Differential Regulation of Proinflammatory and Hematopoietic Cytokines in Human Macrophages After Infection With Human Immunodeficiency Virus

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Cells of the macrophage lineage (MAC) play an important role in human immunodeficiency virus (HIV) infection. However, the knowledge on the extent of macrophage involvement in the pathogenesis of HIV infection is still incomplete. In this study we examined the secretory repertoire of HIV-infected MAC with respect to the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-8, and the hematopoietic growth factors M-CSF, G-CSF, and granulocyte-macrophage colony stimulating factor (GM-CSF). Using a culture system on hydrophobic teflon membranes, blood-derived MO from healthy donors were infected with a monocytotropic HIV-1 isolate (HIV-1DB1791). We therefore contribute to the pathogenesis of HIV-mediated disease.

TNF-α and IL-1β are produced in response to normal immune stimuli such as immune complexes, lipopolysaccharides (LPS), and phorbol esters. Both molecules are highly inflammatory, can cause fever and loss of appetite, and contribute to a catabolic state. The main physiological functions of the proinflammatory cytokine interleukin-6 (IL-6) are the induction of terminal differentiation of activated B cells into antibody producing cells and the induction of the hepatic acute phase response. IL-8 functions as a chemoattractant for neutrophils, basophils, and T lymphocytes, affects the adhesion of neutrophils to the endothelium, and induces the transendothelial migration of neutrophils. Beside chemoattractant activities IL-8 also activates neutrophils. The hematopoietic cytokines also regulate the proliferation and differentiatiation of hematopoietic progenitor cells. In addition to their proliferative role they contribute in maintaining cell viability, and functionally stimulate mature macrophages and granulocytic cells.

In the present study we investigated the secretory repertoire of MO/MAC after infection with HIV. Our data show a differential regulation of proinflammatory cytokines and hematopoietic growth factors. Although the proinflammatory cytokines tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8 are elevated in HIV-infected cultures, MO/MAC down-regulate their hematopoietic activity, indicated by a decreased secretion of macrophage colony-stimulating factor (M-CSF), granulocyte-CSF (G-CSF), and granulocyte macrophage (GM-CSF).

MATERIALS AND METHODS

Isolation and culture of PBMC. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by density gradient centrifugation, and cultured in supplemented RPMI 1640 with 5% heat-inactivated human AB serum cultured in hydrophobic teflon bags (3 x 10^6 PBMC/mL). The MO-derived MAC were subsequently separated by adherence in 24-well tissue culture plates (Costar, Cambridge, MA). They were further cultured in RPMI 1640 with 5% human AB serum. The adherent cell layer (1 to 2 x 10^6 MAC/well) consisted of up to 95% MAC as judged by morphology, nonspecific esterase staining and expression of CD14 antigen.

Virus isolate and infection of PBMC and MAC. Virus preparations were obtained by propagating the virus in PB T cells and harvesting the culture at the peak of reverse transcriptase activity.
The cell suspension (10^6 cells/mL) was stored in aliquots at -70°C until further use. Only mycoplasma-free virus stocks, tested with a mycoplasma tissue culture DNA probe assay (Gen-Probe, San Diego, CA) were used. Mock stock was prepared from uninfected cells corresponding the protocol for stock virus. The cytokine content of stock virus and mock material was analyzed: the cytokines IL-1β, IL-6, IL-8, and the hematopoietic growth factors M-CSF, G-CSF, GM-CSF could be detected in stock virus as well as in mock material in similar amounts. TNF-α could not be detected in stock virus nor in mock material (data not shown). The PBMC and MO-derived MAC, respectively, were infected with 1 mL stock virus (1 × 10^6 PBMC with reverse transcriptase activity of 500,000 to 800,000 cpm/mL/90') per 10 mL cell suspension in teflon bags at different time points. Data presented are the means ± SEM of a representative experiment done in triplicate.

