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Raised serum levels of interleukin-18 is associated with disease progression and may contribute to virological treatment failure in HIV-1-infected patients

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SUMMARY

To gain further insight into the possible role of interleukin (IL)-18 in HIV-1 infection we examined serum levels of IL-18 in various clinical and immunological stages of HIV-1 infection during cross-sectional ($n = 41$) and longitudinal testing ($n = 20$) and during HAART ($n = 21$, 24 months follow-up). Our main findings were that HIV-1-infected patients had significantly raised IL-18 levels comparing healthy controls, particularly in those with advanced disease, that while HAART induced a marked decline in IL-18, virological treatment failure was associated with persistently raised IL-18 levels during such therapy and that our *in vitro* experiments showed an IL-18-mediated up-regulation of the HIV-1 coreceptor CXCR4 and the pro-apoptotic mediator TRAIL in PBMC from HIV-1-infected patients receiving HAART. HIV-1 infection appears to be characterized by persistently raised IL-18 levels and during HAART, such a pattern was associated with virological treatment failure, possibly contributing to immunodeficiency and HIV-1 replication in these patients.

Keywords HIV interleukin-18 HAART chemokine receptors

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by a progressive $CD4^+$ T cell depletion and by defects in T cell function, and several factors may contribute to this immunodeficiency including cytokine dysregulation [1]. Interleukin (IL)-18, mainly produced by monocytes/macrophages, plays an important role in the immune system by enhancing T cell responses, regulating interferon (IFN)γ production and promoting the development of T helper cell (T_H)1 immune responses [2]. Thus, IL-18 is involved in the development of protective immunity against intracellular microbes including several viruses. Indeed, IL-18 has been found to inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMC) [3], but has also been reported to enhance HIV-1 replication in both monocytic and T cell lines [4,5]. Moreover increased IL-18 activation has been implicated in the pathogenesis of various autoimmune disorders [2], and due to its inflammatory properties, this cytokine could potentially also contribute to the inappropriate and harmful immune activation in HIV-1-infected patients. Therefore, the exact role of IL-18 in the pathogenesis of HIV-1 infection is at present unclear.

To gain further insight into the possible role of IL-18 in HIV-1 infection, we examined serum levels of IL-18 in various clinical and immunological stages of HIV-1-infected patients during both cross-sectional and longitudinal testing as well as during highly active antiretroviral therapy (HAART).

MATERIALS AND METHODS

Patients and controls

Forty-one HIV-1-infected patients were included in the study. Clinically, 15 patients were classified as having asymptomatic HIV-1 infection (CDC group A), 13 as having symptomatic non-AIDS HIV-1 infection (CDC group B), and 13 as having AIDS (CDC group C)(Table 1). All patients were included in the cross-sectional study, and 20 were also followed during longitudinal testing (see Results). In the cross-sectional study only the last serum sample from each patient was included. Only blood samples taken in periods without any acute or exacerbation of chronic opportunistic infection were included. Nineteen HIV-1 seronegative, age- and sex-matched healthy blood donors were used as controls (Table 1). Serum samples were collected and stored as previously described [6]. Informed consent for blood sampling was obtained from all subjects. The study was conducted

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Table 1. Immunological and virological parameters in the study group

	HIV-1-infected patients			
	CDC A	CDC B	CDC C	Controls
Number of subjects	15	13	13	19
Age (years)	38 (28–56)	35 (21–54)	43 (29–58)	41 (25–64)
Sex (males/females)	11/4	12/1	11/2	14/5
$CD4^+$ T cells ($\times 10^6$/l)	430 (232–744)*	127 (105–171)**	9 (6–20)**	625 (520–900)
$CD8^+$ T cells ($\times 10^6$/l)	1016 (690–1540)**	617 (467–760)*	230 (130–340)**	400 (290–540)
HIV-1-RNA ($\times 10^3$copies/ml)	23 (6–143)	29 (18–91)	245 (61–758)	–

Data are given as medians and (25–75th percentiles). *$P < 0{\cdot}05$ and **$P < 0{\cdot}001$ *versus* controls.

according to the ethical guidelines at our hospital according to the Helsinki declaration and was approved by the hospital's authorized representative.

