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HIV-1 does not alter *in vitro* and *in vivo* IL-10 production by human monocytes and macrophages

A. BERGAMINI, F. BOLACCHI, E. FAGGIOLI, R. PLACIDO*, S. VENDETTI*, L. CAPPANNOLI, L. VENTURA, G. CERASARI†, I. UCCELLA, M. ANDREONI & G. ROCCHI *Department of Public Health and Cellular Biology, Chair of Infectious Diseases and *Department of Biology, University of Rome 'Tor Vergata', and †First Division L. Spallanzani Hospital, Rome, Italy*

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SUMMARY

The present study analyses the ability of HIV-1 to modulate IL-10 production in cells of monocyte-macrophage lineage cultured in the presence of macrophage colony-stimulating factor (M-CSF). Both monocytes and macrophages spontaneously produced low amount of IL-10. Lipopolysaccharide (LPS) induced a strong IL-10 response in fresh monocytes and in M-CSF-treated macrophages. In contrast, macrophages cultured in the absence of M-CSF exhibited a marked decrease in their susceptibility to LPS stimulation. M-CSF increased the IL-10 response of macrophages to LPS by enhancing both the expression of membrane-bound CD14, the protein that serves as LPS receptor, and the sensibility of CD14-expressing cells to LPS stimulation. Neither spontaneous nor LPS-induced expression of IL-10 was modulated in monocytes and macrophages by infection with eight monocytotropic strains, as demonstrated by ELISA and cytofluorimetric analysis. In contrast, all the HIV-1 strains primed macrophages for an increased IL-6 response to LPS stimulation. To determine whether IL-10 production was associated with *in vivo* infection, monocytes from AIDS individuals were analysed for IL-10 production. We found that neither spontaneous nor LPS-induced IL-10 production were different between healthy controls and HIV-infected patients. Taken together, these data strongly suggest that HIV-1 infection of monocytes-macrophages does not play a significant role in the regulation of IL-10 in infected patients. This study also emphasizes the role of M-CSF activation in the regulation of the cytokine response in macrophages.

Keywords IL-10 monocytes-macrophages macrophage colony-stimulating factor lipopolysaccharide

INTRODUCTION

IL-10 is a recently identified cytokine produced by T and B lymphocytes as well as monocytes-macrophages [1–4]. IL-10 inhibits T cell proliferation and cytokine production [5–8], and it is thought to play an important role in T cell dysfunction and cytokine imbalance observed in HIV-1-infected patients [9–13]. IL-10 levels were found to be significantly elevated in phytohaemagglutinin (PHA)-stimulated peripheral blood lymphocytes and in cell populations isolated from lymph nodes of HIV-infected individuals, compared with healthy controls [13–15]. In addition, serum IL-10 levels were found higher in HIV-infected subjects than in uninfected controls [16].

Correspondence: Alberto Bergamini MD, Cattedra di Malattie Infettive, Dipartimento di Sanità Pubblica e Biologia Cellulare, Università di Roma 'Tor Vergata,' Via di Tor Vergata 135, 00133 Rome, Italy.

Although several studies reported that the major source of IL-10 in HIV-infected patients are T lymphocytes [14,15], it has been recently hypothesized that also monocyte-macrophage cells, a major target for HIV-1 *in vivo* [17–21], could contribute to IL-10 dysregulation. However, while some studies have described an over-production of IL-10 by monocyte-macrophage cells exposed to HIV-1 products (recombinant gp120) or infected by HIV-1 [22–25], others reported contrasting results [26,27]. Resolving which cells are over-producing IL-10 in HIV-1-infected patients may have a potential profound impact on the design of therapeutic strategies.

Following this line of research we have tested, *in vitro*, several HIV-1 isolates (primary clinical isolates and laboratory-adapted strains) for their ability to induce IL-10 in monocytes and macrophages activated by macrophage colony-stimulating factor (M-CSF) which *in vivo* regulates monocyte-macrophage differentiation

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and function [28–31]. Then, to determine whether IL-10 production was associated with *in vivo* HIV-1 infection, monocytes from AIDS individuals were analysed for IL-10 production.

