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Dynamics and Consequences of IL-21 Production in HIV-Infected Individuals: A Longitudinal and Cross-Sectional Study

Alexandre Iannello,*† Mohamed-Rachid Boulasse,‡ Suzanne Samarani,*† Olfa Debbeche,*† Cécile Tremblay,‡ Emil Toma,‡ Jean-Pierre Routy,‡ and Ali Ahmad*†

IL-21 is a relatively newly discovered immune-enhancing cytokine that plays an essential role in controlling chronic viral infections. It is produced mainly by CD4+ T cells, which are also the main targets of HIV-1 and are often depleted in HIV-infected individuals. Therefore, we sought to determine the dynamics of IL-21 production and its potential consequences for the survival of CD4+ T cells and frequencies of HIV-specific CTL. For this purpose, we conducted a series of cross-sectional and longitudinal studies on different groups of HIV-infected patients and show in this study that the cytokine production is compromised early in the course of the infection. The serum cytokine concentrations correlate with CD4+ T cell counts in the infected persons. Among different groups of HIV-infected individuals, only elite controllers maintain normal production of the cytokine. Highly active antiretroviral therapy only partially restores the production of this cytokine. Interestingly, HIV infection of human CD4+ T cells inhibits cytokine production by decreasing the expression of c-Maf in virus-infected cells, not in uninfected bystander cells. We also show that the frequencies of IL-21–producing HIV-specific, but not human CMV-specific, Ag-experienced CD4+ T cells are decreased in HIV-infected viremic patients. Furthermore, we demonstrate in this study that recombinant human IL-21 prevents enhanced spontaneous ex vivo death of CD4+ T cells from HIV-infected patients. Together, our results suggest that serum IL-21 concentrations may serve as a useful biomarker for monitoring HIV disease progression and the cytokine may be considered for immunotherapy in HIV-infected patients. The Journal of Immunology, 2010, 184: 114–126.

Interleukin-21 is a relatively recently discovered multifunctional and pleiotropic cytokine. It is a member of the common γ-chain–using cytokine family that includes IL-2, IL-4, IL-7, IL-9, and IL-15 (1, 2). The cytokine has been shown to exert significant immune-enhancing and immune-regulatory functions. It promotes proliferation and accumulation of Ag-specific CD8+ effector T cells, and increases their survival and cytolytic potential, especially in synergism with other cytokines, such as IL-15 and IL-18 (3, 4). It can also promote differentiation of naive CD4+ T cells into Th17 cells, which play an important role in inducing inflammation and controlling invading pathogens (5, 6). Moreover, the cytokine is needed for homeostatic expansion of Th17 cells (5, 7). IL-21, unlike IL-2, does not support proliferation of anti-CD3–activated regulatory T cells that are involved in suppression of antiviral immunity (8). In fact, the cytokine suppresses expression of FOXP-3, a transcription factor necessary for the development and differentiation of regulatory T cells (9). It is also well known that IL-21 has a significant influence on the regulation of B cell functions: It promotes differentiation of Ag-stimulated B cells into memory and Ab-secreting plasma cells, affects IgE production, and induces Ig switch to IgG1 and IgG3 production (10, 11). This cytokine also inhibits activation and maturation of dendritic cells but increases activation of macrophages and their IL-8 production (12, 13). Several in vivo studies in animal models have shown that IL-21 is essential for controlling chronic viral infections (14–16).

HIV-1 is the etiological agent of AIDS in humans. The infection is usually accompanied by changes in the production of several immunologically important cytokines, such as IL-15, TNF-α, IL-4, IL-12, IL-10, IL-18, and TGF-β1. Today, the production of many of these cytokines is known to be dysregulated in HIV-infected individuals. These cytokine disturbances play an important role in the pathogenesis of AIDS in HIV-infected individuals (17–19). Learning about these disturbances helps in understanding the mechanism of the disease as well as in devising novel, rational, and knowledge-based immunotherapies. Studies have demonstrated that CD4+ T cells are the main producers of IL-21 in the human body (1, 20, 21). It is noteworthy that these cells are also main targets of HIV infection. The virus replicates primarily in CD4+ T cells in HIV-infected individuals. The cells undergo rapid turnover and death even in early asymptomatic stages of the infection (22–24). Depletion of CD4+ T cells in the circulation of HIV-infected individuals is a hallmark of AIDS (25). The depletion of CD4+ T cells is likely to result in a decreased production of IL-21.
Indeed, on the basis of results from a small cross-sectional study, we reported earlier that serum levels of the cytokine were significantly reduced in patients with chronic HIV infection (26). We have now performed much larger cross-sectional and longitudinal studies to better understand the dynamics of cytokine production in different stages of the infection. In this paper, we show that concentrations of this cytokine are decreased in the circulation of HIV-infected individuals soon after infection and in general follow the kinetics of the CD4+ T cell counts of infected persons in all groups. Interestingly, we found that the elite controllers (EC; see below) maintain normal levels of the cytokine in their circulation. Our results also show that highly active antiretroviral therapy (HAART) causes a partial restoration of IL-21 levels in these patients. More importantly, we have discovered that in vitro infection of human PBMCs and purified CD4+ T cells with the virus reduces their production of IL-21 at both mRNA and protein levels. The infection reduces expression of the transcription factor c-Maf, which is known to play a key role in transcriptional activation of the IL-21 gene in CD4+ T cells (27). We found decreased frequencies of IL-21–producing HIV-specific Ag-experienced CD4+ T cells in HIV-infected viremic patients, compared with frequencies of the cells specific for the human CMV (HCMV). We also provide experimental evidence to indicate that IL-21 protects primary human CD4+ T cells in vitro from undergoing spontaneous apoptosis. In vitro, the cytokine restores survival of CD4+ T cells from HIV-infected patients comparable to survival of cells from HIV-seronegative healthy subjects. Finally, we demonstrate that decreased serum concentrations of IL-21 have consequences for the frequency and functionality of HIV-specific CTL in HIV-infected individuals. These results suggest that a reduced production of IL-21 in HIV-infected persons may be at least one reason for the decreased survival of their CD4+ T cells and may adversely affect their antiviral CTL responses as well.

