the following independent variables were included: sex, age, type of infection, and % CD4. For healthy adult participants, the initial models included % CD4 and the variable of interest. The significance level was set at $p < 0.05$; data not provided herein did not reach that level of significance on any type of analysis.

RESULTS

Clinical and demographic characteristics

Clinical and demographic results (Table 1) were not recorded for the asymptomatic, healthy participants. Compared with the HIV^- patients, the HIV^+ patients were significantly older and included a slightly lower proportion of males (Table 1). As expected, acute mortality was higher and bloodstream infections were more common in the HIV^+ patients. All patients with isolated mycobacteremia were HIV infected.[1] Rates of malaria parasitemia and bacteremia did not vary significantly by HIV serostatus.[2] HIV-1 plasma levels did not differ significantly between adult and pediatric patients (medians, 287,091 vs.

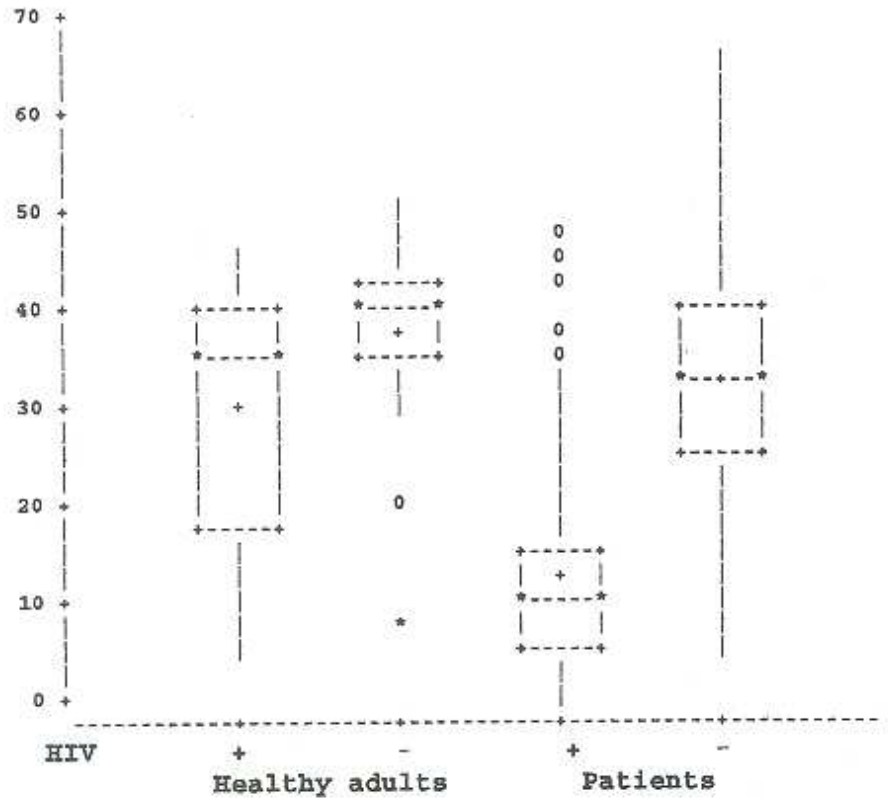


FIG. 1. Percentage of lymphocytes expressing CD4, by HIV serostatus and participant category. Boxes include medians (lines) and values between the 25th and 75th percentiles for the following participant groups: HIV-seropositive (HIV^+) healthy adults ($n = 16$), HIV-seronegative (HIV^-) healthy adults ($n = 24$), HIV^+ patients ($n = 154$), and HIV^- patients ($n = 156$). Lines extend to ±1.5 times the interquartile ranges; outliers within 3 interquartiles are presented as circles, outliers beyond 3 interquartiles are presented as circles, and the mean is presented as a plus sign.

667,954 copies/ml, respectively), but did differ between patients and HIV$^+$ asymptomatic participants (medians, 483,388 copies/ml vs. nondetectable, respectively, $p < 0.001$).