MATERIALS AND METHODS

Cells

Peripheral blood obtained from HIV^- donors was enriched for mononuclear cells (PBMC) by centrifugation over Ficoll–Hypaque. The PBMC were then further enriched for monocytes by elutriation as described by Gerrard *et al.* [32]. Cells obtained by this method are > 90% monocytes and > 80% $CD14^+$ as determined by FACS analysis.

Compounds

Recombinant M-CSF was kindly provided by Genetic Institute (Cambridge, MA). The product concentration was 0·78 mg/ml, and the specific activity $1{\cdot}9 \times 10^6$ U/mg protein (one unit equals half maximal stimulation in the Murine Bone Marrow Colony Assay). Lipopolysaccharide (LPS) from *Escherichia coli* 0111/B4 was purchased from Sigma Chimica (Milan, Italy). LPS (100 ng/ml) was used to stimulate the cells.

Viruses

A laboratory strain (Ba-L) and seven clinical isolates of HIV-1 were used to infect macrophages. Supernatants of infected macrophages were used as the source of HIV-1 Ba-L; these were filtered and stored in liquid nitrogen before use. The clinical isolates of HIV-1 were obtained from seven HIV antibody-seropositive individuals. Isolation of these strains from the plasma was performed in PBMC cultures, and the supernatants of these cultures were used as the source of the virus. Titration to determine infectivity of Ba-L and primary isolates was performed, respectively, in a primary macrophage system or in PBMC as previously described [33,34]. The titre of the virus stocks, expressed as 50% tissue culture infectious dose ($TCID_{50}$), was determined as previously described [35].

Viral detection

HIV-p24 antigen production in supernatants was assessed by a sandwich ELISA (Abbott, Pomezia, Italy).

ELISA immunoassay

Commercially available sandwich ELISA kits (R&D Systems Minneapolis, MN) were used to determine the concentration of IL-10 and IL-6. The detection limits of these ELISAs are 7·8 pg/ml (IL-10) and 3·13 pg/ml (IL-6). According to the manufacturer's specifications, these ELISAs are specific for the relative interleukin. All the samples were determined in duplicate, in a single analytical set. The intra-series variation coefficient was < 15%.

Assessment of interleukin production by monocytes and macrophages infected or not by HIV-1

After purification (day 0), monocytes, stimulated or not with LPS, were cultured in the presence or in the absence of 1000 U/ml of M-CSF in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (complete medium), at 37°C in a humidified atmosphere of 5% CO_2 in air, in 48-well plates (Costar, Cambridge, MA) at a concentration of 2×10^5 cells/well per ml. After 48 h of incubation the supernatants were harvested and stored at –80°C. Alternatively, just after purification monocytes were infected with either 1000 $TCID_{50}$ of the different HIV-1 clinical isolates or with 300 $TCID_{50}$ of HIV-1 Ba-L. After 2 h of incubation the cells were extensively washed to remove excess virus and cultured for 48 h as described above. Then the supernatants were collected and stored at −80°C.

For the determination of IL-10 production by macrophages, 5×10^5 monocytes were cultured for 21 days in 48-well plates in 1 ml of complete medium in the presence or absence of M-CSF. The cells were washed and fed every 7 days. At different time points the cultures were washed, refed with fresh medium (containing or not M-CSF as needed) and stimulated or not with LPS. After 48 h of incubation the supernatants were harvested and stored at −80°C. Alternatively, the cells were cultured for 7 days and then infected as described before. At day 21, cultures were washed and refed with fresh medium (containing or not M-CSF as needed) and stimulated or not with LPS. After 48 h of incubation the supernatants were collected and stored at −80°C.

Cytofluorimetric (FACS) analysis

FACS analysis was performed using a previously described method [36]. In short, macrophages were cultured in 25-cm^2 flasks (Corning 25102-25) and infected as described above. At day 21, the cultures were washed and refed with complete medium containing or not M-CSF and LPS, in the presence of 2·5 μg/ml of the protein transport inhibitor brefeldin A (Sigma Chimica). After 24 h incubation, the cells were washed twice in PBS, detached by gentle scraping, collected by centrifugation and stained for 15 min with R-PE-cyanine 5 (PE-Cy5) conjugated anti-CD14 MoAb (Immunotech, Marseille, France) for determination of their surface phenotype. The cells were then washed twice in PBS and fixed in ice-cold PBS containing 4% paraformaldehyde. After two further washes in PBS, 2×10^5 cells were resuspended for 30 min at room temperature in 30 μl PBS containing 0·1% saponin (Sigma Chimica), 1% bovine serum albumin (BSA) (Sigma Chimica) and 0·5 μg/1×10^6 cells of the following MoAbs: PE-conjugated mouse anti-HIV-p24 antigen (Immunotech) or PE-conjugated MoAb against IL-10 (Pharmingen, San Diego, CA), or both. Paired isotype-specific control antibodies (Pharmingen) were run with each sample. As a last step, the cells were washed twice in PBS containing 0·01% saponin, resuspended in PBS and analysed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Five thousand to 10 000 cells were computed in list mode and analysed using the FACScan research software (Becton Dickinson).