Materials and Methods

Cell cultures and HIV infection

All cells used in this study were cultured at 37°C in humidified atmosphere containing 5% CO2. PBMCs were obtained from the peripheral blood of donors by centrifugation over a Ficoll-Hypaque (Pharmacia, Mail, Quebec, Canada) cushion, as described earlier (28). After washing with PBS, the cells were cultured in RPMI 1640 culture medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated FBS. The cells were activated with PHA (10 µg/ml) and IL–2 (10 IU/ml), and the experiments, human CD4+ T cells were isolated by negative selection using a kit (Stem Cell Technology, Vancouver, British Columbia, Canada). The purified CD4+ T cells, which were 98% positive for CD4 expression, were infected in vitro with a T-tropic HIV-1 strain (NL4.3). The cells were infected for 2 h at 37°C with the Cytofix/Cytoperm Kit (BD Biosciences). The washed cells were resuspended in 2% paraformaldehyde (PFA) and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). CellQuest Pro software (BD Biosciences) was used to collect and analyze the flow cytometry data.

Western blotting

For this purpose, 106 cells were incubated with fluorochrome-conjugated Abs for 45 min on ice and washed three times with PBS containing 0.05% BSA and 0.002% sodium azide. Intracellular stainings were performed with the Cytofix/Cytoperm Kit (BD Biosciences). The washed cells were resuspended in 2% paraformaldehyde (PFA) and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). CellQuest Pro software (BD Biosciences) was used to collect and analyze the flow cytometry data.

Real-time quantitative RT-PCR

The method was used to measure IL-21 mRNA in cells. For this purpose, total RNA was extracted from the cells with a kit (RNeasy Micro Kit, Qiagen, Ontario, Canada), and cDNA was synthesized using the Quantitect Reverse Transcriptase Kit. The primers used to amplify GAPDH were as follows: forward 5′-ACTGGTGCCAAAAATCAAGGCTCCCA-3′ and reverse 5′-TTCTCAGTTGCGCAGAATTCCAG-3′. Samples for RT-PCR were run on the SmartCycler Mx3000P (Stratagene, La Jolla, CA), and expression of the IL-21 gene was normalized to that of a human housekeeping gene, GAPDH. The primers used to amplify GAPDH were as follows: forward 5′-ATCGTGGAAAGACTCATGACCA-3′ and reverse 5′-TAGGAGGAGGGTGATGTTCGAG-3′. Error bars were calculated based on triplicate measurements for each gene.
**siRNA and transfection**

In some experiments, small interfering RNA (siRNA) was used to inhibit expression of c-Maf in purified human CD4+ T cells. Five million CD4+ T cells isolated from PBMCs were transfected with 30 pmol of the c-Maf-specific or control siRNA duplexes (Santa Cruz Biotechnology), using a commercial transfection kit and the Nucleofector device (T Cell Nucleofector Kit; Amxia, Gaithersburg, MD) following the manufacturer’s instructions. The protocol U-014, which ensures high viability, was used.

**Quantification of IL-21—producing Ag experienced HIV- and HCMV-specific CD4+ T cells**

Fifty million frozen PBMCs from each of HAART-naive, HIV-infected viremic patients (five) and HIV-seronegative healthy donors (five) were rapidly thawed and incubated with PBS, HCMV lysate, or HIV-1 lysate. The viral lysates were obtained from Advanced Biotechnologies (Columbia, MD) and were used at 10 μg/ml. Anti-CD28 and anti-CD49d Abs (each at 1 μg/ml from eBioscience) were added to the cultures. The cultures were incubated at 37°C in humidified 5% CO₂ atmosphere for 10 h. Brefeldin A (1 μg/ml) was added to the cultures in the last 4 h of the incubation period. Postincubation, the cells were washed with PBS, and CD4+ T cells were isolated by negative selection using a commercial kit (Stem Cell Technology). The isolated CD4+ T cells (96% pure or more) were stained with APC-conjugated anti-CD27, FITC-conjugated anti-CCR7 (both from eBioscience), and PerCP-conjugated anti-CD45RA (BioLegend, San Diego, CA). The stained cells in each treatment were divided into two aliquots and were stained intracellularly using Cytofix/Cytoperm Kit (BD Biosciences) with PE-conjugated IL-21 (eBioscience) or with PE-conjugated IFN-γ (eBioscience). The cells were washed, resuspended in 2% PFA, and analyzed by flow cytometry using FACSCalibur (BD Biosciences). Live cells were gated for three different Ag-experienced phenotypes (CD27+CD45RA CCR7+, CD27-CD45RA CCR7+ or CD27-CD45RA CCR7-) (30) and analyzed for the intracellular expression of IL-21 or IFN-γ. For each phenotype, 10⁶ events were acquired.