Immune parameters related to the presence or absence of HIV infection

Asymptomatic, actively employed adult participants. Although tending to be lower, the % CD4 did not differ significantly between HIV$^+$ and HIV$^-$, healthy participants (Fig. 1); however, a wide array of cytokine parameters did vary by HIV serostatus (Table 2). In particular, differences involved both spontaneous and induced cytokine production by both lymphocytes and monocytes. They did not involve any parameter related to TNF-α. Except for spontaneous production of IL-8 by lymphocytes, in every instance the median percentage of cells producing a given cytokine was lower among the HIV$^+$ compared with the HIV$^-$, healthy adult participants (Fig. 2A–D). Also among this group, the median ratios of the percentage of T cells making IL-4 to the percentage making IL-2 or IL-6 were greater in the HIV$^+$ healthy adult participants, compared with the HIV$^-$ healthy adult participants, as was the median ratio of T cells making IL-10 to those making IFN-γ (Table 2).

Patient participants. Unlike for healthy adult participants, for both adult and pediatric patients the % CD4 strongly differed between HIV$^+$ and HIV$^-$ persons (Table 3) (Fig. 1). In multivariate analyses in which HIV serostatus was the dependent variable, the % CD4 was usually the only significant immune variable. The only cellular cytokine findings that retained significance in multivariate analyses were the percentages of all lymphocytes, T cells, and CD8$^-$ T cells making induced IFN-γ. Unlike for the healthy adult participants, for patients, the median percentages of these overlapping cell types producing IFN-γ with induction were higher for HIV$^+$ patients than for HIV$^-$ persons (Table 3 and Fig. 2B).

These findings, the opposite of those of healthy adult participants, were not present in all patient subgroups. Specifically, these differences were present in those with mycobacterial/fungal infections; for the 14 HIV$^+$ persons, a median of 56.0% of T cells made induced IFN-γ (range, 6.3–81.1%) compared with 6.6% of the T cells of the single, HIV-negative, fungemic person. They were also present in those with negative blood cultures/malaria smears (medians: HIV$^+$, 22.7% vs. HIV$^-$, 11.7%) and those with malaria parasitemia (medians, 24.0 and 12.3%, respectively). They were not present in those with bacteremia (28.0 and 25.5%, respectively).

In further analyses of patient subgroups, the variables that were significant for healthy adult participants (Table 2) had similar trends in the bacteremic and in the blood culture-negative/malaria smear-negative patients, that is, for each of these two groups, for the variables outlined in Table 2, the HIV$^+$ patients had lower median percentages and higher ratios than did

TABLE 2. IMMUNE FINDINGS BY HIV STATUS OF HEALTHY ADULT PARTICIPANTS, MALAWI, AFRICA

	HIV$^+$ (n = 16)	*HIV$^-$ (n = 24)*	p *Value*[a]
Median percentage of:			
Lymphocytes producing induced IL-2	5.6	12.7	0.002
T cells producing induced IL-2	8.7	16.5	0.007
Lymphocytes producing IL-6 spontaneously	0.4	12.5	0.006
T cells producing IL-6 spontaneously	0.7	11.0	0.003
CD8-negative T cells producing:			
Induced IL-6	0.1	4.0	<0.001
IL-6 spontaneously	0.2	9.5	<0.001
Monocytes producing:			
Induced IL-6	0.2	9.6	0.006
IL-6 spontaneously	0.3	0.8	0.009
Lymphocytes producing IL-8 spontaneously	3.5	1.4	0.005
Monocytes producing induced IL-8	37.8	60.1	0.007
Lymphocytes producing IFN-γ	16.1	27.2	0.018
T cells producing IFN-γ	26.6	32.1	0.047
Lymphocytes expressing CD4	35.2	40.8	NA[b]
Median ratios of the percentage of T cells spontaneously producing:			
IL-4 to:			
The percentage producing IL-2 spontaneously	3.00	1.69	0.007
The percentage producing IL-6 spontaneously	1.29	0.12	0.003
IL-10 to:			
The percentage producing IFN-γ spontaneously	3.83	1.67	0.008

[a]The p value is for the contribution of the variable to a logistic regression analysis that also included the percentage of resting lymphocytes expressing CD4 as an additional independent variable and where HIV serostatus was the dependent variable. For all but two variables, includes only variables for which $p < 0.01$ and the significance of the CD4 variable to the model was >0.05. For the two IFNγ variables, the contribution of the CD4 variable was associated with a p value of 0.01.

[b]Nonapplicable (NA).

