Enhanced Induction of Interleukin-12(p40) Secretion by Human Macrophages Infected with Mycobacterium avium Complex Isolates from Disseminated Infection in AIDS Patients

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Interleukin-12 (IL-12) is a key cytokine in the immune response to infection with Mycobacterium avium complex (MAC). Human immunodeficiency virus infection, a predisposing factor for disseminated MAC infection, causes a drop of bioactive IL-12(p70), mainly by decreasing the constitutive production of p35. IL-12(p40) and tumor necrosis factor-α (TNF-α) levels in supernatants of MAC-infected human monocyte-derived macrophages with intracellular growth rates were compared. Isolates from AIDS patients showed high growth rates and induced low TNF-α but high IL-12(p40) levels. In contrast, environmental isolates with fast growth rates induced only low levels of IL-12(p40) secretion. The increased IL-12(p40) stimulus seen with the clinical isolates could lead to a p40 surplus, which, alone or as the (p40)2 homodimer, has immunosuppressive properties. An immunosuppressive effect of MAC isolates may be an important advantage for their survival in vivo and may explain the contribution of MAC infection to the progression of AIDS.

Mycobacterium avium complex (MAC) is the most common cause of disseminated infection in the terminal phase of AIDS [1, 2]. It is generally assumed that infection occurs via ingestion or inhalation of environmental MAC organisms [1, 3]. Monocytes and macrophages serve as principal host cells for MAC and respond to infection by secreting a wide array of cytokines. In particular, interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) have repeatedly been shown to be of outstanding importance for developing an efficient antimycobacterial immune response [4, 5]. TNF-α has a strong mycobactericidal effect itself [5, 6]. Accordingly, one possible virulence factor of MAC may be the failure by infected macrophages to induce early TNF-α secretion [6].

IL-12 is a 70-kDa (p70) heterodimer, consisting of a 35-kDa (p35) and a 40-kDa (p40) chain [7]. While p35 is expressed constitutively by a wide variety of cells, the secretion of p40 is closely related to the stimulation of phagocytes [7]. Bioactive p70 activates NK cells and cytotoxic T lymphocytes, which then in turn secrete IFN-γ and TNF-α to stimulate the infected macrophage [7]. Only as a heterodimer is IL-12 able to develop its specific bioactivity. p40 alone, or as homodimer (p40)2, is a potent antagonist of IL-12 [8]. The relevance of this finding has been shown very recently in vivo [9], and this effect could become a critical problem in human immunodeficiency virus (HIV) infection, when expression of p35 is substantially reduced while that of p40 is less affected [10].

The induction of p40 expression by intracellular pathogens could, under these circumstances, further accentuate the immunosuppression seen in AIDS patients and pose an advantage for the infecting organism. It could also be effective as a selection factor for virulent isolates in AIDS patients. In this study, we compared the abilities of 11 clinical and environmental isolates of MAC to induce secretion of IL-12(p40) and TNF-α in cultured human monocyte-derived macrophages (HMDM) with their abilities to replicate inside these cells.

Materials and Methods

Bacteria. Seven MAC isolates were from different water sources (provided R. Schulze-Röbbecke, Institut für Hygiene und Umweltmedizin, Aachen, Germany). Patient-derived isolates were primary isolates that had only been subcultured once for differentiation and preparation of inocula. Their exact sources are listed in table 1. All isolates were positive by testing with the MAC Accu-probe assay (Gen-Probe, San Diego). The bacteria were cultured in 100 mL of 7H9 broth at 37°C for 2 weeks, after which 40 mL of this culture was centrifuged at 4000 g for 10 min and resuspended in 1 mL of fresh autologous serum for opsonization. After sonication in a water bath, serial 10-fold dilutions in RPMI 1640 were made, and 1-mL aliquots were snap-frozen at −70°C. One aliquot of each dilution was later quickly thawed, and three 20-μL drops of further dilutions were plated on 7H10 agar for counts of colony-forming units (cfu). Thus the exact number of viable bacteria used for infection and their growth rate in culture medium could be determined.

HMDM model of infection. Peripheral blood monocytes were isolated from healthy, HIV-uninfected donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation [11]. Interphase mononuclear cells were washed repeatedly, resuspended in warm RPMI 1640 (Life Technologies, Paisley, Scotland), sup-
implemented with 5% autologous serum (AS), and plated into 8-well, glass-bottom chamber slides (Nunc, Wiesbaden, Germany). The seeded cultures were incubated overnight at 37°C in 5% CO₂. Nonadherent cells were washed off with warm RPMI 1640, and the remaining monocytes (5 × 10⁴ cells/well on the day of infection) were cultured in RPMI 1640 with 5% AS prior to infection, with daily changes of medium.

After 7 days, HMDM culture supernatants were withdrawn, and MAC suspensions in RPMI 1640 containing adequate numbers of bacilli (MOI 10:1) were added to the wells. After 2 h of incubation at 37°C in 5% CO₂, cell cultures were vigorously washed to remove extracellular bacteria and incubated for 7 days, with a daily change of medium to minimize extracellular bacterial growth. At various intervals (2 h after infection and days 3, 5, and 7), supernatants from 4 wells per bacterial isolate and time point were withdrawn, macrophages were lysed, and viable bacilli were quantified by cfu counts [11]. The numbers of cfu were plotted semilogarithmically against time in days (data not shown), and the mean generation time was determined from the slope between days 0 and 5 [11].