**Measuring frequencies and functionality of HIV-specific CTL**

For this purpose, we selected six HIV-infected individuals who had high (155, 210, and 189 pg/ml) or low (31, 18, and 29 pg/ml) serum concentrations of IL-21. These patients were viremic and HAART naive. Ten million PBMCs from each of the HIV-infected individuals and three HIV-seronegative healthy donors were rapidly thawed and incubated with sterile PBS (pH 7.3), and incubated in the culture medium containing vehicle (DMSO) or 0.20 μg/ml of each of the 48 20-mer overlapping peptides corresponding to the entire amino acid sequence of the HIV-1 (HxB-2) Gag protein (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health; Catalogue # 3992). Anti-CD28 and anti-CD49d Abs (each at 1 μg/ml from eBioscience) were added to the cultures. The cultures were incubated at 37°C in humidified 5% CO₂ atmosphere for 10 h. Brefeldin A (1 μg/ml) was added to the cultures in the last 4 h of the incubation period. Postincubation, the cells were washed with PBS and stained with FITC-conjugated anti-CD8 and PE-Cy7-conjugated anti-CD3 Abs (both from eBioscience). The cells were also stained intracellularly using the Cytofix/Cytoperm Kit (BD Biosciences) with APC-conjugated anti-IL-2 and PE-conjugated IFN-γ Abs (both from eBioscience). The cells were then washed with PBS, resuspended in PBS containing 2% PFA, and analyzed by flow cytometry using FACSCalibur (BD Biosciences). Live cells were gated for CD8+CD3+ T cells and analyzed for frequencies of the cells expressing IL-2, IFN-γ and both IL-2 and IFN-γ from 10⁶ events.

**Statistical analysis**

Group means were compared using ANOVA, and Pearson’s correlation coefficient between two parameters was determined with the software PRISM (GraphPad, San Diego, CA). Differences and correlations were deemed significant at p ≤ 0.05.

**Results**

**Levels of circulating IL-21 are decreased in HIV-infected patients**

First, we conducted a cross-sectional study and measured serum levels of IL-21 in 127 sera from HIV-infected patients and in 38 sera from age-matched HIV-seronegative healthy subjects. The serum samples obtained from HIV/AIDS patients represented different disease conditions, as described in Table I in Materials and Methods. The average concentrations of the cytokine for the control and different groups of the patients are shown in Fig. 1A. Sera from PI patients showed significantly decreased (p < 0.001) levels of IL-21, compared with sera from healthy subjects (91 ± 54 versus 496 ± 189 pg/ml, respectively). The average concentration in this infected group sera was approximately five-fold that of the control sera. Similarly, significantly decreased (p < 0.001) levels of IL-21 were observed for chronic HIV infection patients naïve for HAART, compared with healthy subjects (56 ± 29 versus 496 ± 189 pg/ml, respectively). The average IL-21 level in this infected group sera was approximately one-ninth that of the control sera. Decreased levels of the cytokine were also observed in CI-H patients, compared with healthy subjects (303 ± 143 versus 496 ± 189 pg/ml, respectively). The difference between mean levels of IL-21 for these two groups of sera was statistically significant (p < 0.001). Interestingly, the average IL-21 level of the group receiving HAART was ∼3–4-fold higher than that of CI patients who were naïve for HAART. Sera from the HAART-receiving patients also showed ∼3–4-fold higher average IL-21 content, compared with the average value for sera from PI patients. These results suggest positive restorative effects of the antiviral treatment on the serum levels of IL-21. Nevertheless, levels of IL-21 in patients receiving HAART still remained significantly lower (p < 0.001) compared with those of healthy donors, suggesting that HAART causes a partial restoration of IL-21 production in infected persons. Of note, the average serum IL-21 level for the LTNP group was significantly higher (p < 0.001) than that for either CI or PI patients. The average levels, however, were lower than those of the control group and patients who had received HAART (Fig. 1A). The median values of cytokine concentration, as well the quartile ranges for the study participants, are shown in Supplemental Fig. 1.

On the basis of viral loads and CD4+ T cell counts, we classified LTNP into three groups—NP, SP, and EC—as described in Materials and Methods. A comparison of cytokine levels in the sera of these groups is shown in Fig. 1B. A progressive increase in the average serum levels of the cytokine from NP to EC is quite evident. These levels are lower in NP and SP than in healthy control subjects (p < 0.001). Interestingly, IL-21 concentrations in the EC were not significantly different from those in healthy subjects

<table>
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<tr>
<th>Category</th>
<th>No. of Patients</th>
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<tr>
<td></td>
<td></td>
<td>Median: &lt;50</td>
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</table>

The infection was defined PI if it had been within 6 mo of the time of seroconversion and CI if it had been >6 mo.

A and B indicate CD4+ T cell counts (cells/μl) and viral loads (HIV-1 RNA copies/ml), respectively.
and were ~5-fold higher than those of NP (370 ± 191 versus 71 ± 10 respectively; \( p < 0.05 \)). The average IL-21 concentration in EC was also higher than that in SP (370 ± 191 versus 197 ± 70 pg/ml, respectively; \( p < 0.05 \)). These data suggest that serum IL-21 concentrations remain elevated in HIV-infected patients who are efficiently controlling the infection. This finding may indicate a role for this cytokine in antiviral immunity and in delaying AIDS progression.

CD4+ T cells are recognized as the main producers of IL-21 in the human body. The cell numbers are known to decrease in HIV-infected individuals. This prompted us to determine whether a correlation existed between the serum concentrations of this cytokine and CD4+ T cell counts in these patients. As shown in Fig. 1C–F, we found significant positive correlations between the two parameters for all groups of patients: \( p = 0.0057; r = 0.47 \) for PI patients; \( p < 0.0001; r = 0.66 \) for CI patients, \( p < 0.0001; r = 0.72 \) for CI-H, \( p = 0.01; r = 0.54 \) for LTNP, and \( p = 0.005; r = 0.25 \) for all patients. The dashed lines indicate 90% confidence limit.

The correlations, however, were nonsignificant \( p > 0.05 \) between IL-21 levels and viral loads and between serum cytokine levels and total CD8+ T cell counts (also see below) for all groups of patients (data not shown).