Isolation and stimulation of cells

PBMC were obtained from heparinized blood by Isopaque-Ficoll (Lymfoprep; Nycomed Pharma, Oslo, Norway) gradient centrifugation. Mononuclear cells were resuspended in RPMI 1640 (Gibco, Paisley, UK) with 2 mmol/l L-glutamine and 25 mmol/l HEPES buffer and 10% fetal calf serum (Myoclone, Cibco). PBMC (6×10^6 cells/ml) were seeded in 24-well plates (Costar, 1 ml/well) in culture medium alone or with 10 ng/ml recombinant human IL-18 (PeproTech, London, UK) with or without antihuman IFNγ neutralizing antibodies (final concentration 10 μg/ml, clone #25723; R & D Systems, Minneapolis, MN, USA). Unstimulated and stimulated cells were harvested after culturing for 7 h and stored in liquid nitrogen.

Quantification of IL-18 by enzyme immunoassay (EIA)

IL-18 was quantified by EIA (R & D Systems). All samples from a given patient were analysed in the same microtiter plate to minimize run-to-run variability.

RNase protection array (RPA)

Extraction and storing of total RNA from frozen PBMC pellets were performed as previously described [7]. RPA was performed with the chemokine multiprobe hCK5 and the chemokine receptor multiprobes hCR5 and hCR8 (Pharmingen, San Diego, CA, USA) [7]. The mRNA signal was normalized to the signal from the housekeeping gene GAPDH.

Real-time quantitative RT-PCR

Quantification of mRNA was performed using the ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA) [8]. Primers and probes for tumour necrosis factor (TNF)α, TNF-related apoptosis inducing ligand (TRAIL) and FasL were as previously published [8]. Real-time RT-PCR assays for CXCR4 (forward primer: 5′-GGTTACCATGGAGGGGATCAGTA-3′, reverse primer: 5′-CAGGGTTCCTTCATGGAGTCATAG-3′ and TaqMan probe: 5′-FAM-ACACTTCAGATAACTACACCGAGGAAATGGGC TAMRA-3′) and IFNγ (forward primer: 5′-GAATTGGAAAG AGGAGAGTGACAGA-3′, reverse primer: 5′-GTCTCCACAC TCTTTTGGATGCT-3′ and TaqMan probe: 5′-FAM-TGCAGA GCCAAATTGTCTCCTTTTACTTCAAAC-TAMRA-3′) were designed using the Primer Express software version 1·5 (Applied Biosystems). Gene expression of the housekeeping gene GAPDH was used for normalization.

Miscellaneous

Plasma HIV-1 RNA was measured by quantitative RT-PCR (Amplicor Monitor; Roche Diagnostic Systems, Brancburg, NY, USA; detection limit 50 copies/ml).

Statistical methods

When comparing more than two groups, the Kruskal–Wallis test was used. If a significant difference was found, the Mann–Whitney *U*-test was used to determine differences between each pair of groups. Responses within the same individuals were compared by the Wilcoxon signed-rank test for paired data. Data are given as medians and 25–75th percentiles if not otherwise stated. *P*-values were two-sided and considered significant when <0·05.

RESULTS

IL-18 levels in cross-sectional testing

When examining serum levels of IL-18 in 41 HIV-1-infected patients and 19 healthy controls in cross-sectional testing (see methods), we found that all clinical groups of HIV-1-infected patients had raised IL-18 levels comparing controls, with particularly high levels in the AIDS group [734·7 (484·0–964·3) pg/ml *versus* 285·0 (223·8–417·4) pg/ml; $P < 0{\cdot}001$, HIV-1-infected patients *versus* controls, Fig. 1a].