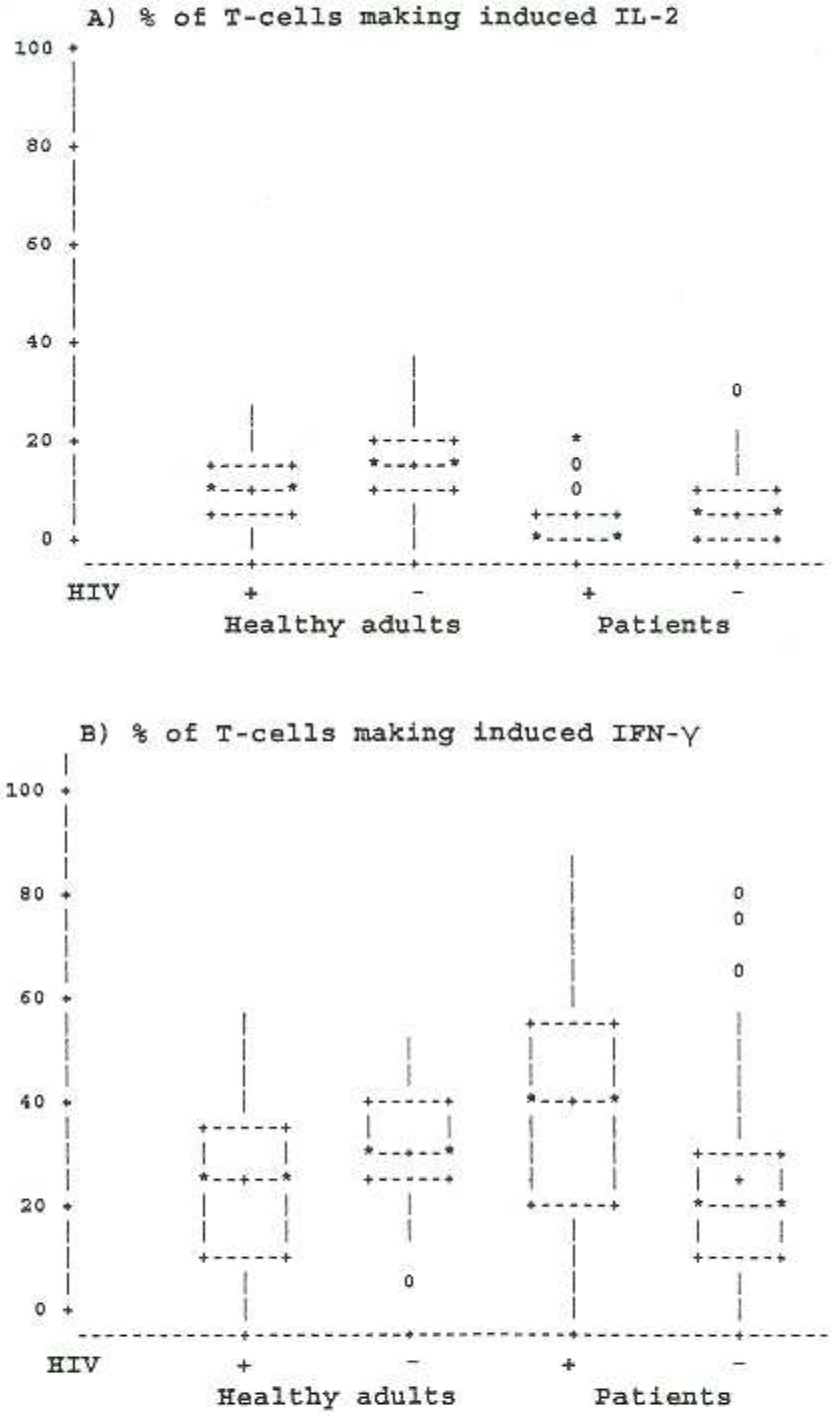
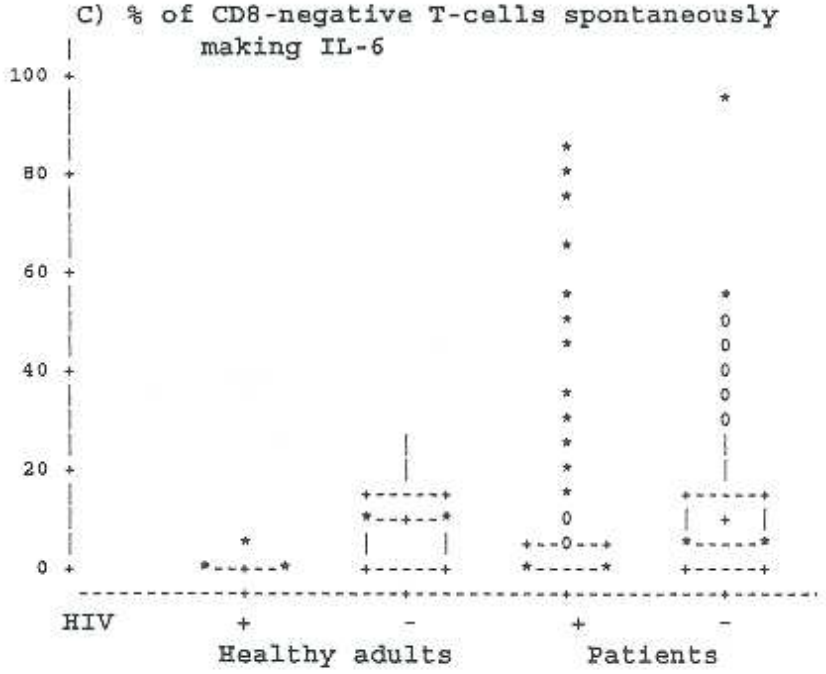