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Serum IL-21 concentrations in HIV-infected patients. IL-21 concentrations were determined in serum samples using a commercial ELISA kit. A, Average concentrations ± SD of IL-21 in the sera of 38 control (CONT) subjects, 32 PI patients, 41 CI patients, 34 CI-H, and 20 LTNP. Average concentrations differed significantly between the different infected groups of patients and CONT subjects \( p < 0.0001 \). B, Average concentrations ± SD of IL-21 in the sera of 38 CONT subjects and the three groups of LTNP representing 6 SP, 8 NP, and 6 EC. Average concentrations of the CONT group differed significantly from those of NP and SP donors \( p < 0.0001 \). Pearson’s correlation between concentrations of IL-21 and CD4+ T cell counts is given for PI patients (C), CI patients naive for HAART (D), CI-H (E), LTNP (F), and all HIV-infected patients (G). The two parameters showed a statistically significant correlation for all groups of patients: \( p = 0.0057; r = 0.47 \) for PI, \( p < 0.0001; r = 0.66 \) for CI, \( p < 0.0001; r = 0.72 \) for CI-H, \( p = 0.01; r = 0.54 \) for LTNP, and \( p = 0.005; r = 0.25 \) for all patients. The dashed lines indicate 90% confidence limit.
IL-21 concentrations start decreasing early in the course of primary HIV infection and correlate positively with CD4+ T cell counts

We investigated the kinetics of IL-21 production in treatment-naive HIV-infected patients by analyzing their sera taken longitudinally at different time points (until 28 mo) after the day of infection. The vertical dotted line indicates end of duration for PI (6 mo postinfection). The letter T on the x-axis indicates start of HAART. Shown are median values for serum IL-21 levels, CD4+ T cell counts, and plasma viral loads. T+1, T+2, and T+3 indicate first, second, and third time points for serum collection after initiation of HAART. T-1 and T-2 indicate time points prior to initiation of HAART.

HAART stops decreases in serum IL-21 concentrations and increases them progressively after beginning treatment

To investigate the impact of HAART on IL-21 concentrations in the serum, we followed up and measured cytokine levels in the sera of eight HIV-infected patients before and after HAART. The changes observed in serum IL-21 concentrations, plasma viral loads, and CD4+ T cell counts are summarized in Fig. 3. Mean values and values for each HIV-infected individual are shown in Supplemental Figs. 4 and 5, respectively. These data suggest that levels of the cytokine increased progressively with time after beginning HAART.
antiviral treatment. This rise in IL-21 concentration is accompanied by an increase in the number of CD4+ T cells in most patients posttherapy. Interestingly, at the time points at which IL-21 concentrations start augmenting posttreatment, we also observe the beginning of a decline in plasma viral loads in these patients.

**CD4+ T cells from HIV-infected patients have a decreased ability to produce IL-21**

A significant positive correlation between CD4+ T cell counts and serum IL-21 concentrations, observed above, suggests that a depletion of CD4+ T cells plays a role in decreased serum concentrations of the cytokine. However, decreased serum concentrations may also result from reduced production of the cytokine by CD4+ T cells. Therefore, we compared the cytokine-producing capacities of cells from HIV-infected subjects and from healthy subjects. For this purpose, we isolated PBMCs from HIV-infected and healthy control subjects, then cultured them in vitro in the presence of ionomycin (1 μg/ml). Next, we stained the cells for CD3, CD4, and intracellular IL-21 and examined them by flow cytometry, as shown in Fig. 4A. As seen in Fig. 4B, the expression of IL-21 was very low in PI and CI patients, compared with healthy subjects (p < 0.001). The average level of the cytokine in CI-H patients was elevated. Nevertheless, the levels of IL-21 still remained significantly lower (p < 0.01) in patients receiving HAART than in healthy donors. The average IL-21 level for LTNP was significantly higher (p < 0.01) than that for either the CI or the PI patients. A progressive increase in the average intracellular levels of the cytokine in CD4+ T cells from NP to EC was also observed (Fig. 4C). The SP and EC patients also had elevated levels of IL-21 in CD4+ T cells, compared with those seen in other groups (Fig. 4C). No positive signal for intracellular IL-21 was observed in CD3+CD4+ and CD3+CD8+ cells in the PBMCs from HIV-infected as well as from HIV-seronegative healthy individuals (data not shown).

We also measured IL-21 mRNA expression by quantitative real-time RT-PCR in PBMCs obtained from different groups of patients, and normalized it with respect to the mRNA of a housekeeping gene, GAPDH. As shown in Fig. 4D, the expression of IL-21 mRNA is very low in PI and CI patients compared with healthy subjects (p < 0.001). The average level of the cytokine mRNA in CI-H patients was elevated and was not statistically different (p > 0.05) from that found in NP patients. Of note, SP and EC patients, compared with other groups of patients, had elevated levels of the cytokine mRNA. Again, EC expressed the same amount of IL-21 mRNA as that seen in healthy donors (Fig. 4D). In general, the IL-21 mRNA-producing abilities of the PBMCs are in accord with intracellular expression of the cytokine in CD3+CD4+ T cells in all groups of donors. Taken together, these data suggest that low serum levels of IL-21 are also due to an attenuated ability of the PBMCs, and more specifically of CD3+CD4+ T cells, to produce IL-21 in HIV-infected patients. The results also indicate that these cells in the EC group maintain their ability to produce IL-21 at normal levels.