IL-18 levels during longitudinal testing

We next analysed IL-18 levels in serial serum samples from patients in which follow-up data for > 15 months were available ($n = 20$) (median observation time 36 months, range 16–54; median numbers of blood samples 4, range 3–4). During the study period 9 patients received single nucleoside analogue therapy, but none received nucleoside analogs in combination or HAART. Patients were defined as clinical progressors if they fulfilled one of the following criteria during the study:

- Death from HIV-1 complication.
- Clinical progression as reflected in altered CDC classification.
- A new AIDS defining event (for patients in CDC group C at baseline).

Of the nine patients who received monotherapy with one nucleoside analogue, 5 belonged to the progressors and 4 to the nonprogressors. At inclusion, nonprogressors ($n = 10$) and

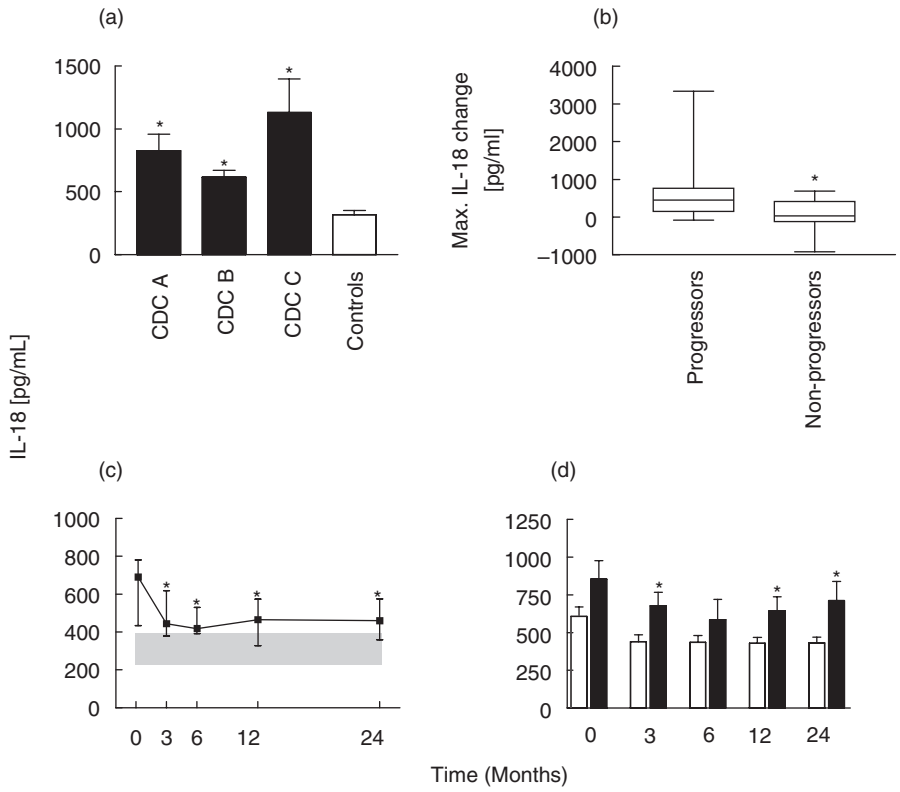

Fig. 1. (a) IL-18 levels in cross-sectional testing. IL-18 levels in three clinical groups of HIV-1-infected patients and 19 healthy blood donors. CDC A, asymptomatic HIV-1-infected patients ($n = 15$); CDC B, symptomatic non-AIDS HIV-1-infected patients ($n = 13$) and CDC C, AIDS patients ($n = 13$). Data are given as means ± SEM. *$P < 0{\cdot}001$ *versus* controls. (b) IL-18 levels during longitudinal testing. Changes in IL-18 levels during the study in two defined clinical groups of HIV-1-infected patients classified as progressors ($n = 10$) and nonprogressors ($n = 10$, median observation time 36, range 16–54 months; see Results). *$P < 0{\cdot}05$ *versus* nonprogressors. Data are given as medians, 25–75th percentiles and minimum and maximum. (c) IL-18 levels during HAART. The effect of HAART on serum levels of IL-18 in 21 HIV-1-infected patients. IL-18 levels were analysed at baseline and after 3, 6, 12 and 24 months of therapy. The shaded area represents 25–75th percentiles in healthy controls. Data are given as medians and 25–75th percentiles. *$P < 0{\cdot}01$ *versus* baseline. (d) IL-18 levels in relation to virological treatment failure (HIV-1 RNA > 50 copies/ml) after 12 months of HAART. Six patients were classified as virological nonresponders (■) and 15 as virological responders (□). Data are given as means ± SEM. *$P < 0{\cdot}05$ *versus* responders.