Assessment of IL-10 production by monocytes from HIV-infected individuals

Twenty $HIV\text{-}1^+$ individuals (13 male and seven female, mean age 37 years, range 25–49 years) were enrolled in this study, 10 healthy HIV^- donors were included as controls. All the seropositive subjects had had at least one AIDS-defining illness, but had no active opportunistic diseases. All patients were repeatedly positive for HIV-1 antibodies by ELISA, confirmed by Western blot analysis. Patients were classified according to their absolute T CD4 cell counts: < 200/mm^3 ($n = 10$, mean T CD4 cell counts 84 ± 63, range 11–183), > 200/mm^3 ($n = 10$, mean T CD4 cell counts 317 ± 73, range 250–442). All patients were receiving antiretroviral drug therapy but not cytokines. Informed consent was obtained from all participants.

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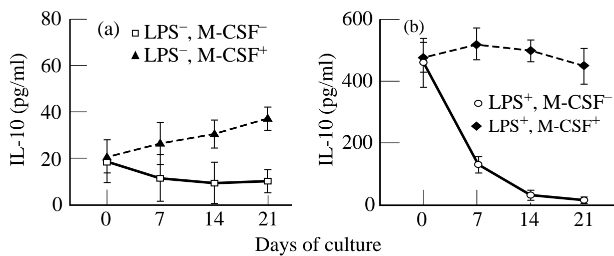

Fig. 1. IL-10 production by monocytes and macrophages stimulated or not with lipopolysaccharide (LPS) and macrophage colony-stimulating factor (M-CSF). At given time points, macrophage cultures were washed, refed with fresh medium with or without LPS and M-CSF. After 48 h of incubation, the supernatants were obtained for IL-10 determination. The data represent the mean of three experiments each carried out in duplicate. Each experiment was performed with cells from a single donor. The error bars represent s.e.m.

Peripheral blood was obtained by venipuncture. Monocytes were obtained as described above and then further purified by immunomagnetic depletion of T and B lymphocytes, using Dynabeads M-450 Pan-T (CD2) and Pan-B (CD19) (Dynal AS, Oslo, Norway). The procedure was carried out as indicated by the manufacturer. Cells obtained by this method are >99% pure as determined by FACS analysis. After purification, 2×10^5 monocytes were cultured for 48 h as described above in the presence or absence of LPS. Then the supernatants were harvested and stored at −80°C.

Statistical analysis
Student's *t*-test was used to analyse data.

RESULTS

Effect of M-CSF on IL-10 production by monocytes and macrophages
We initially evaluated the effect of M-CSF on IL-10 production by freshly elutriated monocytes (day 0) and macrophages at different stages of maturation, in the absence of LPS stimulation (Fig. 1a). M-CSF treatment did not modify IL-10 production by monocytes, yet it induced some cytokine release in macrophages starting from day 7 of culture. Since M-CSF causes proliferation of macrophages [28], we wondered if the augmented IL-10 levels found in the supernatants were simply due to the M-CSF-induced increase in cell number. Indeed, the ratio between the number of cells and the levels of IL-10 was quite similar in the cultures exposed to M-CSF compared with those unexposed (not shown).

We then analysed the ability of M-CSF to modulate the response of monocytes and macrophages to LPS. As shown in Fig. 1b, M-CSF significantly primed macrophages, but not monocytes, to an enhanced IL-10 response to LPS. It should be noted that IL-10 levels in M-CSF-treated macrophages at days 7, 14 and 21 were, respectively, 4, 26 and 28 times greater than in untreated cultures, whereas the increase in cell number was 1·5-, 2·4- and 3·4-fold. Thus, it is unlikely that the increase in cell number induced by M-CSF can account for the enhanced IL-10 production.

