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Increased soluble CD14 serum levels and altered CD14 expression of peripheral blood monocytes in HIV-infected patients

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SUMMARY

Serum levels of soluble CD14 were elevated in HIV-infected asymptomatic patients or those with lymphadenopathy (CDC II/III) $2{\cdot}9 \pm 0{\cdot}8$ mg/*l* compared with normal controls with $2{\cdot}2 \pm 0{\cdot}47$ mg/*l*, $P < 0{\cdot}001$. A further rise was seen in patients with ARC (CDC IVA) $3{\cdot}8 \pm 1{\cdot}1$ mg/*l*, $P < 0{\cdot}01$ and patients with AIDS (CDC IVB–D) $5{\cdot}7 \pm 2{\cdot}5$ mg/*l*, $P < 0{\cdot}01$. Although absolute numbers of $CD14^+$ cells decrease in the AIDS group, the percentage of $CD14^+$ monocytes did not change. In contrast, levels of soluble T cell antigens sCD4 and sCD8, which are higher in HIV-infected patients compared with normal subjects, showed no increase with disease progression. Serum levels of sCD14 were correlated positively with β_2-microglobulin levels ($r_s = 0{\cdot}63$, $P < 0{\cdot}0001$). Whereas the percentage of $CD14^+$ monocytes did not change, an increase in monocytic CD14 expression in HIV-infected patients was observed ($P < 0{\cdot}01$). The percentage of a monocyte subset expressing both CD14 and CD16 increased from 6% in normal healthy persons to 13% in HIV-infected patients ($P < 0{\cdot}001$), and did not vary between the HIV patient groups. Incubation of cultured peripheral blood monocytes with azidothymidine had no effect on either normal or LPS-induced or IL-4-inhibited sCD14 release *in vitro*. Therefore, an effect of AZT on sCD14 serum values *in vivo* is considered to be unlikely. Our data further provide evidence that monocytes/macrophages are engaged in HIV infection.

Keywords HIV infection CD14 antigen expression soluble monocyte antigen serum parameters monocyte subpopulation

INTRODUCTION

The 53-kD membrane antigen CD14 is a glycosyl-phosphatidylinositol (GPI)-anchored cell surface molecule expressed primarily on peripheral blood monocytes and macrophages [1,2]. Granulocytes and neutrophils also show a weak CD14 expression which can be stimulated by several factors [3,4]. While most peripheral blood monocytes express CD14 at a high level, a subpopulation coexpresses low levels of CD14 together with the CD16 antigen (FcγRIII), and shares other phenotypic markers of mature tissue macrophages [5].

A soluble form of CD14 has been detected in cell culture supernatants [1,6] and in human serum and urine [7,8]. Recently, the release of two different forms of sCD14 from a monocyte-like cell line has been reported [9]. Elevated serum levels of sCD14 have been observed under pathological conditions in which activated macrophages may play an important role such as in inflammatory [10] or autoimmune diseases [11].

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The exact physiological function of the CD14 antigen within the immunological network is still under investigation. Early observations, indicating that CD14 may be involved in monocyte activation by lipopolysaccharide (LPS) are supported by various studies showing binding of LPS or LPS/LPS-binding protein complex to CD14 [12–14]. Moreover, LPS-induced activation of cells (endothelia or epithelia) that do not themselves express CD14 is also mediated by soluble CD14 [15–17]. Other studies also suggest involvement of CD14 in monocyte–T cell or monocyte–endothelia interactions [18,19].

Mononuclear phagocytes are major participants in the development of HIV infection: macrophages are primary targets during initial phases of HIV infection due to expression of the CD4 membrane antigen [20]. HIV-infected monocytes/macrophages show some immunological alterations including increased expression of IgG Fc receptor type I (FcRI) [21], a defect in Fc receptor-dependent phagocytosis [22], as well as augmented release of IL-1 activity [23] and IL-1 inhibitory factors [24]. Recently the involvement of CD14 in the control of HIV-1 expression in a latently infected monocyte-macrophage-like cell line was described [25]. Here we report changes in

serum levels of soluble CD14 antigen as well as differential expression of CD14 on peripheral blood monocytes during different progressive stages of HIV infection.