**HIV infection decreases the ability of human CD4+ T cells to produce IL-21**

The decreased ability of CD4+ T cells to produce IL-21 in HIV-infected individuals prompted us to investigate the effect of HIV infection on these cells. For this purpose, we performed in vitro infection of human CD4+ T cells purified from PBMCs after their activation with PHA and IL-2 for 72 h. The PBMCs were obtained from HIV-seronegative healthy donors. The cells were infected with a T-tropic HIV-1 strain (NL4.3) with a multiplicity of infection of 1. The infected cells were cultured for different lengths of time in the presence of ionomycin (1 μg/ml). At each time point, the numbers of viable cells were counted by Trypan blue exclusion, and the culture supernatants were collected and centrifuged to remove debris. The IL-21 concentrations of the

**FIGURE 5.** Effect of HIV infection on IL-21 production in CD4+ T cells. A, Human CD4+ T cells were purified from PHA and IL-2-activated PBMCs, infected in vitro with a T-tropic HIV strain (NL4.3), washed, and cultured for different lengths of time in the presence of ionomycin (1 μg/ml) with and without 3TC (200 nM). The IL-21 secretion was measured in the culture supernatants with an ELISA kit. At each time point, the numbers of live cells were counted in the microcultures. The cytokine concentration was normalized with the number of live CD4+ T cells at each point. Concentrations of the cytokine in the culture supernatants are shown at the indicated time points. B, IL-21 mRNA levels in HIV-infected and mock-infected CD4+ T cells normalized to the GAPDH mRNA levels and the number of live CD4+ T cells in arbitrary units. C, Effect of in vitro HIV infection and 3TC (200 nM) on IL-21 production (72 h postinfection) from human CD4+ T cells. The cytokine concentration was normalized with the number of live CD4+ T cells. D, Effect of the infection and 3TC on IL-21 mRNA expression in human CD4+ T cells normalized to the GAPDH mRNA levels and the number of live CD4+ T cells in arbitrary units. Essentially, similar results were obtained with three different healthy donors.
culture supernatants were measured by ELISA, and IL-21 mRNA was measured by real-time RT-PCR. As shown in Fig. 5A, we observed a progressive decrease of IL-21 secretion from HIV-infected PBMCs, especially 48 h (p < 0.01) and 72 h postinfection (p < 0.001). We also measured IL-21 mRNA expression in vitro postinfection at each time point and observed a progressive decline in IL-21 mRNA production in the infected cells (p < 0.001) (Fig. 5B). To determine whether HIV replication was necessary for decreased IL-21 production, we infected PBMCs with and without 3TC; a nucleoside reverse transcriptase inhibitor known to block viral replication (31). The treatment with 3TC restored IL-21 production in the cell culture supernatants (p < 0.01) as well as
IL-21 mRNA production in PBMCs ($p < 0.001$) (Fig. 5C, 5D). A reduced production of the cytokine was also observed when activated PBMCs from HIV-seronegative healthy subjects were infected in vitro with HIV-1 (shown in Supplemental Fig. 6). Taken together, these data suggest that HIV replication has a direct suppressive effect on IL-21 production in CD4$^+$ T cells at mRNA and protein levels.

HIV infection decreases the expression of the transcription factor c-Maf

We next wanted to investigate the mechanism involved in the HIV-induced downregulation of IL-21 in CD4$^+$ T cells. Recent studies have shown that expression of c-Maf, the cellular homolog of the avian viral oncogene v-Maf and the first identified Th2-type cytokine-specific transcription factor, is needed for activation of the IL-21 gene in CD4$^+$ T cells (27, 32). The lack of expression of this factor has been shown to cause a drastic downregulation of IL-21 production in CD4$^+$ T cells (27). Therefore, we sought to determine whether HIV infection affected c-Maf expression. To this end, we performed in vitro infection of CD4$^+$ T cells purified from PHA and IL-2–activated PBMCs obtained from healthy donors with HIV-1, as described in Materials and Methods. After 24 h, the cells were analyzed for their intracellular expression of c-Maf, IFN-γ, and IL-21. As shown in Fig. 6A, infection with HIV-1 decreased the production of c-Maf as well as of intracellular IL-21, compared with mock-infected cells, but not of intracellular IFN-γ. We also verified the decreased expression of c-Maf in the infected cells by Western blots at different time points postinfection (Fig. 6B). In these experiments, we measured the expression of c-Maf relative to the expression of the housekeeping gene GAPDH by densitometry of their respective bands. The ratios between the expression of IL-21 and GAPDH at each time point are depicted in Fig. 6C. These data clearly show that the expression of c-Maf increased with time in mock-infected cells, which correlates with a progressive increase in IL-21 levels secreted in the cell supernatants (Fig. 5A). Note that these cells have been cultured in the presence of ionomycin. However, the infection caused a progressive decrease in the expression of this factor in purified CD4$^+$ T cells. Fig. 6A also shows that HIV infection of CD4$^+$ T cells does not affect their IFN-γ production. Furthermore, it reveals that in HIV-infected CD4$^+$ T cell cultures, noninfected cells (CD3$^+$ p24$^-$ cells) retain their ability to produce IL-21 and maintain normal expression of c-Maf. Taken together, these data suggest that HIV-induced decreased expression of IL-21 is mediated, at least in part, by decreased expression of c-Maf, and it happens only in virus-infected cells, not in noninfected bystander cells.

Inhibition of c-Maf expression by siRNA downregulates intracellular expression of IL-21

We further sought to determine whether inhibition of c-Maf expression results in decreased expression of IL-21 in human CD4$^+$ T cells. For this purpose, we isolated human CD4$^+$ T cells from PBMCs obtained from HIV-seronegative healthy subjects. We transfected them with a c-Maf–specific or control siRNA (both from Santa Cruz Biotechnology), as described in Materials and Methods. Transfection of the siRNA vector resulted in ∼80–90% decrease in the expression of c-Maf in the transfected cells (Fig. 7A and data not shown). We cultured the transfected cells in vitro in the presence of ionomycin (1 μg/ml) for 24 h and then harvested the cells. The cells were washed and divided into aliquots. The aliquots were stained intracellularly for IL-21, IL-2, or IL-4. As shown in Fig. 7B, the c-Maf siRNA–transfected cells downregulated their expression of IL-21 and IL-4, but not of IL-2. These and previous results suggest that c-Maf–specific siRNA and HIV-1 infection both induced downregulation of c-Maf, which specifically downregulated the expression of c-Maf–regulated genes (IL-21 and IL-4) but not of c-Maf–unaffected genes (IL-2 and IFN-γ).