Table 1. Cytofluorimetric evaluation of surface CD14 and intracellular IL-10 expression in macrophages stimulated by lipopolysaccharide (LPS)

	Percent of anti-CD14-reacting cells and % (shown in parentheses) of cells reacting with both anti-CD14 and anti-IL10	
	Treatment	
Day of culture	None	M-CSF
7	42 ± 15 (5 ± 2)	71 ± 13 (15 ± 7)
14	16 ± 4 (<2)	65 ± 9 (13 ± 4)
21	<2	67 ± 11 (12 ± 5)

At different time points cultures were stimulated with LPS in the presence of the protein transport inhibitor brefeldin A. After 24 h incubation cells were fixed and stained for intracellular IL-10 and surface CD14. Five thousand to10 000 cells were analysed for each sample. Less than 2% of the $CD14^-$ cells stained positive for intracellular IL-10 at each time point, both in normal and macrophage colony-stimulating factor (M-CSF)-stimulated cells. The data represent the mean ± s.d. of three different experiments.

Effect of M-CSF on surface CD14 and intracellular IL-10 expression
Cytofluorimetric analysis was carried out to determine whether the ability of M-CSF to enhance macrophage IL-10 response to LPS might be due to the up-regulation of CD14, the cellular receptor for LPS [37], to a different ability of macrophages to respond to the LPS signal, or both. Membrane CD14 expression and intracellular IL-10 production were studied at single-cell level by using a recently developed cytofluorimetric technique [36]. As shown in Table 1, M-CSF treatment increased both the percentage of cells expressing CD14 and the percentage of CD14-bearing cells that produced IL-10 in response to LPS compared with untreated cells. Taken together, these data suggest that M-CSF modulates IL-10 production in LPS-stimulated macrophages by increasing both the expression of surface-bound CD14 and the sensitivity of the CD14-expressing cells to LPS stimulation.

HIV-1 infection of monocytes and macrophages
A productive infection (HIV-p24 antigen production) was consistently obtained with all the HIV-1 isolates in both normal and M-CSF-treated macrophages. However, both the HIV-p24 antigen production in the supernatants and the percentage of HIV-producing cells were higher in M-CSF-treated macrophages than in normal cells (Table 2). These data agree with those from other groups [38] and indicate that M-CSF strongly up-modulates HIV-1 replication in macrophages.

IL-10 production by HIV-infected monocytes-macrophages
Just purified monocytes (day 0) were infected with the HIV-1 isolates in the presence or absence of LPS. Alternatively, the cells were infected on day 7 and then stimulated by LPS when a productive infection became established (day 14 after infection).

None of the eight HIV-1 isolates significantly modulated the

Table 2. HIV-1 infection of normal and macrophage colony-stimulating factor (M-CSF)-treated macrophages

	Percent of anti-HIV p24 antigen-reacting cells and (shown in parentheses) titre of HIV-p24 antigen (ng/ml) in the supernatant	
	Cells	
HIV-1 isolates	Untreated	M-CSF-treated
None	< 2 (< 0·12)	< 2 (< 0·12)
Ba-L	25 ± 6 (7 ± 3)	87 ± 15 (117 ± 28)
MA107	11 ± 4 (3 ± 1)	57 ± 9 (56 ± 21)
RA12	19 ± 6 (6 ± 1)	63 ± 18 (87 ± 16)
DG10	19 ± 5 (6 ± 3)	68 ± 13 (95 ± 25)
GI24	ND (8·4 ± 1·5)	ND (43 ± 16)
GF2	ND (6·2 ± 2·2)	ND (31 ± 10)
FA26	ND (6·1 ± 1·8)	ND (39 ± 8·8)
KG5	ND (1·5 ± 0·7)	ND (15 ± 6·1)

On day 21 supernatants were harvested for HIV-p24 titration by ELISA testing. Then the cultures were washed and refed with fresh medium containing the protein transport inhibitor brefeldin A. After 24 h incubation cells were fixed and stained for intracellular HIV-p24 and analysed by FACS. Five thousand to 10 000 cells were analysed for each sample. ND, Not done. The data are the arithmetic mean ± s.e.m. of three independent experiments.

production of IL10 in monocytes or in macrophages, both in the presence or absence of LPS or M-CSF stimulation (Fig. 2a,b).