progressors ($n = 10$) had similar median $CD4^+$ T cell counts (220 (range 140–370) × 10^6/l and 250 (range 120–410) × 10^6/l) and median viral load (90 (range 55–170) × 10^3 copies/ml and 100 (range 35–150) × 10^3 copies/ml), progressors and nonprogressors, respectively. However, while nonprogressors had stable IL-18 levels throughout the study period, there was a gradual and significant increase in IL-18 concentrations among progressors, resulting in a significant difference in changes in IL-18 levels between these two groups (Fig. 1b).

IL-18 levels during HAART

We next examined whether HAART had any effect on IL-18 levels in 21 HIV-1-infected patients. During HAART there was a significant increase in $CD4^+$ T cell counts (maximum increase: 150 (85–240) 10^6/l, $P < 0{\cdot}001$) and a marked decrease in HIV-1 RNA copy numbers in plasma (maximum decrease 2·78 (1·94–3·33) $\log_{10}$ copies/ml, $P < 0{\cdot}001$). Notably, these changes were accompanied by a significant decrease in IL-18 levels (Fig. 1c). However, there was no normalization of IL-18 during the study period comparing levels in healthy controls (Fig. 1c). During HAART we found no correlation between changes in $CD4^+$ T cell counts and changes in IL-18 levels. However, the decrease in IL-18 levels tended to be correlated with the decrease in viral load, although the correlation did not reach statistical significance ($r = 0{\cdot}38$, $P = 0{\cdot}1$). Even more importantly, when the patients were classified as virological responders ($n = 15$) or nonresponders ($n = 6$) after 12 months of therapy according to viral load at this time point (i.e. < or >50 HIV-1 RNA copies/ml plasma), we found that nonresponders had significantly higher IL-18 levels at several time points during the study period (Fig. 1d). Importantly, these groups of patients were comparable with regard to prior treatment regimen and baseline viral load (110 (range 70–540) × 10^3 copies/ml and 180 (range 90–650) × 10^3 copies/ml, responders and nonresponders, respectively], and there was no differences in compliance between these groups of patients.

Effect of IL-18 on gene expression of chemokines, chemokine receptors and cytokines

Chemokines and cytokines such as members of the TNF superfamily and IFNγ, seem to be involved in HIV pathogenesis [1]. By using RPA and real-time quantitaive RT-PCR we therefore next examined the effect of IL-18 on gene expression of several of