MATERIALS AND METHODS

Subjects

We studied 115 patients (69 male/46 female, mean age 36·3 years) with documented HIV infection. The diagnosis was made on the basis of positive testing for the presence of anti-HIV antibodies, confirmed by Western blot analysis (HIVAG-1 ELISA; Abbott (Wiesbaden, Germany) and New LAV-Blot I; Diagnostics Pasteur (Paris, France)). Using the classification of the Centre for Disease Control (CDC) 25 patients were asymptomatic or had persistent generalized lymphadenopathy (PGL; CDC II/III); 53 patients had ARC (CDC IV A) and 37 patients had AIDS (CDC IV B–D). All patients received 500–1500 mg azidothymidine (AZT) daily. Seventy-eight healthy persons from our clinical staff (38 male/40 female, mean age 35·9 years) were recruited as normal controls.

Reagents

The medium for monocyte culture consisted of RPMI 1640 (GIBCO, Life Technologies, Eggenstein, Germany) with 10% fetal calf serum (FCS), endotoxin not detectable (GIBCO, Life Technologies) and 200 mM glutamine without any antibiotics. LPS (*Escherichia coli* serotype 0127:B8; Sigma, München, Germany) was prepared at a stock concentration of 10 μg/ml in RPMI/FCS. Human recombinant IL-4 with a specific activity of 2×10^6 U/mg was purchased from R&D (Abingdon, UK). AZT (Retrovir; Wellcome) in a solution of 20 mg/10 ml was a gift from Dr K. Schumann (Wellcome, Burgwedel, Germany).

Measurement of sCD14, sCD4, sCD8, IL-1β and β_2-microglobulin

To determine levels of soluble CD14 in serum specimens and cell culture supernatants, a sandwich ELISA (IBL, Hamburg, Germany) was used as described previously [11].

Serum levels of soluble T cell antigens sCD4 and sCD8 were measured using two sandwich ELISAs (CellFree CD4 and CD8) from T Cell Sciences (Cambridge, MA). The sCD4 test was slightly modified to enhance sensitivity by deleting the highest standard point (160 U/ml) and creating an additional standard point in the low concentration range (10 U/ml). The final incubation time for colour development was extended to 30–40 min.

Serum β_2-microglobulin (β_2-M) was determined using a solid-phase time-resolved fluoroimmunoassy based on competition between europium-labelled β_2-M and sample β_2-M for monoclonal anti-β_2-M antibodies (Delfia β_2-micro kit; Pharmacia-Wallac, Freiburg, Germany). Europium fluorescence was measured with a Delfia fluorometer (Delfia, Pharmacia). The assay was done according to the manufacturer's specifications.

IL-1β in culture supernatants was measured with a sandwich ELISA from Immunotec (Dianova, Hamburg, Germany) according to the manufacturer's instructions. In this ELISA an acetylcholinesterase-conjugated MoAb against IL-1β was used for colour development.

Immunofluorescence staining and flow cytometry analysis

Cell phenotyping of peripheral blood mononuclear cells (PBMC) from whole blood collected in tubes containing ethylendiamine tetraacetic acid (EDTA-Monovetten; Sarstedt, Nümbrecht, Germany) was performed by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany). After lysis of erythrocytes, PBMC were incubated with FITC-labelled (CD14, CD4, Becton Dickinson) or PE-labelled (CD16, CD8, Becton Dickinson) MoAbs. After appropriate preparation of cells, monocytes and lymphocytes were gated and analysed separately by two-colour immunofluorescence. CD14 fluorescence intensity was expressed as mean channel of fluorescence (MCF) after subtracting the fluorescence of a corresponding isotype control (IgG2a) from the CD14 antibody staining. All specimens were measured within 1 h after venipuncture, since a time-dependent up-regulation in CD14 expression was observed. Within 60 min after venipuncture the increase was negligible (< 10%). Absolute numbers of monocytes/lymphocytes were calculated using leucocyte counts derived from an automated cell counter (Coulter Counter VS plus; Coulter Electronics Inc., Hialeah, FL).