For comparison, we evaluated under our experimental conditions the ability of HIV-1 to induce the production of another LPS-induced cytokine such as IL-6 [39]. IL-6 production was measured in the same macrophage culture supernatants that were used to determine IL-10 levels. We found that all the HIV-1 isolates primed these cells for an augmented IL-6 response to LPS stimulation (Fig. 3).

IL-10 production by HIV-1-infected macrophages was also analysed at single-cell level by FACS analysis (Fig. 4). Both untreated and M-CSF-treated cells were stimulated by LPS and analysed for contemporary HIV-p24 antigen and IL-10 production at day 14 after infection. Intracellular p24 antigen was detected in both untreated and M-CSF-treated cells. The percentage of HIV-$p24^+$ cells was 21% (Fig. 4b) and 93% (Fig. 4d), respectively. HIV-1 infection did not induce IL-10 production in both untreated and M-CSF-treated cells. Indeed, the percentage of IL-10-producing cells in uninfected and infected cultures was < 2% *versus* < 2% in untreated macrophages (Fig. 4a,b) and 14·3 ± 4% *versus* 15·8 ± 8% in M-CSF-treated macrophages (Fig. 4c,d).

IL-10 production by monocytes from HIV-1-infected patients

The studies described above demonstrate that monocytes-macrophages did not produce IL-10 in response to HIV-1 infection *in vitro*. To determine whether IL-10 production was associated with *in vivo* infection, we analysed highly purified monocytes from AIDS patients.

IL-10 production by monocytes, both in the absence and presence of LPS stimulation, did not differ between 10 controls and 20 patients, whether they were stratified according to their $CD4^+$ T cells or not (Table 3). However, as shown in Table 3, IL-10 plasma levels were significantly lower in controls than in HIV-1-infected patients. Moreover, in the HIV-infected group, plasma IL-10 levels were particularly high in patients with low $CD4^+$ T cell counts.