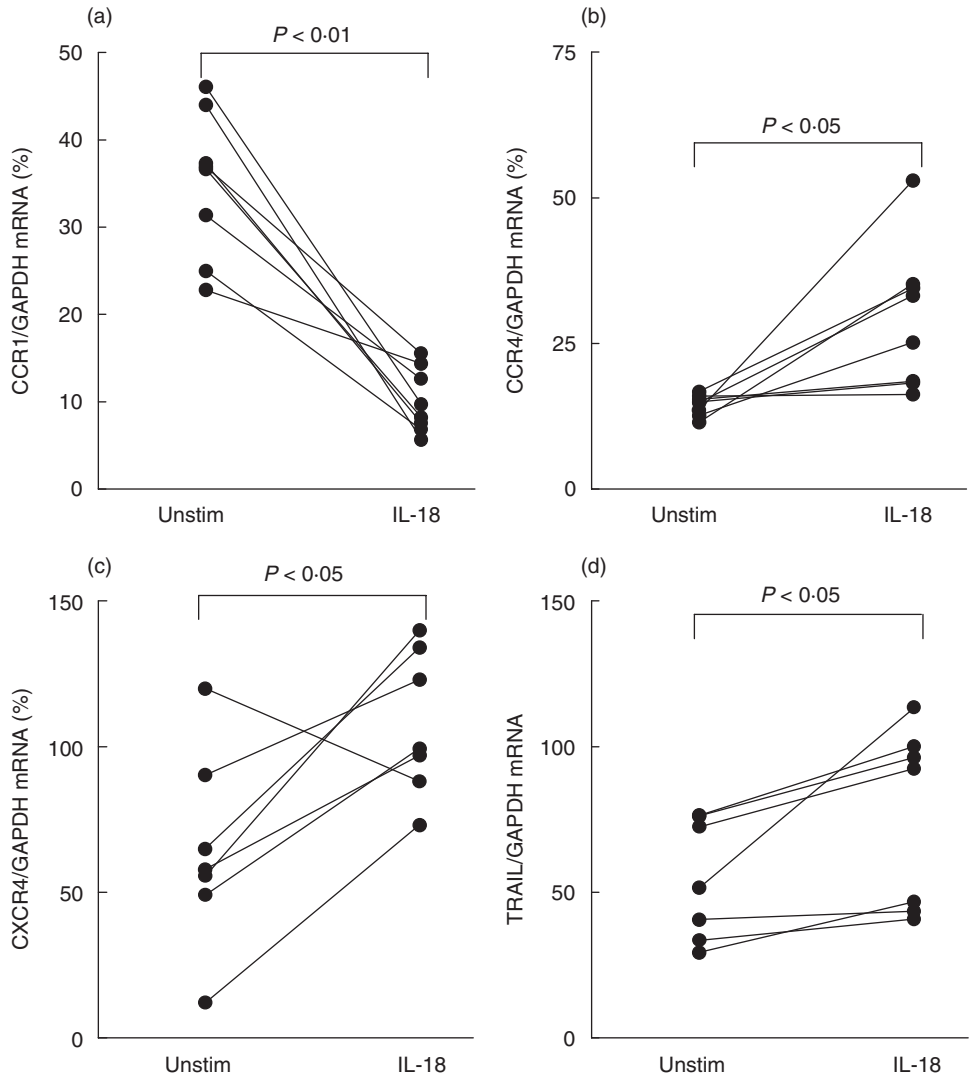

Fig. 2. Effect of IL-18 on gene expression of chemokine receptors and TRAIL. Relative mRNA levels in relation to the control gene GAPDH of CC receptor 1 (CCR1) (a), CCR4 (b), CXCR4 (c) and TRAIL (d) in unstimulated (unstim) and IL-18 (10 ng/ml) stimulated PBMC from 7 HIV-1-infected patients on HAART. Only 6 patients were examined for TRAIL expression. As for CXCR4 expression, only data from the RPA analyses are presented (see Materials and Methods).

these mediators in PBMC from 7 HIV-1-infected patients receiving HAART. As shown in Fig. 2, IL-18 induced a significant up-regulation of CCR4, CXCR4 and TRAIL and a down-regulation of CCR1. In contrast, IL-18 did not influence CCR5 expression and had no significant effect on the expression of various chemokines (i.e. RANTES, IL-8, MIP-1β, MIP-1α, IP-10, MCP-1 and I-309) or cytokines (i.e. TNFα, IFNγ and FasL). Based on the roles of CXCR4 as a coreceptor for HIV-1 and TRAIL as a pro-apoptotic mediator [1,9], these responses were examined in more detail. Notably, while the addition of neutralizing antibodies to INFγ did not influence the IL-18-induced CXCR4 expression, it reduced the IL-18-mediated TRAIL expression by approximately 50% ($P < 0{\cdot}05$) suggesting that the latter response at least partly depends on endogenous IFNγ production.