Preparation of peripheral blood monocytes

Peripheral blood monocytes from normal donors were prepared using a gradient medium with high osmolarity (NycoPrep 1·068; Nycomed, Norway) in which leucocytes expel water and increase their density. Lymphocytes are more sensitive than monocytes and thus sediment further during centrifugation, whereas the monocytes remain at the top of the gradient layer [26]. Blood was collected in EDTA tubes and 1 ml of 6% Dextran T500 (Pharmacia, Freiburg, Germany) in PBS was added to 9 ml EDTA–blood. After standing 40 min at room temperature the leucocyte-rich plasma was removed. A 15 ml centrifugation tube was filled with 4 ml of NycoPrep 1·068 and then the leucocyte-rich plasma was carefully overlaid. After centrifugation for 15 min and 600 **g** at room temperature the monocyte ring was aspirated and the pooled monocyte fractions were then washed twice with ice-cold PBS. The purity of $CD14^+$ monocytes was then evaluated by immunofluorescence microscopy. Cells were labelled with a rhodamine (RD1)-conjugated anti-CD14 MoAb (My 4; Coulter, Krefeld, Germany) and counted in a Neubauer microchamber. On average 77% (69–88%) of the cells were positive for CD14 immunofluorescence.

Treatment of cultured monocytes with AZT, LPS and IL-4

Isolated peripheral blood monocytes were cultured at a density of 2×10^5 cells/ml in RPMI/FCS. Cultures were performed in 48-well plates (Falcon, Heidelberg, Germany) with 0·5 ml cell suspension per well. At this cell density sCD14 values in the culture supernatants fell within the detection range of the sCD14-ELISA (3–100 ng/ml) without any previous dilution. Dilutions of AZT, IL-4 and LPS in RPMI were added in 20 μl/well to give the desired concentration. Culture supernatants from each well were collected, centrifuged at 500 **g** for 5 min and frozen until measurement of sCD14 was performed.

Statistical analysis

All statistical analyses were performed with a software package 'BIAS' version 3.1, established by Dr Hanns Ackermann (Department of Biomathematics, University of Frankfurt/

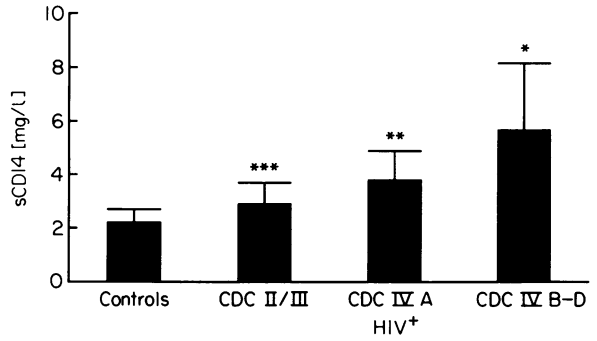

Fig. 1. Serum levels of soluble CD14 (sCD14) in 78 normal controls and 115 HIV^+ patients according to Centres for Disease Control (CDC) classification divided into CDC II/III ($n = 25$), CDC IV A ($n = 53$) and CDC IV B–D ($n = 37$). Statistical analysis was performed according to the Mann–Whitney test. All CDC groups *versus* controls $P < 0{\cdot}001$. ***CDC II/III *versus* controls $P < 0{\cdot}001$; **CDC IV A *versus* CDC II/III $P < 0{\cdot}01$; *CDC IV B–D *versus* CDC IV A $P < 0{\cdot}01$.

Main, Germany). Differences in parameters between two groups were evaluated with the Wilcoxon Mann–Whitney *U*-test. Spearman's rank correlation test was applied to evaluate possible correlations between different study parameters within a group.