Loss of IL-21–producing HIV-specific CD4$^+$ T cells in HIV-infected individuals

We compared percentages of IL-21– and IFN-γ–producing Ag-experienced HIV-1 and HCMV-specific CD4$^+$ T cells after in vitro stimulation of PBMCs with viral lysates, as detailed in Materials and Methods. The results from five HIV-infected HAART-naive viremic individuals (different from those participating elsewhere in this study) and five HIV-seronegative healthy controls are shown in Table II. The mean percentages of all subsets of HIV-specific IL-21–producing Ag-experienced CD4$^+$ T cells are significantly reduced ($p < 0.01$) compared with HCMV-specific cells in HIV-infected individuals. However, we did not observe a significant difference between mean percentages of HIV-specific and HCMV-specific IFN-γ–producing cells in HIV-infected individuals. Only one subset of HCMV-specific Ag-experienced CD4$^+$ T cells (CD27$^+$ CD45RA$^-$CCR7$^+$) were significantly reduced ($p < 0.01$) in HIV-infected individuals, compared with the same subset in healthy donors.
IL-21 increases survival of CD4+ T cells

Studies have shown that members of the γ-chain–using cytokine family (like IL-7 and IL-15) play a crucial role in the survival of T cells (33, 34). Furthermore, it is also known that circulating lymphocytes (including both CD4+ T cells and CD8+ T cells) from HIV-infected individuals undergo accelerated spontaneous apoptosis, compared with cells from HIV-seronegative healthy individuals, when these cells are cultured ex vivo under suboptimal conditions (e.g., reduced serum concentrations) (33–35). Therefore, we sought to investigate whether IL-21 could promote CD4+ T cell survival in HIV-infected individuals. For this purpose, PBMCs from healthy and HIV-infected patients were isolated and cultured ex vivo for 72 h, the cells were harvested and stained for CD3, CD4, and IL-21R. After 72 h, the cells were harvested and stained for CD3, CD4, and IL-21R. The percentages of dead (positive for 7-AAD) CD3+CD4+ cells were determined by flow cytometry. Fig. 8B shows cell death in CD3+CD4+ T cells with and without IL-21 addition in three healthy and three HIV-infected donors. The addition of IL-21 reduced cell death in CD3+CD4+ T cells in all healthy and HIV-infected donors. The average percentages of live CD4+ T cells with and without the cytokine addition for healthy and HIV-infected donors are shown in Fig. 8C. It is noteworthy that IL-21 restores the percentage of live CD4+ T cells comparable to that seen in IL-21–treated PBMCs obtained from HIV-seronegative healthy donors. The cytokine increased the percentages of live CD4+ T cells by 2.5-fold in PBMC cultures from healthy donors, whereas the increase was ~6.0-fold in PBMC cultures from HIV-infected donors. Taken together, these data show that IL-21 exerts a prosurvival effect on CD4+ T cells from both healthy and HIV-infected patients.

Expression of IL-21R increases on CD4+ T cells in HIV-infected individuals

Decreased production of a cytokine often results in increased expression of its receptor on target cells. Therefore, we investigated whether a decreased production of IL-21 in HIV-infected individuals affects the expression of the cytokine receptor on CD4+ T cells. It is noteworthy that CD4+ T cells not only are major producers of the cytokine but also are its major target cells in the body. We compared the expression of the IL-21R on PBMCs obtained from HIV-infected and control subjects by flow cytometry after staining them for different fluorochrome-conjugated Abs against CD3, CD4, and IL-21Rα-chain. As shown in Fig. 9, CD3+CD4+ T cells from HIV-infected individuals demonstrate increased expression of the receptor, compared with cells from HIV-seronegative healthy individuals. Expression is significantly higher (p < 0.01) on CD4+ T cells from PI and CI patients than on cells from HIV-seronegative healthy controls.

HIV-infected individuals with higher serum levels of IL-21 have higher frequencies of HIV-specific CTL

We measured the frequencies of HIV-specific CTL (CD3+CD8+ T cells) expressing IL-2, IFN-γ, and both together, as described in Materials and Methods. The HIV-specific CTL responses were clearly detectable in the PBMCs of all six HIV-infected individuals (Fig. 10). Frequencies of CTL responding to HIV-1 peptides in the control donors were almost similar to those seen in vehicle-treated PBMCs (data not shown). Furthermore, frequencies of the HIV-specific CTL producing single (IFN-γ or IL-2) as well as double cytokines (IFN-γ and IL-2) were significantly higher (p < 0.05) in HIV-infected patients with high levels of serum IL-21 than in HIV-infected individuals with low serum cytokine levels (Fig. 10B, 10C). These data suggest that IL-21 promotes higher frequencies, as well as functionality, of HIV-specific CTL in HIV-infected individuals.

Discussion

The present study confirms earlier results from our laboratory (26), in which we showed decreased serum levels of IL-21 in HIV-infected individuals compared with those observed in HIV-seronegative healthy subjects. However, the earlier study was based only on a limited number of sera taken from a cross section of patients with chronic HIV infection. We extended our investigations to larger cohorts of patients, encompassing those with PI, CI, CI-H, and different categories (NP, SP, and EC) of LTNP. We also performed longitudinal studies on patients with PI who were or were not receiving HAART. Our results show for the first time that IL-21 production becomes compromised in early stages of the infection. Sera from all groups of HIV-infected patients showed decreased concentrations of the cytokine, compared with sera from HIV-seronegative healthy subjects. In this regard, only EC did not show a significant decrease in IL-21 levels in their sera. Longitudinal studies, as well as comparison between CI patients with and without HAART, revealed that HAART tended to increase serum concentrations of the cytokine, but only partially. Consistent with our results, partial restoration of other cytokines like IL-2 and IL-15 has also been observed in HIV-infected patients upon receiving HAART (36–39).