**Results**

**Infection of human MO/MAC with HIV-1D1171.** In vitro cultures offer the opportunity to study the interaction of MO/MAC with HIV. For this investigations we used a culture system on hydrophobic teflon membranes for blood-borne transmission. Both freshly isolated MO as well as MO-derived MAC from healthy donors were infected with the monocytotropic HIV-1 strain D1171. Successful infection of MO/MAC cultures was monitored by assay for viral antigen or reverse transcriptase activity in the supernatant. Figure 1 shows a representative time course of infection. Virus replication reached a maximum after 3 weeks and was constant for several weeks at a high level with approximately 80 ng/mL HIV-antigen produced within a 24-hour period (24-hour supernatant). A corresponding reverse transcriptase activity of 5.9 × 10^3 cpm/mL/90' was measured on day 22. Immunoperoxidase staining methods using anti-HIV-1-p24 antibodies revealed that early after infection no HIV-1-p24+ cells were detectable (Fig 1B). After 3 weeks, when a productive infection had been established 70% to 80% of MAC were HIV-1-p24+ (Fig 1B). To compensate the decline of cell counts with time of cultivation, the cytokine concentration was normalized to the cell number determined in the culture when harvesting the supernatant and compared to mock-infected cells of the same donor.

**Secretion of proinflammatory cytokines.** When the con-

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*Fig 1. HIV-antigen in HIV-1D1171-infected MO cultures. The cells were infected with HIV-1D1171 at day 1 after start of culture. HIV-antigen concentration was determined in the 24-hour supernatant at the indicated time points. Data presented are the means ± SEM of a representative experiment done in triplicates. (A) HIV-antigen determined with ELISA in the supernatant. (B) HIV-p24+ cells after immunostaining.*
Constitutive and LPS-stimulated secretion of proinflammatory cytokines was analyzed in long-term cultured, virus-replicating cells at day 22 after start of culture. Constitutive secretion could only be detected for IL-8 (Fig 2). This constitutive production of IL-8 was increased in the HIV-infected cultures. On LPS-stimulation for 24-hour MAC released high amounts of TNF-α, IL-6, and further increased the secretion of IL-8 (Fig 2). Comparing the HIV-infected with the uninfected MO/MAC cultures a marked increase in secretion of TNF-α, IL-6, and IL-8 could be observed. IL-1β is downregulated during the initial phase of maturation and remains low in long-term cultured MO/MAC as described. An upregulated proinflammatory reaction could also be demonstrated early after infection when a productive infection has not yet been established. Figure 3 shows the LPS-stimulated secretion of proinflammatory cytokines at day 4. The maximum TNF-α secretion could be observed after 6 hours LPS stimulation (Fig 3A); the IL-8 amount is similar in the 6 hour and 24 hour supernatant (Fig 3D); IL-6 increased from 6 hours to 24 hours (Fig 3C) and low amounts of IL-1β were only detectable in the 24-hour supernatant (Fig 3B). The HIV-infected MO/MAC constantly showed an increased secretion of all four cytokines. Secretion of proinflammatory cytokines was analyzed during the course of cultivation and infection. Figure 4A shows the secretion of TNF-α and HIV-antigen production at day 4, 8, and 22 after start of culture (corresponding to day 3, 7, and 21 after infection with HIV). Early after infection when HIV-antigen production in the infected cultures could not be detected yet (day 4) or is still low (day 8) as well as in later stages when a productive infection...
infection has been established (day 22), secretion of TNF-α was increased in the infected cultures (Fig 4A). Similar results were obtained with IL-6 and IL-8 (data not shown). When MO-derived MAC were infected at day 6 after start of culture, again an elevated secretion of TNF-α, IL-6, and IL-8 in the HIV-infected MAC was observed (Fig 5).

**Secretion of hematopoietic growth factors.** The stimulated secretion of hematopoietic growth factors was investigated early during MO to MAC maturation as the secretion of M-CSF is suppressed by LPS beyond day 6 in culture. Table 1 shows the secretion of hematopoietic growth factors by MO on day 2. Only control MO secreted M-CSF constitutively whereas in HIV-infected MO cultures no M-CSF could be detected without stimulation. When LPS was added, an increasing response to LPS during the time of stimulation was observed in the uninfected cultures whereas the HIV-infected MO showed no M-CSF secretion. No constitutive production of G-CSF and GM-CSF was seen neither in control nor in HIV-infected MO, respectively. On LPS stimulation G-CSF and GM-CSF were secreted by HIV-infected MO at a significantly lower amount compared to the uninfected control cells. Secretion of hematopoietic growth factors G-CSF and GM-CSF was also analyzed during the course of cultivation and infection. Figure 4B shows the secretion of G-CSF and HIV-antigen production at day 4, 8, and 22 after start of culture (corresponding to day 3, 7, and 21 after infection with HIV). As the upregulation of proinflammatory cytokines (shown for TNF-α in Fig 4A) the downregulation of hematopoietic growth factors (shown for G-CSF in Fig 4B) after infection with HIV could be observed early after infection when HIV-antigen production in the infected cultures could not be detected yet (day 4) or is still low (day 8) as well as in later stages when a productive infection has been established (day 22). Similar results were obtained with GM-CSF (data not shown). The response of LPS-induced hematopoietic growth factor production was even more pronounced in mature MAC (Fig 6).

