Alterations in Expression of Monocyte Chemotactic Protein–1 in the Simian Immunodeficiency Virus Model of Disseminated *Mycobacterium avium* Complex

Elizabeth E. Hendricks,1,2 Kuei-Chin Lin,2 Karen Boisvert,2 Douglas Pauley,2 and Keith G. Mansfield2

1Department of Pathobiology and Veterinary Sciences, University of Connecticut, Storrs; 2New England Regional Primate Research Center, Harvard Medical School, Southborough, Massachusetts

*Myobacterium avium* complex (MAC) infection is the most common disseminated bacterial infection in untreated patients with acquired immunodeficiency syndrome (AIDS). We investigated the potential role of monocyte chemotactic protein–1 (MCP-1) in the pathogenesis of disseminated MAC, using the simian immunodeficiency virus (SIV)/macaque model of AIDS. Macaques were inoculated with SIV, followed by challenge with a pathogenic AIDS isolate of *M. avium* 14 days later. After challenge with *M. avium*, marked increases in serum MCP-1 levels were detected in SIV-infected macaques, a finding that was duplicated in coinoculated bronchoalveolar macrophages. MCP-1 levels were significantly higher in SIV-infected macaques than in non-SIV-infected controls (327.1 vs. 151.5 pg/mL, respectively; \( P < .04 \)), suggesting that up-regulation of MCP-1 may play a role in the development of progressive mycobacterial disease. Similarly, morphometric analysis revealed increased expression of MCP-1 in hepatic microgranulomas from SIV-infected macaques. We conclude that the pronounced increases in MCP-1 levels demonstrated in tissue and serum samples after *M. avium* inoculation may play a role in the development of disseminated mycobacterial disease.

*Myobacterium avium* complex (MAC) infection is the most common disseminated bacterial infection in untreated patients with AIDS [1, 2]. In contrast, disseminated MAC infection is rare in individuals without HIV infection, and the precise mechanism for the unique susceptibility to MAC, in patients with AIDS, has not been fully elucidated. Recent evidence suggests that host, lentiviral, and mycobacterial factors all contribute to the development of MAC infection [3]. The hallmark of disseminated MAC infection is the formation of granulomas in multiple organs—including the liver, spleen, lymph nodes, bone marrow, and lung—and sheets of epithelioid macrophages within the lamina propria of the small and large intestines [1, 2, 4–6]. Formation of granulomas is dependent on sequential release of specific cytokines and chemokines, which activate and chemoattract T lymphocytes and monocytes, the cellular components of granulomas.

A potent chemoattractant of monocytes and T lymphocytes is the \( \beta \)-chemokine (CC-chemokine) monocyte chemotactic protein–1 (MCP-1; CCL2) [7–10]. MCP-1 has been implicated in the recruitment of monocytes and T lymphocytes in a variety of diseases in which these inflammatory cells play a critical role in the development of disease. Most notable among these conditions are multiple sclerosis, atherosclerosis, and rheumatoid arthritis, which are characterized by inflammation and in which the primary cell type is of monocyte lineage [11–13]. In addition, recent studies have demonstrated a significant connection between increased levels of MCP-1 in the cerebrospinal fluid (CSF) of human patients with AIDS-related dementia and HIV-associated encephalitis.
rapidly induce [22–24]. We previously described an experimental system to clinical and pathologic findings and a similar source of infection with the disease in human patients with AIDS, including similar Disseminated MAC in macaques shares extensive similarities Naturally disseminated MAC disease also occurs in rhesus manta.

in SIV-infected macaques [6]. This experimental model of MAC in AIDS provides an excellent tool to study the pathogenesis of this disease process. In the present study, we investigate the potential role of MCP-1 in the development of experimentally disseminated MAC, using this simian model of AIDS.