FIG. 2. (A–D) Representative cytokine parameters differing significantly between HIV-seropositive (HIV^+) and HIV-seronegative (HIV^-) healthy adult participants, by HIV serostatus and participant category. Boxes include medians (lines) and values between the 25th and 75th percentiles for the following participant groups: HIV^+ healthy adults ($n = 16$), HIV^- healthy adults ($n = 24$), HIV^+ patients ($n = 154$), and HIV^- patients ($n = 156$). Lines extend to ± 1.5 times the interquartile ranges; outliers within 3 interquartiles are presented as circles, and the mean is presented as a plus sign. Outliers beyond 3 interquartiles are presented as asterisks (graphs A and C).

the HIV^- patients. These differences were statistically significant in univariate analyses of the IL-2 variables, the lymphocyte and T-cell IL-6 variables, the monocyte-induced IL-6 and IL-8 variables, and the IL-4 ratio variables (see Table 2 for list of variables and healthy participant data; patient data not shown). For patients with negative blood cultures/negative malaria smears, the difference between HIV^+ and HIV^- patients in the percentage of $CD8^-$ T cells producing induced IL-6 was significant on univariate analysis but did not retain significance in multivariate analyses; the difference in the median



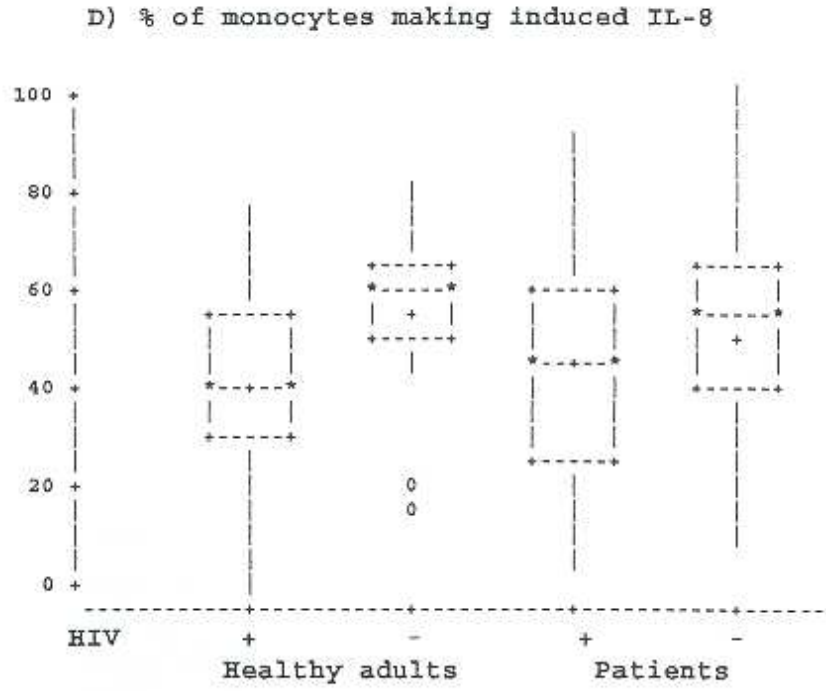


FIG. 2. (continued)

percentage of T cells producing induced IL-2 and the median percentage of lymphocytes spontaneously producing IL-6 did retain significance on multivariate analyses. Thus, the mycobacteremic/fungemic and malaria smear-positive patients differed from the healthy adult participants, whereas the patients with bacteremia or both negative blood cultures and negative malaria smears were, or tended to be, similar to the healthy adult participants.