**DISCUSSION**

We have shown that MO and MAC, which have been infected with HIV in vitro showed an increased secretion of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) in long-term cultured, virus-replicating cells, as well as early after infection when a productive infection has not yet been
Table 1. Secretion of Hematopoietic Growth Factors by HIV-Infected MAC*

<table>
<thead>
<tr>
<th></th>
<th>M-CSF (pg/10^5 MOI)</th>
<th>G-CSF (pg/10^5 MOI)</th>
<th>GM-CSF (pg/10^5 MOI)</th>
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<tbody>
<tr>
<td></td>
<td>Control†</td>
<td>HIV</td>
<td>Control†</td>
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<td>With LPS</td>
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<td>1 h</td>
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<td>&lt;20</td>
<td>&lt;10</td>
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<tr>
<td>6 h</td>
<td>79 ± 3</td>
<td>&lt;20</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>24 h</td>
<td>1,955 ± 191</td>
<td>25 ± 1</td>
<td>270 ± 22</td>
</tr>
<tr>
<td>Without LPS§</td>
<td>444 ± 45</td>
<td>&lt;20</td>
<td>&lt;10</td>
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<td>24 h</td>
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* After isolation the MO were infected or mock-infected with HIV-1D117111 (day 0 after start of culture), cultured for 2 days and then stimulated with 100 ng/mL LPS (S abortus equi).
† Data presented are the mean ± SEM of a single representative experiment done in triplicate out of three experiments.
‡ The control MO were mock-infected.
§ For constitutive secretion the medium was changed 24 hours before obtaining the supernatant.

established. Concomitant with this upregulation of proinflammatory cytokines, HIV-infected MO/MAC downregulate their hematopoietic activity, reflected by a decreased secretion of M-CSF, G-CSF, and GM-CSF.

Proinflammatory cytokines, which may increase host resistance to viral or bacterial infections when released in moderate amounts, are detrimental in the context of AIDS. HIV replication can be upregulated by proinflammatory cytokines like TNF-α, IL-1β, and IL-6. In addition alterations in the inflammatory gene expression caused by HIV infection could alter immune responses, cause tissue damage by increasing oxidant release from leukocytes, inducing expression of acute-phase proteins, leading to a cachectic state and even upregulate virus replication.

Our results about the secretion of proinflammatory cytokines by HIV-infected MO/MAC add to the controversy that arises from the conflicting results published. Several reports have shown induction of proinflammatory cytokines by in vitro infected MO/MAC by HIV infection. However, others have presented conflicting data and do not find an altered mRNA expression or cytokine release in MO infected with HIV in vitro. Roy et al. reported a decreased LPS-stimulated IL-1 secretion by HIV-infected MO. Several differences in experimental techniques may account for these discrepancies, eg, culture conditions, different HIV isolates, the use of cell lines rather than primary MAC, the point of time for infection as well as for cytokine analysis, etc. In our system MO and lymphocytes were cultivated together only for the first days but the virus inoculum and the lymphocytes were removed after the infection. This procedure has proven to yield very reproducible data comparing cells of the same donor. We found a variability between primary cells isolated from different donors concerning viral replication and level of cytokine secretion, but comparison of uninfected and infected cultures from cells of the same blood donation resulted in reproducible findings concerning the kind of effect (upregulation of proinflammatory cytokines and downregulation of hematopoietic growth factors). Furthermore, the infection of primary MO/MAC should yield responses that are much closer to the natural situation than studies with cell lines.

Mononuclear phagocytes are key effectors of the inflam-
matory component of the cellular immune response and, through cytokine production, interact with all phases of the immune system. During a normal immune response proinflammatory cytokines are involved in coordinating activities of T cells and B cells, as well as in directing cytotoxicity. Therefore, reduced expression of any of these inflammatory proteins could lead to an impaired immune response whereas their overexpression may induce fever, cachexia and neurological symptoms. The fever and wasting syndrome seen in AIDS patients may be well related to enhanced production of TNF-α and IL-1. In addition, IL-1 and TNF-α may also be involved in the pathogenic mechanisms of Kaposi sarcoma. Furthermore the upregulated IL-6 secretion might explain the chronic B-cell activation and elevated Ig production in HIV-infected patients. Gastrointestinal disorder like chronic diarrhea and malabsorption syndrome are often observed in AIDS patients in the absence of any known pathogens. HIV infection of enterochromaffin cells in the intestinal mucosa might deregulate motility and digestive functions of the intestine. In addition, the possible involvement of MAC-derived cytokines in these disorders in the lamina propria should be considered. IL-8 as a chemoattractant may contribute to perivascular leukocytic accumulations observed in various tissues (kidney, heart, gastrointestinal tract, and central nervous system [CNS]) of HIV-infected patients. An elevated IL-8 production especially by alveolar macrophages of HIV patients with nonspecific interstitial pneumonitis was reported.