MATERIALS AND METHODS

Macaques and inoculations. Rhesus macaques (Macaca mulatta) were housed at the New England Primate Research Center, in a centralized biolevel 3 animal-containment facility, in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and Harvard Medical School’s Animal Care and Use Committee. Macaques were tested before assignment to experimental protocols and were found to be free of simian retrovirus type D, SIV, simian T-lymphotropic virus–1, and herpes B virus. The macaques were fed a commercially available monkey chow and were supplied with autoclaved water ad libitum, to reduce environmental exposure to mycobacteria. Macaques were inoculated intravenously (iv) with 25 ng of p27 of SIVmac on day 14 (SIVmac251 and related clones) and then were challenged iv with 10^6 cfu of a simian AIDS M. avium isolate (88415) 14 days later (day 0), as described elsewhere [6]. This time point was chosen to coincide with the period of peak viremia and reproducibly results in progressive mycobacterial disease. An additional 4 non-SIV-infected macaques were similarly challenged with M. avium. When the macaques became moribund, they were euthanized by the veterinary staff. A cohort of 12 SIV-infected macaques not challenged with M. avium served as controls.

All macaques had liver biopsies performed before M. avium inoculation and at days 14 and 42 after M. avium inoculation. Blood samples were obtained from macaques before SIV inoculation (day 14), before M. avium inoculation (day 0), and on days 14, 28, and 42 after M. avium inoculation. Tissue samples were processed for routine histopathologic analysis, acid-fast stains, and immunohistochemical analysis. Blood samples were processed for complete blood counts, serum chemical analysis, virus isolation, lymphocyte subset analysis, and MCP-1 ELISA. Immunologically healthy macaques (n = 4) were inoculated with M. avium and served as controls. All macaques were euthanized between days 42 and 50. Complete postmortem examinations were performed on all macaques, and representative tissue samples were obtained for formalin-fixation, freezing at optimum cutting temperature, and snap-freezing. Tissue samples were stained with hematoxylin-eosin, for routine evaluation, and were stained with Ziehl-Neelson acid-fast stain, for evaluation of the distribution of mycobacterial organisms.

Coinfection of macaque BAL macrophages with SIV and M. avium. BAL fluid was obtained from 3 healthy macaques, and alveolar macrophages were isolated by use of a protocol described elsewhere [25]. The macrophages were then plated into 24-well plates (VWR Scientific Products), at concentrations of 0.25 × 10^6 cells/mL and 2 mL/well. Macrophages were infected with SIVmac239 or SIVmac251, at concentrations of 20 ng/well, by use of a technique described elsewhere [26]. Macrophages were inoculated 96 h later with 1 × 10^7 cfu/well of the pathogenic AIDS isolate of M. avium, 88415. Cell-free supernatant was obtained for the MCP-1 ELISA, on days 0, 4, 7, 11, 14, 21, and 28 after M. avium inoculation and before SIV inoculation.

MCP-1 ELISA. The ELISA for MCP-1 was performed using a commercially available kit provided by R&D Systems. In accordance with the manufacturer’s protocol, MCP-1 was measured in serum from SIV-infected and immunologically healthy macaques infected with M. avium and in culture supernatant from BAL-derived macrophages coinoculated with SIV and M. avium. In brief, serum samples and culture supernatant were diluted 10-fold in the provided diluent. A total of 200 μL of each sample was added to individual wells coated with the specific antibody, followed by a 2-h incubation at room temperature. The plates were then washed, and the specific antibody for MCP-1 was then added to each well and incubated for 2 h at room temperature. The substrate solution was then added.
In vitro and in vivo alterations in monocyte chemotactic protein–1 (MCP-1) production. A, In vitro simian immunodeficiency virus (SIV) infection of bronchoalveolar lavage (BAL) macrophages induced a slight but statistically significant increase in MCP-1 production. In contrast, Mycobacterium avium inoculation of BAL macrophages induced a marked, acute increase in MCP-1 production. B, In rhesus macaques coinoculated with SIV and M. avium, there were no significant differences in mean serum MCP-1 levels between 2 weeks (day of SIV infection) and 0 weeks (day 14 after SIV infection; day of M. avium challenge [day 0]). There were significant increases in mean serum MCP-1 levels at 4 and 6 weeks after M. avium infection. C, Significant differences were observed in serum MCP-1 levels between SIV-infected macaques and non-SIV-infected macaques challenged with M. avium, suggesting a role for MCP-1 in the development of progressive disease. Box plots representing the 25th and 75th percentiles of data points: *, Student’s t test; ‡, Mann-Whitney rank sum test. MAC, M. avium complex.