DISCUSSION

In the current study we have demonstrated that IL-10 production in

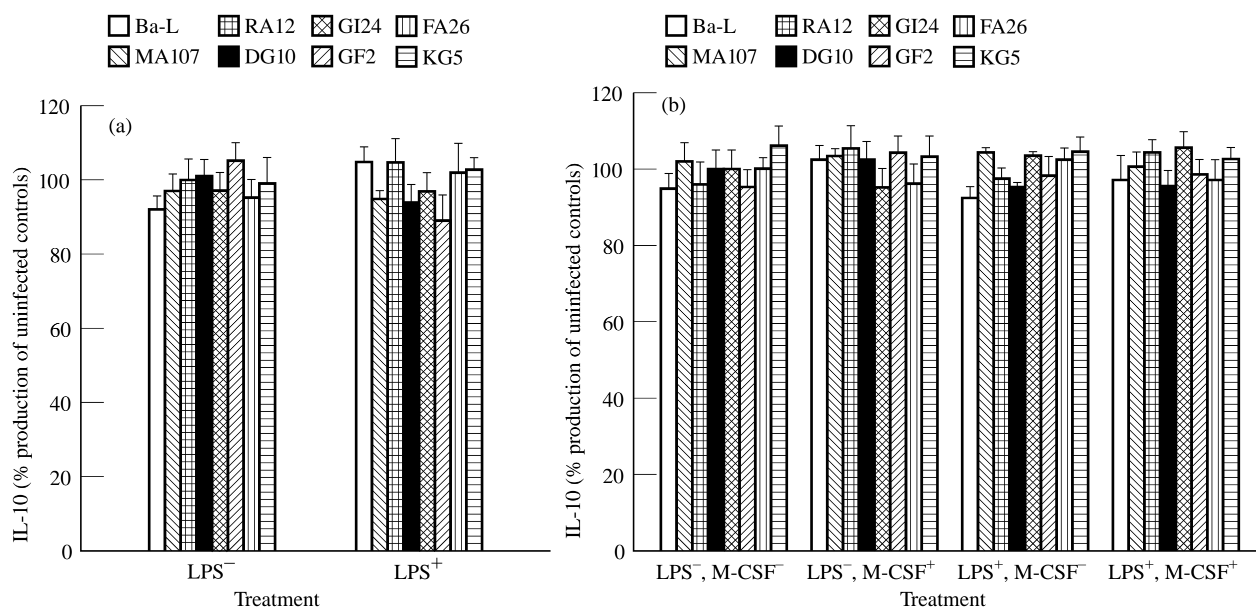

Fig. 2. IL-10 production by monocytes and macrophages infected with different HIV-1 isolates. (a) Monocytes. (b) Macrophages. The levels of IL-10 in uninfected controls were the same as in Fig. 1a,b. Cell cultures were considered infected if the levels of HIV-p24 antigen in the supernatants were equal to or more than those presented in Table 2. The data represent the mean of three experiments each carried out in duplicate. The error bars represent s.e.m.

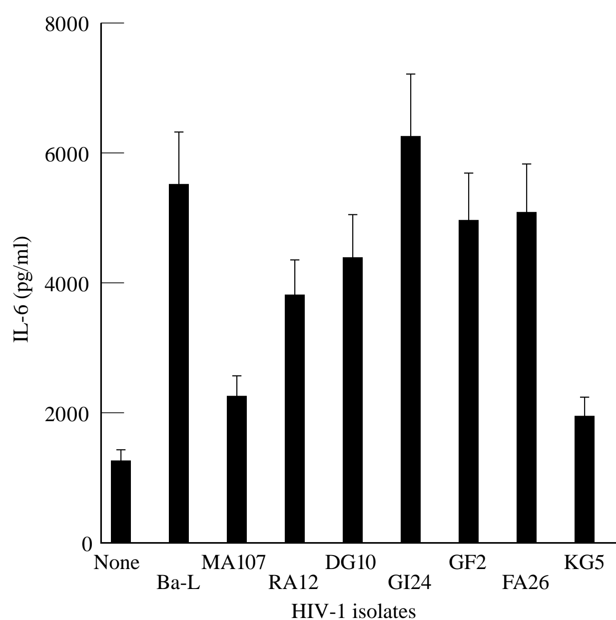

Fig. 3. IL-6 production by HIV-infected macrophages. The cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF) and stimulated by lipopolysaccharide (LPS) when HIV-p24 antigen in the supernatants was equal to or more than that presented in Table 2. The supernatants were harvested after 24 h of incubation. The data represent the mean of three experiments each carried out in duplicate. The error bars represent s.e.m.

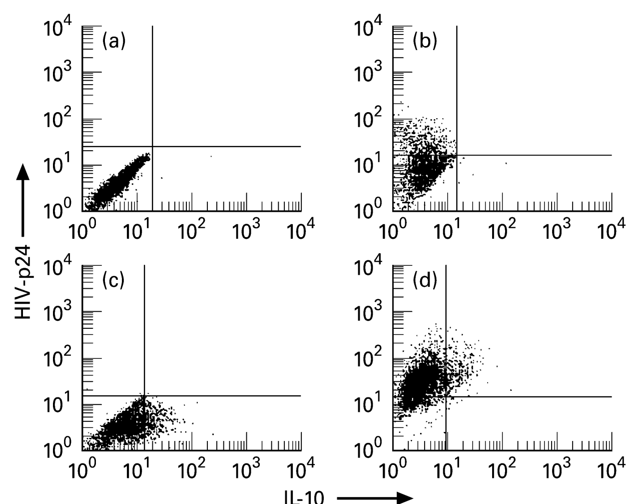

Fig. 4. Cytofluorimetric assessment of individual IL-10- and HIV-p24 antigen-producing cells in lipopolysaccharide (LPS)-stimulated macrophages by specific two-colour, intracellular staining. (a,b) Uninfected and HIV-Ba-L-infected macrophages cultured in the absence of macrophage colony-stimulating factor (M-CSF). (c,d) Uninfected and HIV-Bal-infected macrophages cultured in the presence of M-CSF. The data are displayed as bivariate dot plots. The quadrants were set according to the negative controls (< 1% of the isotype control cells appeared positive). Low left quadrants, unstained cells; upper left quadrants, HIV-p24-stained cells; low right quadrants, IL-10-stained cells; upper right quadrants, cells stained for both IL-10 and HIV-p24. Five thousand to 10 000 cells were analysed for each sample. The data refer to a typical experiment of three performed with similar results.