Immune parameters related to HIV-1 plasma RNA levels

For both healthy adults and patients, the vast majority of correlations with HIV-1 RNA plasma levels were in a positive direction, involved T2/T1 ratios, and were stronger with nontransformed than with $\log_{10}$-transformed variable data. For healthy adult participants, these ratios involved induced cyto-

TABLE 3. IMMUNE FINDINGS BY HIV STATUS OF PATIENTS, MALAWI, AFRICA

	HIV^+	HIV^-	p Value[a]
Percentage of lymphocytes expressing CD4 (n = 155, 155)[b] Median	9.0	32.4	<0.001
Percentage of lymphocytes producing induced IFN-γ (n = 155, 152) Median	19.5	12.3	<0.001
Percentage of T cells producing induced IFN-γ (n = 155, 154) Median	38.8	20.7	0.003
Percentage of $CD8^+$ T cells producing induced IFN-γ (n = 149, 150) Median	51.1	31.4	0.017

[a]The p value is for the contribution of the variable to a logistic regression analysis that also included the percentage of resting lymphocytes expressing CD4 as an additional independent variable and where HIV serostatus was the dependent variable. For all models the p value for the contribution of the CD4 variable was <0.001.

[b]Individuals lacked data because of changes in tube protocols between study phases or because of poor quality of data from particular tube(s); therefore, actual numbers with data are given in parentheses.

kine production only, involving IL-10 more often than IL-4, T cells (not monocytes), and IL-2, IFN-γ, and the proinflammatory chemokine IL-8 (Table 4). For adult patients, all ratios involved IL-4, spontaneous (as well as induced) cytokine production, IL-6 and TNF-α (as well as IL-8 and IFN-γ), and monocytes as well as T cells, but did not involve IL-2. For pediatric patients, only the percentage of B cells making IL-4 contributed to a multivariate model, and then only weakly and in a direction opposite to that for adult patients. Only two mycobacteremic/fungemic patients had viral loads assessed; therefore, this subgroup could not be further evaluated. Virtually all findings for the entire adult patient group were present in the subgroup with negative blood cultures/negative malaria smears (data not shown). For those with bacteremia, the only significant correlation was with the T cell IL-10-to-IL-2 ratio, a finding also present in the healthy adult participants. For those with

TABLE 4. CYTOKINE FINDINGS ASSOCIATED WITH HIV-1 PLASMA RNA LEVELS, BY PARTICIPANT CATEGORY

	Pearson correlation coefficient	p Value[a]
Healthy adults (n = 13)		
Percentage of T cells producing induced IL-10	+0.66	0.008
Ratio of the percentage of T cells producing induced:		
IL-10 to the percentage producing induced IL-2	+0.98	<0.001
IL-10 to the percentage producing induced IL-8	+0.70	0.002
IL-10 to the percentage producing induced IFN-γ	+0.83	<0.001
IL-10 to the percentage producing induced IL-2	+0.72	0.002
HIV^+ adult patients (n = 47–48)		
Percentage of B cells spontaneously producing IL-4	−0.31[b]	0.032
Percentage of T cells producing induced IL-4	+0.46	0.001
Percentage of T cells spontaneously producing IL-4	+0.50	<0.001
Percentage of monocytes producing induced IL-6	+0.79	<0.001
Percentage of monocytes spontaneously producing IL-6	+0.85	<0.001
Ratio of the percentage of T cells producing induced:		
IL-4 to the percentage producing induced IL-6	+0.77	<0.001
IL-4 to the percentage producing induced IL-8	+0.39	0.007
IL-4 to the percentage producing induced IL-10	+0.65	<0.001
IL-4 to the percentage producing induced IFN-γ	+0.64	<0.001
IL-4 to the percentage producing induced TNF-α	+0.63	<0.001
Ratio of the percentage of T cells spontaneously producing:		
IL-4 to the percentage spontaneously producing IL-6	+0.61	<0.001
IL-4 to the percentage spontaneously producing IL-8	+0.32	0.027
IL-4 to the percentage spontaneously producing IL-10	+0.32	0.024
IL-4 to the percentage spontaneously producing TNF-α	+0.65	<0.001
HIV^+ pediatric patients (n = 35)		
Percentage of B cells spontaneously producing IL-4	+0.39	0.017

[a]The p value is for the contribution of the variable to a regression analysis. In many instances, the percentage of lymphocytes expressing CD4 and/or the persons' sex also contributed to the model. Correlations were highly dependent on outlier values.