Figure 1. In vitro and in vivo alterations in monocyte chemotactic protein–1 (MCP-1) production. A, In vitro simian immunodeficiency virus (SIV) infection of bronchoalveolar lavage (BAL) macrophages induced a slight but statistically significant increase in MCP-1 production. In contrast, Mycobacterium avium inoculation of BAL macrophages induced a marked, acute increase in MCP-1 production. B, In rhesus macaques coinoculated with SIV and M. avium, there were no significant differences in mean serum MCP-1 levels between 2 weeks (day of SIV infection) and 0 weeks (day 14 after SIV infection; day of M. avium challenge [day 0]). There were significant increases in mean serum MCP-1 levels at 4 and 6 weeks after M. avium infection. C, Significant differences were observed in serum MCP-1 levels between SIV-infected macaques and non-SIV-infected macaques challenged with M. avium, suggesting a role for MCP-1 in the development of progressive disease. Box plots representing the 25th and 75th percentiles of data points: *, Student’s t test; ‡, Mann-Whitney rank sum test. MAC, M. avium complex.
Table 1. Comparison of mean number of colony-forming units, mean microgranuloma (MG) area, and mean percentage of area of microgranulomas positive for monocyte chemotactic protein–1, between simian immunodeficiency virus (SIV)–infected and immunologically healthy macaques infected with Mycobacterium avium.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SIVmac positive</th>
<th>SIVmac negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Liver biopsy specimen, cfu/mg</td>
<td>$6.67 \times 10^4$</td>
<td>$6.08 \times 10^2$</td>
</tr>
<tr>
<td>MG area, mean, $\mu m^2$</td>
<td>7442.90</td>
<td>14,413.30</td>
</tr>
<tr>
<td>MG area positive for MCP-1, %</td>
<td>1.48</td>
<td>0.29</td>
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RESULTS

**Increased production of MCP-1 from BAL macrophages inoculated with M. avium.** To examine the effects of *M. avium* and SIV infection on the production of MCP-1 by macrophages, we collected BAL macrophages from healthy rhesus macaques and inoculated them in vitro with SIVmac251 or SIVmac239, followed by mycobacterial challenge 96 h later. SIV-infected macrophages not inoculated with *M. avium* and normal macrophages inoculated and not inoculated with *M. avium* served as controls. Cell-culture supernatants were tested for MCP-1 by use of ELISA (figure 1A). In replicates (n = 6), BAL-derived macrophages had a slightly but statistically significant increase in production of MCP-1 at the time of *M. avium* inoculation 4 days after SIV infection (82.4 vs. 92.0 pg/mL, respectively; P = .03, Mann-Whitney rank sum test). In contrast, there was a marked increase in the production of MCP-1 in macrophages infected with *M. avium* by 3 days after inoculation (98.7 vs. 1045.0 pg/mL, respectively; P < .001, t test; figure 1A). After this acute increase in MCP-1 levels, levels temporarily decreased in *M. avium*–inoculated groups before increasing again at later time points. *M. avium* infection induced a more pronounced increase in MCP-1 production than did SIV infection alone. MCP-1 levels began to increase in all treatment groups by days 14–21, and the increased levels were then sustained throughout the culture period. Similar results were obtained regardless of the timing of mycobacterial challenge or the SIV strain used.

**Increased production of MCP-1 in SIV-infected macaques inoculated with M. avium.** All macaques inoculated with *M. avium* developed disseminated mycobacterial disease, evident by granulomatous hepatitis, by day 14 after inoculation. Histologic examination of hepatic biopsy specimens from immunologically healthy macaques demonstrated multifocal microgranulomas that were negative for acid-fast organisms, by a Ziehl-Neelson stain, and negative for mycobacterial organisms, by quantitative culture, by day 42 after *M. avium* inoculation (table 1). Conversely, SIVmac-inoculated macaques had demonstrable acid-fast bacilli within hepatic microgranulomas and positive quantitative culture results, at all time points after *M. avium* inoculation (table 1). Serum MCP-1 levels did not increase acutely after SIV inoculation (P = .69, t test; figure 1B). In contrast, after *M. avium* challenge, serum MCP-1 levels increased significantly at 4 and 6 weeks after inoculation (P = .016 and P = .008, respectively, t test; figure 1B).