Conflicting results have also been published on the cytokine activity in vivo. Elevated serum levels of proinflammatory cytokines were detected in HIV-infected patients. In addition PB cells from HIV-infected patients cultured in vitro also release high levels of cytokines as do tissue macrophages. However, Jones et al. reported normal concentration of cytokines in serum. Also a decreased production of TNF-α by alveolar MAC or IL-1 by MO from patients with AIDS is reported there.

Apart from these conflicting results on the influence of HIV infection on proinflammatory cytokines there are also conflicting reports about the regulation of hematopoietic growth factor production during HIV infection. In contrast to our results Molina et al. described an unchanged GM-CSF production by in vitro HIV-infected MO/MAC. However, Molina et al. analyzed the total cytokine content (cell-associated and secreted). Probably the decrease of GM-CSF in the supernatant is based on a dysregulated secretion. Intra-cellular detection by immunochemistry showed that GM-CSF and G-CSF are produced constitutively but released only upon stimulation (Esser et al., 1996, submitted). The results of Molina et al. could be due to the unchanged, cell-associated GM-CSF. Conflicting results exist also on the production of GM-CSF in HIV-infected patients. Although some investigators described low levels of GM-CSF production, Hober et al. found normal or even high levels of GM-CSF. In contrast to in vitro studies the cause of cytokine dysregulation in HIV-infected patients could be more complex: donor-dependent factors as well as opportunistic infections can contribute to the dysregulated cytokine response. When cells of healthy donors were infected with HIV in vitro the observed cytokine dysregulation could be attributed to HIV-infection.

Hematologic abnormalities have been documented in all stages of HIV-infection and constitute of anemia, granulocytopenia, and thrombocytopenia. GM-CSF plays a critical role in pulmonary homeostasis. Pathologic analysis of knockout mice lacking GM-CSF showed a progressive accumulation of surfactant lipids and proteins in the alveolar space, the characteristic of the idiopathic human disorder pulmonary alveolar proteinosis also seen in HIV-infected patients. This alveolar proteinosis is secondary to a deranged surfactant catabolism or clearance.

The mechanism by which HIV mediates the differential regulation of cytokine production as described here is not clear. Some reports showed that productive infection of the target cells is not necessary to observe an increased cytokine production after infection with HIV. Inactivated virus as well as lymphocytotropic HIV-1 isolate are able to induce cytokine production. Blocking experiments with sCD4 could show that the viral binding to the MAC surface is necessary and sufficient to account for a signal transduction pathway resulting in cytokine expression. Induction of cytokine production by native gp120 and monoclonal antibody to CD4 as well as their inhibition by neutralizing antibodies against gp120 reveal evidence for the important role of binding of HIV-1–gp120 to the cellular CD4 molecule. In summary a biphasic impact of the virus on monocyte cytokine production appears to occur: initial interaction of the monocyte–CD4 with HIV-1–gp120 results in a signal leading to transient monocyte activation and a subsequent upregulation during viral replication, possibly mediated by viral products. Viral proteins other than gp120 also contribute to cytokine induction after HIV infection. Recent reports showed that, eg, HIV-1–tat, the viral transactivating protein can not only be released from infected cells and taken up by uninfected cells, but may also induce TNF-α, IL-1α, IL-1β, and IFN-γ in a dose-dependent manner. Another regulatory protein, HIV-1–nef was shown to induce IL-6 mRNA in PBL.

Our findings that cytokine production is altered early after infection with HIV as well as in long-term cultured, virus replicating cells are consistent with the theory of a biphasic impact of the virus on cytokine production described.

To conclude, our results show the differential regulatory effects of HIV on proinflammatory cytokines and hematopoietic growth factor. Such effects on cytokines could alter overall immune function as well as virus reactivation and pathogenesis, including virus-associated immunodeficiency states.

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