RESULTS

Serum levels of sCD14 compared with sCD4/sCD8 and β_2-M

The CD14 antigen level in serum specimens from 78 normal controls was $2{\cdot}2 \pm 0{\cdot}47$ mg/*l* (median 2·2 mg/*l*). The mean sCD14 level in serum specimens from 25 HIV^+ patients with lymphadenopathy was $2{\cdot}9 \pm 0{\cdot}8$ mg/*l* (median 2·6 mg/*l*), which differed statistically ($P < 0{\cdot}001$) from the control value (Fig. 1). A further rise was observed in patients with ARC ($3{\cdot}8 \pm 1{\cdot}1$ mg/*l*, median 3·4 mg/*l*) and patients with AIDS ($5{\cdot}7 \pm 2{\cdot}5$ mg/*l*, median 5·1 mg/*l*). Similar increases have been shown in serum levels of β_2-M (Table 1). A significant correlation between the two parameters could be demonstrated ($r_s = 0{\cdot}63$, $P < 0{\cdot}0001$). Serum values of the soluble T cell antigens CD4 and CD8 were also significantly elevated in HIV^+ patients compared with normal controls, but showed no increase with disease progression. No correlation between sCD4/sCD8 serum values and the number of $CD4^+/CD8^+$ lymphocytes could be detected. Due to the continuous decline in $CD4^+$ cells during progressive HIV infection the constantly high sCD4 levels resulted in an increasing sCD4/$CD4^+$ cell ratio.

Expression of CD14 and CD14/CD16 of peripheral blood monocytes in HIV-infected patients and normal controls

All FACS analyses were performed within 60 min after venipuncture as described above. Representative examples are shown in Fig. 2. The total monocyte counts and $CD14^+$ monocytes did not differ between the normal control group and the CDC II/III and CDC IV A group. However, in the AIDS group (CDC IV B–D) a significant drop in total and $CD14^+$ monocytes was observed (Table 2), whereas the percentage of $CD14^+$ monocytes remained the same in all groups. No correlation was found between the number of $CD14^+$ monocytes and serum levels of sCD14. Determination of mean fluorescence intensity revealed a significantly higher CD14 expression by peripheral blood monocytes in all HIV groups compared with normal controls (Fig. 3). No difference in CD14 fluorescence intensity was found between various HIV groups.

The subpopulation of monocytes expressing both CD14 and CD16 increased significantly. $CD14^+/CD16^+$ cells accounted for 13% of all monocytes in HIV-infected patients compared with 6% in healthy controls. The percentage of $CD14^+/CD16^+$ monocytes did not vary between the different HIV patient groups. In the AIDS group (CDC IV B–D) a minor decline in absolute numbers of $CD14^+/CD16^+$ monocytes was observed. $CD14^+/CD16^+$ monocytes normally express lower levels of CD14 compared with $CD14^+/CD16^-$ monocytes (Fig. 2b). However, in a few cases (four of 41) CD14 expression of this subpopulation was equal to that of regular $CD16^-$ monocytes (Fig. 2c).

Table 1. CD4/CD8 lymphocyte count, soluble CD4 (sCD4), soluble CD8 (sCD8) and β_2-microglobulin (β_2-M) serum levels in subjects with HIV infection according to the Centres for Disease Control classification and normal controls.

	No of subjects	Lymphocytes/μl		sCD4 (U/ml)	sCD8 (U/ml)	β_2-M (mg/*l*)
		$CD4^+$	$CD8^+$			
Normal controls	78	1040 ± 342 Median 923	683 ± 215 Median 640	10·2 ± 6·1 Median 7·6	559 ± 187 Median 555	1·6 ± 0·26 Median 1·6
HIV^+ CDC II/III	25	562 ± 210 Median 568	1112 ± 532 Median 1052	23·7 ± 13·3 Median 21·3 ($P < 0{\cdot}001$)*	1572 ± 564 Median 1439 ($P < 0{\cdot}001$)*	3·3 ± 1·4 Median 2·9 ($P < 0{\cdot}001$)*
CDC IV A	53	224 ± 149 Median 182	843 ± 591 Median 735	20·7 ± 13·5 Median 19·3 ($P < 0{\cdot}001$)* (NS)†	1458 ± 648 Median 1379 ($P < 0{\cdot}001$)* (NS)†	3·8 ± 1·4 Median 3·7 ($P < 0{\cdot}001$)* (NS)†
CDC IV B–D	37	101 ± 159 Median 41	585 ± 141 Median 582	31·2 ± 22·8 Median 25·6 ($P < 0{\cdot}001$)* (NS)‡	1463 ± 624 Median 1287 ($P < 0{\cdot}001$)* (NS)‡	4·4 ± 1·6 Median 4 ($P < 0{\cdot}001$)* (NS)‡

*CDC group *versus* controls; †CDC IV A *versus* CDC II/III; ‡CDC IV B–D *versus* CDC IV A. NS, Not significant.