Table 3. IL-10 production by monocytes and plasma IL-10 levels in HIV-infected patients

Subjects	No. of cases	IL-10 (pg/ml) (monocytes) LPS$^-$	IL-10 (pg/ml) (monocytes) LPS$^+$	IL-10 (pg/ml) (plasma)
HIV$^-$	10	30 ± 26	553 ± 278	10·8 ± 9·2*
HIV$^+$				
(CD4$^+$ T cells/ml)	20	34 ± 31	578 ± 267	29·8 ± 15·6*
> 500 < 200	10	33 ± 27	568 ± 267	24 ± 5
< 200	10	34 ± 36	588 ± 284	35·6 ± 20·4

* $P < 0{\cdot}01$.

monocytes-macrophages is dramatically modulated by LPS and M-CSF stimulation. In the absence of M-CSF, macrophages exhibited a marked decrease of both the expression of membrane CD14 and the sensibility of CD14-expressing cells to LPS stimulation. These results are consistent with those from other authors which reported that monocytes show a time-dependent CD14 loss, and a decreased ability to produce cytokines in response to LPS activation [40–42]. M-CSF treatment significantly enhanced IL-10 production by macrophages by increasing both the expression of membrane-bound CD14 and susceptibility to LPS stimulation. These data confirm and extend the results of a recent report which shows that M-CSF may prime macrophages to an enhanced cytokine release under LPS stimulation, possibly by influencing the expression of CD14 receptor [43].

M-CSF is a haematopoietic growth factor that supports the proliferation and differentiation of bone marrow progenitor cells and enhances the function of mature cells such as macrophages [28–31]. Bioassays performed on blood have shown that endogenous M-CSF levels are very similar to those used here [44,45]. Also, recombinant M-CSF is currently undergoing clinical trials as an anticancer drug [46]. In light of these observations, our data suggest that the effect of M-CSF should be taken into account when cytokine production is studied in monocyte-macrophage cells.

A great number of observations now suggest the critical role of cytokines in the pathogenesis of HIV-1 infection [9–16,39,47,48]. In particular, recent *in vitro* observations have indicated a potential role for IL-10 in HIV-induced immune dysfunction [13–15], and some studies have described the induction of IL-10 during *in vitro* infection of monocytes-macrophages [23–25]. We have tested several viral isolates (primary clinical isolates and laboratory-adapted strains) for their ability to induce IL-10 in monocytes and macrophages, under a broad range of experimental conditions. Our results clearly show that HIV-1 infection did not modulate IL-10 production in these cells. Such differences may rest on the mode of isolation and activation of monocytes-macrophages. Indeed, differently from all the other authors who isolated cells by adhesion, a procedure which led to the recovery of mature and activated monocyte subpopulations, we obtained monocytes by elutriation. Since elutriation is a gentle process that uses physiological media, normal cell viability and function are maintained. Consequently, the cells are not activated or artificially stimulated. Thus, cells purified by elutriation are suitable for studies, such as those on cytokine induction, where cell purity and unaltered cell function are critical.

Another possible explanation for the discrepancy between our results and those reported by others might be that the frequency of infected macrophages was below the threshold required for measurable IL-10 release. This is improbable, however, since IL-10 secretion was not increased also in cultures with $>90\%$ of productively HIV-1-infected macrophages.

Additional experiments with cells from HIV-1-infected individuals were carried out to find out if monocytes contribute or not to IL-10 dysregulation *in vivo*. In accordance with previously reported data [16], we detected significant changes in IL-10 plasma levels in HIV patients. However, monocytes from the same subjects were not found to produce more IL-10 than the uninfected controls. Thus, other cells, such as T lymphocytes, may be responsible for the increased IL-10 levels found in HIV-infected subjects, as previously reported [14,15]. In this regard, in preliminary experiments with multiparametric, three-colour cytofluorimetric analysis, we found that CD8 T cells are the main source of IL-10 in infected patients (A. Bergamini *et al*., unpublished observations).

Taken together, our data do not support the hypothesis of a direct role of HIV-1 in modulating IL-10 production in monocytes-macrophages both *in vitro* and *in vivo*. Moreover, our study emphasizes the role of growth factor activation in the regulation of the cytokine response in monocytes-macrophages.

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