To further examine this relationship, serum MCP-1 levels were compared with those for both immunologically healthy macaques challenged with *M. avium* and SIVmac-infected macaques not challenged with *M. avium*. At 4 weeks after inoculation, coinoculated macaques had significantly higher MCP-1 levels (327.1 pg/mL) than did immunologically healthy *M. avium*–infected macaques (151.5 pg/mL; P = .042, Mann-Whitney rank sum test; figure 1C). Similarly, coinoculated macaques had higher MCP-1 levels than did SIVmac-infected macaques not inoculated with *M. avium* (103.1 pg/mL; P = .024, Mann-Whitney rank sum test; figure 1C). Evaluation of a historical cohort of SIV-inoculated macaques that had died from AIDS (n = 19) revealed a wide range of serum MCP-1 levels (107.9–588.9 pg/mL) at death, which were statistically higher than preinoculation levels (P = .003; Mann-Whitney rank sum test).

**Increased expression of MCP-1 within hepatic microgranulomas of SIV-infected macaques inoculated with M. avium.** After mycobacterial challenge, microgranulomas were found disseminated throughout the hepatic parenchyma in both SIV-infected (n = 10) and healthy control macaques (n = 4). Quantitative culture demonstrated higher numbers of mycobacterial colony-forming units in liver samples from macaques coinoc-
ulate with SIV and *M. avium*, compared with immunologically healthy macaques inoculated with *M. avium* (table 1). We next compared the in vivo expression of MCP-1 in hepatic microgranulomas from these immunologically healthy macaques and SIV-infected macaques inoculated with *M. avium*, by use of immunohistochemical and morphometric analysis. Immunohistochemistry revealed granular cytoplasmic MCP-1 staining within discrete cells that was morphologically consistent with that in macrophages. Double-label confocal laser microscopy demonstrated colocalization of the macrophage marker CD68 and MCP-1, confirming macrophages as the cell type producing MCP-1 (figure 2).

Morphometric analysis also demonstrated a temporal expression and down-regulation of MCP-1. In all examined macaques, MCP-1 expression was highest at day 14 after inoculation and then decreased significantly between days 14 and 42 (day 14 [1.48% area positive] vs. day 42 [0.66% area positive]; *P* < .001, Mann-Whitney rank sum test). The expression of MCP-1 in hepatic microgranulomas at day 14 was significantly higher in SIV-infected macaques (1.48% area positive) than in immunologically healthy macaques (0.29% area positive) (*P* < .001, Mann-Whitney rank sum test; table 1). There was minimal expression of MCP-1 in uninfected liver samples, with rare positive cells located only within the portal areas.

**DISCUSSION**

In the present study, we have examined the effects of SIV and *M. avium* infection on MCP-1 production, both in vitro and in vivo. Using macaque BAL-derived macrophages, we have shown that SIV and *M. avium* infection induce MCP-1 production by mononuclear phagocytes in vitro. Similarly, *M. avium* induces increased production of MCP-1 in vivo, and this increase is higher in SIV-infected macaques than in immunologically healthy macaques. We have demonstrated that MCP-1 is expressed in hepatic microgranulomas from macaques inoculated with *M. avium* and that this expression is higher in SIV-infected macaques than in immunologically healthy macaques. Furthermore, we have demonstrated that there is a temporal pattern to this expression of MCP-1 in *M. avium*-associated hepatic microgranulomas, with higher levels seen at day 14 after inoculation, compared with those at day 42 after inoculation.

MCP-1 is produced by a variety of cells—including macrophages, microglia, and endothelial cells—and its actions are executed through the binding of its receptor, CCR2 [7, 8]. CCR2 is expressed on monocytes, immature dendritic cells, T lymphocytes, basophils, NK cells, fibroblasts, and endothelial cells [7, 8]. Although CCR2 is expressed on many different cell types, MCP-1 is primarily chemotactic for cells of monocyte lineage, and it has been found to be the most potent chemotactic agent for these cells [7, 8].