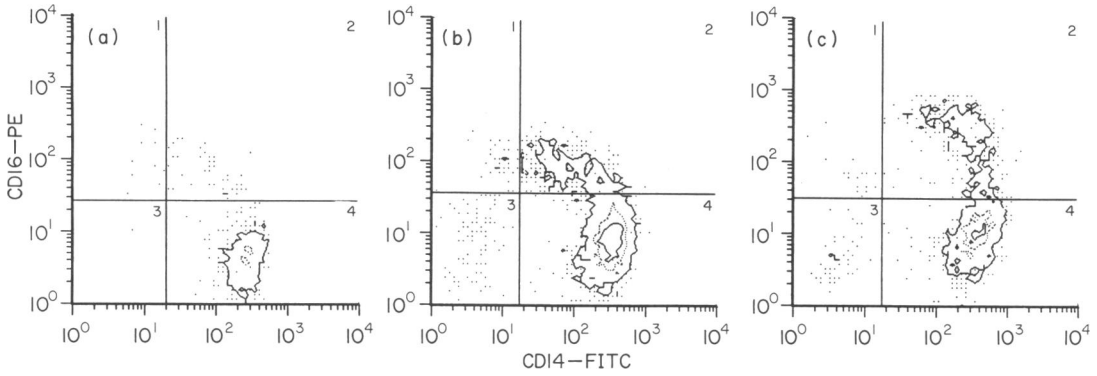

Fig. 2. Monocyte subsets in two HIV-infected patients and a healthy control. Whole blood was stained with CD14–FITC and CD16–PE antibodies and after lysis of erythrocytes, monocytes were first gated by their light scatter profile. Within the monocyte population the gates of CD14–FITC andCD16–PE fluorescence were set as indicated. (a) Healthy subject, 98% $CD14^+$ monocytes (gates 4 and 2), 6% $CD14^+/CD16^+$ monocytes (gate 2). Mean CD14 fluorescence intensity:260 MCF in gate 4 and 41 MCF in gate 2. (b) HIV-infected patient, 96% $CD14^+$ monocytes (gates 4 and 2), 17% $CD14^+/CD16^+$ monocytes (gate 4). Mean CD14 fluorescence intensity: 356 MCF in gate 4 and 48 MCF in gate 2. (c) HIV-infected patient, 96% $CD14^+$ monocytes, 25% $CD14^+/CD16^+$ monocytes. Mean CD14 fluorescence intensity: 389 MCF in gate 4 and 346 MCF in gate 2.

Effect of AZT on sCD14 release of untreated and LPS- or IL-4-treated peripheral blood monocytes

In untreated peripheral blood monocytes sCD14 increased from 24 to 72 h (Table 3). Addition of AZT (1–100 μg/ml) had no effect of the sCD14 shedding at any time. Incubation with LPS resulted in a rise of sCD14 release which was statistically significant at 72 h compared with the control. In contrast, IL-4 down-regulated sCD14 production even at 24 h. Addition of AZT to LPS- or IL-4-treated monocytes had no effect on the stimulating effect of LPS or the inhibitory signal of IL-4 on sCD14 release.

DISCUSSION

An important goal in clinical immunology is to find laboratory parameters to monitor disease progression in HIV infection. Due to the stage-dependent increase in soluble CD14 serum values, sCD14 may represent a reliable candidate. A significant correlation between sCD14 and β_2-M, an accepted index of disease activity, was found. Further studies, however, including longitudinal monitoring, are necessary to support our results.