DISCUSSION

Raised serum levels of IL-18 have recently been reported during HIV-1 infection in a cross-sectional study [10], and in the present study we extend these finding by showing enhanced levels also during longitudinal testing with particular high levels in those with advanced clinical and immunological disease. Moreover, we show that while HAART induces a marked decline in IL-18, virological treatment failure is associated with persistently raised IL-18 levels during such therapy. Furthermore, our *in vitro* experiments show an IL-18-mediated up-regulation of the HIV-1 coreceptor CXCR4 and the pro-apoptotic mediator TRAIL in PBMC from HIV-1-infected patients, suggesting that IL-18 is not only one of several inflammatory markers that increases during HIV-1

infection, but may also be involved in the pathogenesis of this disorder.

Based on its ability to augment T_H1-mediated immune responses [2], IL-18 has been considered as a candidate for immunomodulation in HIV-1-infected patients [4]. However, in the present study we found no effect of IL-18 on IFNγ expression in PBMC from HIV-1-infected patients. We have previously reported deficient IL-12 production in HIV-1-infected individuals even during HAART [11], and it is possible that the lack of IL-18-mediated induction of IFNγ and other inflammatory cytokines in these patients may reflect the importance of IL-12 as a costimulus for such responses. In fact, it has been suggested that IL-18 in the presence of low IL-12 levels, as in HIV-1-infected patients, may promote a Th2 rather than a Th1 response [12]. Herein we extend these findings by showing that IL-18 without any exogenous addition of IL-12 also enhances the expression of CXCR4 and TRAIL in PBMC from HIV-1-infected patients.

Enhanced apoptosis has been suggested to contribute to $CD4^+$ T cell depletion during HIV infection, and such mechanisms seem also to persist during HAART [13]. TRAIL is a mediator of apoptosis and has also been implicated in the pathogenesis of HIV-1 infection [8,14]. In the present paper we for the first time report that IL-18 enhances the expression of TRAIL in PBMC from HIV-1-infected patients, which could potentially contribute to $CD4^+$ T cell depletion and immunodeficiency in these patients.

It has been reported that IL-18 can induce HIV-1 replication in monocytic and T cell lines partly mediated by TNFα and IL-6 [4]. By showing an IL-18-mediated up-regulation of the HIV-1 coreceptor CXCR4 in PBMC from HIV-1-infected patients, we suggest another mechanism by which IL-18 might promote HIV-1 replication. Impaired response to HAART has previously been reported to be associated with a cAMP/protein kinase A-mediated up-regulation of CXCR4 [15], and in the present study we suggest a similar enhancing effect of IL-18. The *in vivo* relevance of this finding is at present not clear. However, while we found that HAART induced a significant decrease in IL-18, a lack of decline in IL-18 levels was associated with virological treatment failure in these patients. While relatively few patients were studied, these preliminary findings may suggest that this association between virological treatment failure and persistently raised IL-18 levels could at least partly involve CXCR4-related mechanisms. Furthermore, the increased expression of CXCR4 could also possibly lead to the emergence of syncytium-inducing HIV-1 variants that have been associated with a more-rapid decline of $CD4^+$ T cells and high HIV-1 RNA levels [16].

While IL-18 may promote activation of T_H1 cells with potential beneficial effects in HIV-1-infected patients, this was not found in the present study as assessed by IFNγ production in PBMC. In fact, IL18 was found to increase CXCR4 and TRAIL expression in PBMC from HIV-1-infected patients receiving HAART possibly contributing to immunodeficiency and HIV-1 replication in these patients.

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