In all groups of HIV-infected individuals studied, we observed a strong correlation between CD4+ T cell counts and serum IL-21 concentrations. The correlation was also observed when serum IL-21 levels and CD4+ T cell counts of all HIV-infected patients were taken into consideration irrespective of clinical stage and HAART. These results strongly suggest that serum IL-21 levels could be used as surrogate markers for monitoring loss of CD4+ T cells, disease progression, and immune reconstitution in HIV-infected individuals.
These surrogate markers could be of great value when researchers and clinicians may have access only to serum samples from the patients. Furthermore, these results also indicate that depletion of CD4+ T cells, which is a hallmark of AIDS, contributes toward decreased concentrations of the cytokine in the circulation of HIV-infected individuals.

In this paper, we have shown that CD4+ T cells from HIV-infected individuals have a reduced ability to produce this cytokine, compared with their counterpart cells from healthy donors. In HIV-infected individuals, the frequencies of all major Ag-experienced HIV-specific IL-21–producing CD4+ T cell subsets (CD27+CD45RA-CCR7+, CD27+CD45RA-CCR7-, and CD27-CD45RA-CCR7+) were significantly decreased compared with the same subsets of HCMV specificity. Interestingly, the IFN-γ–producing HIV-specific Ag-experienced CD4+ T cell subsets did not differ in frequency from their counterpart cells of HCMV specificity. These data suggest that HIV infection causes depletion of IL-21–producing Ag-experienced cells of only HIV specificity.

FIGURE 8. IL-21 is a prosurvival factor for CD4+ T cells. A, PBMCs cultured in the medium supplemented with 10% FCS were stained with 7-AAD and used in a flow cytometer to set gates for live CD3+CD4+ T cells. B, PBMCs from three different healthy donors (D1, D2, and D3) and three HIV-infected viremic patients naïve for HAART (P1, P2, and P3) were isolated and cultured for 72 h in RPMI 1640 supplemented with 1% FCS with or without IL-21 (15 μM). The cells were then harvested, stained with a mixture of Abs containing FITC-conjugated anti-CD4, PE-conjugated anti-CD3 Abs, and 7-AAD. The cells were washed, resuspended in PBS, and subjected to analysis by flow cytometry, counting 10,000 gated events. Cell death (positivity for 7-AAD) was measured by flow cytometry in CD3+CD4+ cells. The percentages of CD3+CD4+ T cells stained for 7-AAD are shown in each panel. C, Average percentages and SD of live CD3+CD4+ T cells with or without treatment with IL-21 are shown for HIV-infected and CONT donors.
creased production of this cytokine from human CD4+ T cells was (32). Therefore, we sought to determine whether HIV-induced de-

m, mean fluorescence intensities (MFI) of IL-21R expression on CD3+CD4+ T cells from 10 individuals in each of the indicated donor groups: HIV-

a reduction in viral load but is very slow in restoring CD4+ T cell counts to their physiological levels. This finding has led researchers to con-

This may explain, at least in part, the compromised production of IL-21 as well as c-Maf (Fig. 6). This is supported by our results that heat-

A decreased ex vivo survival of different lymphocytes from HIV-infected individuals has been well documented (33, 34, 43). CD4+ T cells are also targeted by the virus; therefore, depletion of these cells in the circulation of HIV-infected individuals is considered an AIDS-defining condition (25, 43). The restoration of these cells in HIV-infected AIDS patients is a real challenge. The use of HAART is highly effective in these patients in reducing viral load but is very slow in restoring CD4+ T cell counts to their physiological levels. This finding has led researchers to consider using immune-enhancing cytokines like IL-2, IL-15, IL-7 as therapeutic agents (17, 44–47). In this regard, our in vitro results demonstrate that IL-21 has the ability to prevent cell death in CD4+ T cells, and in ex vivo cultures it maintains the numbers of live cells to the levels seen in cultures of HIV-seronegative healthy donors (Fig. 8B). The prosurvival effects of the cytokine for CD4+ and CD8+ T cells have been demonstrated earlier (48). These workers showed that IL-21 activates PI-3K, increases Bcl-2 expression, and prevents decrease in cell size and spontaneous cell death in human T cells when cultured ex vivo. Our results demonstrate that the prosurvival effects of the cytokine on CD4+ T cells from HIV-infected individuals are greater than those on cells from HIV-seronegative healthy subjects. The cytokine induces a larger (6-fold) increase in the number of live CD4+ T cells from HIV-infected patients than that seen (2.5-fold) in HIV-seronegative healthy donor cultures. At least in part, this could be due to upregulated expression of the cytokine receptor on CD4+ T cells in HIV-infected individuals. These results suggest that decreased ex vivo survival of CD4+ T cells from HIV-infected patients may be a consequence of their decreased production of this cytokine. The cytokine may represent an appropriate immunotherapeutic tool for restoring survival of CD4+ T cells and other lymphocytes, hence enhancing their immune reconstitution in HIV-infected patients.

Studies have shown that IL-21 has many immune-enhancing effects. It increases the cytotoxic potential of CD8+ effector T cells by inducing perforin expression. The cytokine also activates NK cells and increases their degranulation ability (49, 50). Lower levels of the cytokine in HIV-infected individuals could result in compromised effector functions of these immune cells. Indeed, a decreased expression of perforin in these cells in HIV-infected individuals has been described (40). Further studies are needed to appreciate and understand the full spectrum of effects exerted by reduced production of this cytokine in HIV-infected individuals. Reduced levels of the cytokine may adversely affect survival, as well as the proliferation and functions of other immune cells such as CD8+ T cells and NK cells. Indeed, we have shown in this paper that increased serum levels of IL-21 are associated with higher frequencies and better functioning of HIV-specific CTL in HIV-infected individuals. Thus, a deficiency of the cytokine may adversely affect the ability of an individual to control the infection. Recent in vivo studies in mice have clearly demonstrated the indispensability of IL-21 in controlling chronic viral infections (14–16). A compromised production of IL-21 may be at least one reason why humans cannot efficiently control HIV infection.