Recent studies have demonstrated that MCP-1 is an important mediator in macrophage-mediated diseases, such as atherosclerosis, multiple sclerosis, HIVE, and SIVE [11–15]. For example, increased levels of MCP-1 in CSF from SIV-infected macaques predicts the development of SIVE [15]. Furthermore, HIV-infected individuals possessing the MCP-1 Δ2578G allele have been shown to be at increased risk for development of HIVE and disseminated MAC [14]. Although the mechanism is unknown, the MCP-1 Δ2578G allele is a genetic marker for greater MCP-1 transcriptional activity and protein production, suggesting that enhanced expression of MCP-1 may play a role in the development of macrophage-associated disease. We noted a wide range of serum MCP-1 levels in a historical cohort of macaques that had been inoculated with SIV and progressed to AIDS. The reason for this variation is probably multifactorial but, certainly, could include polymorphisms, as described for humans.

Other studies have demonstrated that MCP-1 production is under negative-feedback control induced by interferon (IFN)–γ, but not by tumor necrosis factor (TNF)–α [29, 30]. It is possible that production of MCP-1 by macrophages is under similar negative-feedback control induced by IFN-γ, which would explain the decrease in levels of MCP-1 at later time points in vivo. In contrast, in our in vitro experiment, BAL macrophages were not stimulated with IFN-γ but were exposed to TNF-α produced in response to both SIV and *M. avium* infection. MCP-1 production induced by TNF-α does not result in a refractory state of the MCP-1 gene, as does IFN-γ induction [30]; therefore, continued exposure to TNF-α could result in the persistent increase in levels of MCP-1 that we observed in vitro.

Infection with HIV or SIV before or coincident with *M. avium* infection considerably enhances susceptibility to the development of disseminated MAC. As we have demonstrated here, coinfection with SIV and *M. avium* results in significantly increased levels of MCP-1 in serum and tissue samples. Acute SIV infection has been shown to induce systemic immune activation, with increased production of inflammatory cytokines, including TNF-α [31], which would result in increased macrophage activation and MCP-1 production. The central role of MCP-1 in monocyte and macrophage trafficking may contribute to the recruitment of infected cells to different sites throughout the body, consequently contributing to the development of disseminated MAC in coinfected macaques.

The differential expression of MCP-1 in hepatic microgranulomas from SIV-infected and non-SIV-infected macaques inoculated with *M. avium* is consistent with the serum MCP-1 levels. SIV-infected macaques had higher expression of MCP-1 in tissue samples than did immunologically healthy macaques, and expression was highest at day 14 after *M. avium* inoculation. The temporal pattern of MCP-1 expression is consistent with findings of previous studies, which demonstrate that MCP-
1 production peaks early in granulomatous disease and then decreases [32]. This finding supports the hypothesis that MCP-1 plays a role in the development of MAC and that its expression is highest early in infection, when the recruitment of macrophages is at its peak.

In addition to contributing to the development of MAC, increased production of MCP-1 in MAC may also contribute to the pathogenesis of SIV or HIV infection [14, 15]. Recent studies of mycobacterial infection in HIV-infected patients have demonstrated that increased production of MCP-1 induced by mycobacteria enhances HIV infection through the recruitment of new hosts for the virus and through activation of HIV replication within these cells [33, 34]. Furthermore, individuals infected with both MAC and HIV have increased numbers of macrophages within lymphoid tissues, compared with individuals infected with HIV alone, and this increase can be attributed to mycobacteria-induced production of chemokines, including MCP-1 [33]. Increased levels of MCP-1 in serum and in tissue result in the recruitment of activated macrophages to sites of infection, where they may become infected by macrophage-tropic strains of SIV, as well as M. avium.

To summarize, M. avium induces a consistent increase in the production of MCP-1, which can be demonstrated both in vitro and in vivo. Furthermore, increased levels of MCP-1 can be demonstrated in serum and in tissue at active sites of inflammation. We conclude that the pronounced increases in levels of MCP-1 in tissue and serum after M. avium inoculation may play a role in the development of disseminated mycobacterial disease.

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References

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