[b]Correlation coefficient for $\log_{10}$-transformed HIV-1 plasma titers.

malaria parasitemia, the only significant correlations were with the T cell IL-4-to-IL-2 ratio, also present in the healthy adult participants, and with the T cell spontaneous IL-4-to-IL-6 ratio and the T cell IL-4-to-TNF-α ratio; the last two of these findings were also present in those with negative blood cultures/negative malaria smears (data not shown).

DISCUSSION

There is a plethora of literature on the relationship between HIV and various cytokines.[3,4,12] However, most of the available data concern *ex vivo* cytokine production by pooled cells,[15–18] cultured or cloned cells/cell lines,[19–21] or *in situ* hybridization of lymphoid tissue.[22] Little has been published on immune cell-specific cytokine profiles in the context of untreated HIV infection, perhaps because intracellular cytokine technology did not become widely available until after HAART became the standard of care in developed countries. Sadly, this is not the case in Malawi.

Because cytokines function in a microenvironmental, cell-specific fashion, cell-specific cytokine profiles may provide important information about both the immune network at various stages of clinical HIV infection and also the immune response of HIV-infected persons to associated coinfections. Our goal herein was to examine the relationships between a wide array of type 1, type 2, proinflammatory, and antiinflammatory cell-specific cytokines and HIV infection/viral load in untreated persons. We measured spontaneous cytokine production—presumably due to *in vivo* stimulation—by specific peripheral blood cell populations as well as the ability of these cells to produce cytokines with *ex vivo* induction. We evaluated healthy, asymptomatic, HIV-infected persons as well as acutely ill persons and took the specific acute infections into consideration in our evaluations of the latter group. A number of our findings were consistent with those in the literature, but several have not been noted previously.

For the asymptomatic, actively employed study participants, the median percentage of $CD4^+$ cells did not yet vary significantly by HIV serostatus, but T cell and monocyte cytokine production were already broadly impaired. First, a significantly lower median percentage of the peripheral blood T cells of the HIV^+, compared with the HIV^-, healthy persons could be induced to make IL-2, a type 1 cytokine that stimulates both T cell and HIV proliferation and is produced by $CD4^+$ lymphocytes. This difference remained significant in a multivariate analysis taking % CD4 into consideration. Second, lower proportions of the peripheral blood T cells and monocytes of HIV^+ compared with HIV^-, healthy persons produced IL-6 spontaneously (*in vivo* stimulated) or with induction. Given the role of IL-6, this might be associated with defects in terminal B cell, macrophage, and cytotoxic T cell differentiation. Third, although the median percentage of lymphocytes spontaneously producing IL-8 was higher in the HIV^+ compared with the HIV^-, healthy adult participants, the percentage of monocytes making IL-8 with *ex vivo* induction was lower in those who were HIV^+, suggesting that the cells producing this cytokine were already maximally induced *in vivo* and had moved on to apoptosis and death, either before or with *ex vivo* stimulation. Fourth, when we examined T2/T1 balances in the healthy HIV^+ participants, the ratios were significantly shifted in those who were HIV^+, toward spontaneous production of the type 2 cytokines IL-4 and IL-10, relative to IL-6, and also relative to the type 1 cytokines IL-2 and IFN-γ. This is consistent with a shift toward a type 2 or type 0 cytokine predominance with HIV infection, as has been reported by others.[15,20,23,24] To our knowledge, it has not previously been shown that this shift precedes statistically significant alterations in $CD4^+$ cell percentages. Also novel was our finding that the HIV-1 plasma RNA levels in these healthy adults were more strongly associated with IL-10 than IL-4. This may have important clinical implications, because IL-10 counteracts IL-12 and IFN-γ, whereas IL-4 does not. Presumably, this effect would not be desirable, because HIV infection is best controlled by a type 1 immune response. In summary, the cytokine alterations related to HIV infection in these healthy adults were broad and involved both cellular and humoral immunity. They were present in these asymptomatic individuals even before HIV-related differences in the percentages of $CD4^+$ T cells became statistically significant.