Expression of membrane-bound CD14 on peripheral blood monocytes was significantly increased during the course of HIV infection. The higher expression of CD14 together with the enhanced release of the CD14 antigen apparently causes the rise in sCD14 serum values, which is consistent with the previous notions that shedding of the soluble antigen reflects a mechanism to modulate CD14 expression [27]. Absolute numbers of $CD14^+$ monocytes did not change in the earlier stages of the HIV infection, and decreased in the AIDS group where sCD14 values are highest. This suggests that sCD14 is released from the monocytic membrane by a shedding mechanism. Similarly, the elevated sCD8 serum concentrations may also stem from an increased release of the soluble antigen by activated $CD8^+$ T

Table 2. Monocyte count, $CD14^+$ and $CD14^+/CD16^+$ subset of peripheral blood monocytes in patients with HIV infection classified by the CDC classification and normal controls.

	No of subjects	Monocytes/μl			Per cent $CD14^+$ monocytes	Per cent $CD14^+/16^+$ monocytes
		Total	$CD14^+$	$CD14^+/16^+$		
Normal controls	19	495 ± 169 Median 493	444 ± 156 Median 425	26 ± 16 Median 20	91 ± 5·1 Median 92	5·4 ± 2·6 Median 5
HIV^+ CDC II/III	13	633 ± 262 Median 572 (NS)*	590 ± 242 Median 546 (NS)*	77 ± 37 Median 68 ($P < 0·001$)*	93·4 ± 2·6 Median 94 (NS)*	12·5 ± 4·7 Median 13 ($P < 0·001$)*
CDC IV A	11	512 ± 244 Median 555 (NS)* (NS)†	494 ± 241 Median 539 (NS)* (NS)†	83 ± 45 Median 70 ($P < 0·01$)* (NS)†	95·5 ± 2·9 Median 95·5 (NS)* (NS)†	13·2 ± 4·8 Median 11·5 ($P < 0·01$)* (NS)†
CDC IV B–D	17	286 ± 145 Median 299 ($P < 0·01$)* ($P < 0·05$)‡	280 ± 120 Median 278 ($P < 0·05$)* ($P < 0·05$)‡	54 ± 36 Median 56 ($P < 0·05$)* (NS)‡	91·2 ± 6·6 Median 93·5 (NS)* (NS)‡	15·8 ± 8·2 Median 13 ($P < 0·001$)* (NS)‡

*CDC group *versus* controls; †CDC IV A *versus* CDC II/III; ‡CDC IV B–D *versus* CDC IV A. NS, Not significant.

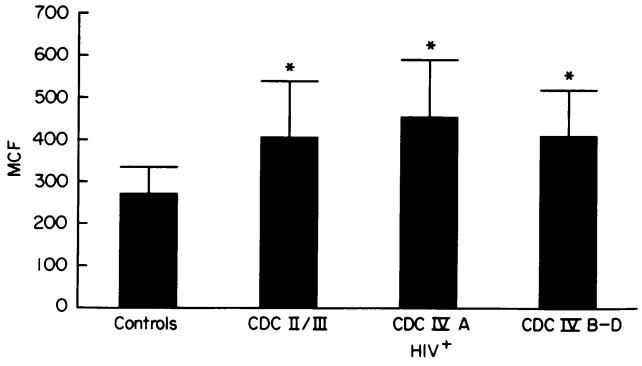

Fig. 3. CD14 expression on peripheral blood monocytes in HIV-infected subjects and normal controls. Blood samples were subjected to FACS analysis within 60 min after venipuncture. Values were expressed as mean channel of fluorescence (MCF). Statistical analysis according to the Mann–Whitney test. *CDC group *versus* controls $P < 0.01$.

cells [28,29]. The pathophysiological background for the elevated sCD4 levels which parallel the dramatic decline in $CD4^+$ T cells remains unknown.

The increased monocytic CD14 expression in HIV infection suggests a direct influence of the HIV virus on infected monocytes. On the other hand, CD14 expression of *in vitro* infected monocytes from healthy blood donors was diminished [30]. Myeloid-monocytic cell lineages infected with HIV produced divergent results concerning CD14 expression [31].