Finally, it may be worth mentioning that IL-21, compared with other cytokines, is well tolerated by humans and is currently being tested for immunotherapy against melanoma and renal cell carcinoma in clinical trials (51, 52). Our studies suggest that it should be considered an immunotherapeutic tool in HIV-infected individuals.
FIGURE 10. HIV-infected individuals with higher serum levels of IL-21 have higher frequencies of HIV-specific CTL. Ten million PBMCs from each HIV-infected or CONT subject were incubated for 10 h in the culture medium containing a pool of 20-mer overlapping peptides corresponding to the HxB2 Gag protein. Postincubation, the cells were stained with FITC-conjugated anti-CD8 and PE-Cy7–conjugated anti-CD3 Abs. The cells were also stained intracellularly with APC-conjugated anti–IL-2 and PE-conjugated IFN-γ Abs, and analyzed by flow cytometry. A, Gating of live cells for CD3⁺CD8⁺ T cells and their analyses for the intracellular expression of IL-2 and IFN-γ are shown. The panels show flow cytometric analysis for a typical HIV-infected individual and a typical CONT donor with HIV-1 peptide stimulation. B, Frequencies of HIV-specific CTL producing IL-2, IFN-γ, and both IL-2 and IFN-γ in response to HIV-1 peptides or a vehicle for HIV-infected individuals with high IL-21 serum levels (patient 1, 2, and 3 with 155, 210, and 189 pg/ml of IL-21, respectively) and low IL-21 serum levels (patient 4, 5, and 6 with 31, 18, and 29 pg/ml of IL-21, respectively). C, Comparisons are shown between HIV-infected individuals with low (L) levels of serum IL-21 and those with high (H) levels with regard to frequencies of different cytokine-producing, HIV-specific CD8⁺ T cells in response to HIV-1 peptides.
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Disclosures
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Supplementary Figures

(S1) Presentation of serum IL-21 concentrations in the Box-and-whisker plots. The Figure summarizes the concentrations for 38 control subjects (CTL), 32 HIV-infected patients in primary infection (PI), 41 HIV-infected chronically infected patients (CI), 34 chronically infected patients receiving HAART (CI-H) and 20 long-term non-progressors (LTNP). The line at the middle is the median and the box extends from the 25th to 75th percentile. The error bars extend down to the lowest and up to the highest values, respectively.

(S2) Kinetics of serum IL-21, CD4⁺ T cell counts and viral loads in treatment naïve HIV-infected patients following infection. Serum IL-21 concentrations from 9 patients naïve for HAART were determined at indicated time points from their day of infection (time zero) until twenty-eight months. The Figure shows mean values from nine patients for serum IL-21, CD4⁺ T cell counts and plasma viral loads. The arrow in the graph indicates end of duration of primary infection (six months after the day of infection).

(S3) Kinetics of serum IL-21, CD4⁺ T cell counts and viral loads in chronically infected patients (P1-P9). Serum IL-21 concentrations from nine patients naïve for HAART (P1-P9) were determined at indicated time points until twenty-eight months after their first day of infection (time zero). Figure shows longitudinal changes in serum IL-21 concentrations, CD4⁺ T cell counts and plasma viral loads in each of the patients. The broken, unbroken and dotted lines indicate IL-21 concentrations, CD4⁺ T cell counts and plasma viral loads (pVL), respectively.
(S4) Kinetics of serum IL-21, CD4⁺ T cell counts and viral loads in chronically infected patients before and after HAART. IL-21 concentrations, CD4⁺ T cell counts and plasma viral loads were determined longitudinally at the indicated time points for 28 months following the day of infection. The vertical dotted line indicates end of the duration for primary infection (six months after the day of infection). The letter T on x-axis indicates start of HAART. The Figure shows mean values for serum IL-21 levels, CD4⁺ T cell counts and plasma viral loads. T+1, T+2 and T+3 indicate first, second and third time points for serum collection after the initiation of HAART. T-1 and T-2 indicate the time points prior to the initiation of HAART.

(S5) Kinetics of serum IL-21, CD4⁺ T cell counts and viral loads in chronically infected patients (P1-P8) before and after HAART. IL-21 concentrations, CD4⁺ T cell counts and plasma viral loads were determined longitudinally at the indicated time points for 28 months following the day of infection (time zero). The arrows indicate end of the duration for primary infection (six months post-day of infection). T indicates the start of HAART. The Figure shows serum IL-21 concentrations, CD4⁺ T cell counts and plasma viral loads in individual patients.

(S6) Effect of HIV infection on IL-21 production in PBMC. (A) Human PBMC were activated with PHA and IL-2 for 3 days. PBMC were then infected in vitro with a T-tropic HIV strain (NL4.3), washed and cultured for different lengths of time in the presence of ionomycin (1 μg per ml) with and without 3TC (200 nM). The IL-21 secretion was measured in the culture supernatants with an ELISA kit. At each time point, the numbers of live cells were counted in the microcultures. The cytokine concentration was normalized with the numbers of live PBMC at each point. The Figure shows concentrations of the cytokine in the culture supernatants at the indicated time points. (B) The Figure shows IL-21 mRNA levels in HIV-infected and mock-
infected PBMC normalized to the GAPDH mRNA levels and the number of live PBMC in arbitrary units. (C) The Figure shows effect of in vitro HIV infection and 3TC (200 nM) on IL-21 production (72 hours post-infection) from human PBMC. The cytokine concentration was normalized with the number of live PBMC. (D) The Figure shows the effect of the infection and 3TC on IL-21 mRNA expression in human PBMC normalized to the GAPDH mRNA levels and the number of live PBMC in arbitrary units. Essentially, similar results were obtained with three different healthy donors.