On cursory examination, the findings for acutely ill patients differed from those for healthy adults in three respects. First, the percentage of $CD4^+$ cells strongly differentiated HIV^+ and HIV^- patients, consistent with the HIV-infected patients being at a more advanced stage of HIV infection compared with the healthy, HIV^+ participants. Second, even when the strong relationship between the percentage of $CD4^+$ cells and HIV serostatus was taken into consideration, the percentage of T cells, especially of $CD8^+$ T cells, producing induced IFN-γ strongly differentiated the HIV^+ and HIV^- patients. Third, HIV^+ patients had a lower median IL-10-to-IFN-γ ratio than did the HIV^- patients. The specificity of these findings became clearer when patients were divided into subgroups on the basis of the organism found in their blood. Those with bacteremia or negative blood cultures had findings similar to those found in the healthy adult participants. Findings for those with mycobacterial/fungal bloodstream infections or malaria parasitemia differed most from those of the healthy participants, especially in terms of IFN-γ. We suggest that this is because both malaria and mycobacterial bloodstream infections induce and require a strong IFN-γ response[2,25,26] and that these coinfections, in combination with HIV infection,[12,16,25] caused IL-10 suppression and induced a maximal IFN-γ responsiveness by the peripheral blood T cells of these patients, despite a predominantly negative effect of HIV infection on type 1 cytokine production.

Four of our findings concerning relationships with plasma HIV-1 RNA levels are both novel and noteworthy. First, TNF-α variables were significant only as inverse correlates of viral RNA, although high serum levels of TNF-α are common in HIV-infected persons.[12,16,17] We did not find evidence of increased TNF-α production by individual cells, that is, fluorescence intensity was not increased (data not shown). These findings suggest that the source of serum TNF-α in HIV^+ persons is not peripheral blood mononuclear cells but, rather, sequestered immune cells or other cell types, for example, leukocytes or endothelial cells. Second, the majority of correlations between viral RNA and TNF-α-related cellular variables were with ratio variables and not with individual cytokines, suggesting a relatively complex interaction between the peripheral blood cell-specific TNF-α and control of the virus. Third, as

with our findings noted above concerning HIV serostatus, the significant correlations with viral RNA were consistent with the findings of previous studies suggesting a relative T2/T1 or T0/T1 shift with more severe HIV infection: for the statistically significant ratio variables, IL-4 and IL-10 were in the numerator and the correlations were positive with viral load. However, the cytokine involved varied by the health status of the participant: for the healthy, HIV^+ participants most ratio variables involved IL-10, whereas for the hospitalized patients (especially blood culture-negative/malaria smear-negative adult patients) only IL-4 ratio variables were positively correlated with viral RNA. Fourth, for the healthy, HIV^+ persons, significant correlations involved only T cells and only induced cytokine production. For the acutely ill, HIV^+ persons, especially those with negative blood cultures or malaria smears, the correlations with viral titer included both spontaneous and induced production and involved monocytes and B cells, as well as T cells, suggesting that a more severe level of dysregulation occurred with or led to intercurrent infections.

In summary, these findings support that in persons with untreated, asymptomatic HIV infection, broad defects in cell-specific cytokine profiles precede a decline in $CD4^+$ cell proportions. However, in symptomatic, acutely ill, HIV^+ patients with confirmed, acute, type 1, systemic coinfections, IFN-γ production, especially by $CD8^+$ T-cells, could still be induced and dominated the cytokine profile. In healthy persons, HIV-1 plasma viral RNA levels were associated with IL-10, an anti-IL-12/anti-IFN-γ cytokine; in acutely ill patients who had only HIV isolated from their blood, HIV-1 plasma viral RNA levels were associated with IL-4. This constellation of findings suggests that the cell-specific cytokine effects of HIV infection may be more important clinically than previously thought and that cytokine therapies, even beyond periodic administration of IL-2, may hold promise for HIV-infected persons.[12] In particular, therapy to restore a type 1 dominance may improve the ability of some HIV-infected persons to respond to both HIV and type 1 coinfections.

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