AZT is an antiviral drug administered at relatively high doses (500–1500 mg/day). Since AZT inhibits release of functional IL-1 from cultured monocytes [32], an effect on expression and release of CD14 could not be excluded. However, our *in vitro* studies indicate that AZT does not influence sCD14 levels. LPS is shown to be a potent stimulator of CD14 expression and shedding of the soluble antigen from monocytes *in vitro*. Therefore, elevated LPS concentrations from bacterial infections may also account for increased CD14 expression and release *in vivo*. Opportunistic infections are major criteria for the clinical definition of 'full blown AIDS', which is also characterized by the highest sCD14 values. Chronic exposure to LPS shifts a subpopulation of $CD14^+/CD16^-$ monocytes to express $CD14^+/CD16^+$ [33]. Recently an increase of the $CD14^+/CD16^+$ monocyte subset in sepsis patients was reported [34]. Therefore, the elevated percentage of $CD14^+/CD16^+$ monocytes in HIV infection indicates a further impact of LPS. On the other hand, the significant increase of sCD14 during earlier stages of HIV infection, during which bacterial infections are rare, appears to contradict this view. We have shown, however, that only trace amounts of LPS are necessary to activate monocytes and stimulate sCD14 release *in vitro* (stimulation of cultured monocytes with 1 pg/ml LPS resulted in a significant rise in sCD14 release and IL-1β secretion; unpublished data). We therefore speculate that monocytes in HIV-infected patients are more suspectible to the influence of very low concentrations of LPS.

Expression of membrane-bound CD14 and release of soluble CD14 by monocytes is down-regulated by T cell-derived IL-4 and interferon-gamma (IFN-γ) [35,36]. T lymphocytes from HIV-infected patients express [37,38] and secrete [39] IFN-γ at an increased rate, but are unable to produce IL-4 [39]. In this regard it is of interest that both cytokines modulating CD14 derive from different subsets of T helper cells, IFN-γ from Th1 and IL-4 from Th2 cells. Thus, the recently described Th1 to Th2 switch in T helper cells in the course of an HIV infection [40] may be involved in the change of lymphokine-regulated CD14 expression in HIV-infected patients.

Taken together our results indicate that *in vivo* regulation of CD14 expression and release in HIV-infected patients appears to be a complex issue requiring further investigation.

Table 3. Effect of AZT on the release of soluble CD14 (sCD14) from human blood monocytes. Freshly isolated monocytes from normal donors were cultured either in RPMI alone or in the presence of AZT, lipopolysaccharide (LPS) (*Escherichia coli* serotype 127:B8), IL-4 or a combination of AZT and LPS or IL-4. Soluble CD14 values in culture supernatants represent the mean ± s.d. of four parallel experiments. Statistical analysis was performed according to the Mann–Whitney test. **Versus* control.

	sCD14 release (ng/ml)		
Treatment	24 h incubation	48 h incubation	72 h incubation
Control	19·7 ± 3·4	34·0 ± 4·5	40·2 ± 5·0
AZT (100 μg/ml)	20·1 ± 2·2	34·1 ± 3·3	43·2 ± 2·9
(10 μg/ml)	19·1 ± 2·5	32·4 ± 1·5	43·4 ± 2·4
(1 μg/ml)	20·7 ± 1·2	32·5 ± 1·2	40·3 ± 3·1
LPS (1 ng/ml)	18·9 ± 4·6	37·7 ± 2·7	55·3 ± 1·4 ($P < 0.001$)*
LPS (1 ng/ml) + AZT (10 μg/ml)	19·1 ± 4·9	38·9 ± 3·0	51·4 ± 1·3 ($P < 0.001$)*
IL-4 (50 U/ml)	4·8 ± 0·2 ($P < 0.001$)*	6·2 ± 0·3 ($P < 0.001$)*	7·3 ± 0·3 ($P < 0.001$)*
IL-4 (50 U/ml) + AZT (10 μg/ml)	4·6 ± 0·6 ($P < 0.001$)*	6·4 ± 0·4 ($P < 0.001$)*	7·2 ± 0·2 ($P < 0.001$)*

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