ELISA. Cytokines in culture supernatants were measured by ELISAs for TNF-α (Endogen, Cambridge, MA) and for IL-12(p40) (Amersham, Amersham, UK) according to the manufacturers’ instructions. HMDM culture supernatants of triplicate wells were diluted, and stored at −70°C until used.

Statistics. Significance of differences between groups of isolates was tested using the t test for cfu counts at different time points for growth rates and for optical densities for cytokine ELISAs. P ≤ .05 was regarded as significant.

### Results

**Mycobacterial growth rates.** All tested MAC isolates grew equally well in standard bacteriologic medium (7H9 broth) as was determined from cfu numbers in the infection inoculum. Environmental MAC isolates possessed differing abilities to multiply inside HMDM. For mean generation times (±SE), see table 1. In some experiments, slow-growing environmental isolates 1817, 2391, and 2895 either showed no significant intracellular replication or were killed. To establish a generation time value for these isolates, only nondeclining growth curves were considered. The resulting position in table 1 does, however, represent their rank, if the actual slope of the growth curves is used for evaluation. Isolates 1816 and 2521 grew significantly faster than isolates 3045 and 2442, which in turn differed significantly from slow growers 2895, 2391, and 1817 (P < .001 and .03 for cfu counts on days 3 and 5 after infection). Isolates of AIDS-related MAC multiplied at a rate comparable to that of fast-growing environmental isolates (P ≤ .002 vs. isolates 3045 and 2442), with surprising homogeneity and low SEs.

The predominant colony morphology of all isolates was domed, with a frequency of ≤3% flat-transparent, except for AIDS-MAC isolate SCH144, which had a 20% fraction of transparent colonies (as determined with the frozen aliquots used for infection of the macrophage cultures). No attempt was made to isolate different colony forms. These results thus represent the isolate as a whole, rather than only one colony

### Table 1. Phenotype of cytokine induction, mean generation time, and source of M. avium complex isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Mean generation time, h ± SE</th>
<th>IL-12(p40), pg/mL ± SE</th>
<th>TNF-α, pg/mL ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS isolates</td>
<td></td>
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</tr>
<tr>
<td>SCH 235</td>
<td>Blood, DI</td>
<td>16 ± 2</td>
<td>185 ± 85</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>SCH 228</td>
<td>BM, DI</td>
<td>18 ± 3.6</td>
<td>190 ± 77</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>SCH 132</td>
<td>Blood, DI</td>
<td>20 ± 1.2</td>
<td>25 ± 0.25</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>SCH 144</td>
<td>BM, DI</td>
<td>20 ± 1.2</td>
<td>460 ± 157</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>SCH 193</td>
<td>Liver, DI</td>
<td>21 ± 1.6</td>
<td>175 ± 24</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Environmental isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2521</td>
<td>Water</td>
<td>19 ± 2.4</td>
<td>65 ± 9</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>1816</td>
<td>Water</td>
<td>23 ± 3</td>
<td>15 ± 0.56</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>3045</td>
<td>Water</td>
<td>58 ± 8.9</td>
<td>50 ± 8</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>2442</td>
<td>Water</td>
<td>84 ± 26</td>
<td>205 ± 22</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>2895</td>
<td>Water</td>
<td>&gt;150 ± NA</td>
<td>420 ± 272</td>
<td>84 ± 39</td>
</tr>
<tr>
<td>1817</td>
<td>Water</td>
<td>&gt;190 ± NA</td>
<td>585 ± 378</td>
<td>50 ± 15</td>
</tr>
<tr>
<td>2391</td>
<td>Water</td>
<td>&gt;200 ± NA</td>
<td>566 ± 431</td>
<td>151 ± 95</td>
</tr>
</tbody>
</table>

NOTE. Interleukin (IL)-12(p40) and tumor necrosis factor (TNF)-α levels in human monocyte-derived macrophage (HMDM) supernatants 24 h after infection were determined by ELISA. Generation time is time needed for doubling of bacillary numbers after phagocytosis and was determined from slope between means of days 0 and 5, assuming exponential growth between these time points. Supernatants from HMDM infected with AIDS-related isolates contained significantly more IL-12(p40) than HMDM that were infected with comparably fast-growing environmental isolates 2521 and 1816 (P = .05). BM, bone marrow; DI, disseminated infection; NA, not applicable (i.e., observed bactericidal effect in ≥1 experiments).
type. The percentage of flat-transparent colonies did not change during the course of macrophage infection experiments.

**TNF-α and IL-12 ELISA.** Levels of TNF-α and IL-12 in HMDM supernatants were assayed immediately before infection with MAC organisms and 24 and 48 h thereafter. The relation between mean generation time of phagocytized bacteria and levels of IL-12(p40) and TNF-α in HMDM supernatants 24 h after infection is shown in table 1. Base levels of cytokines before infection were 80 pg/mL IL-12(p40) and 0 pg/mL TNF-α.

IL-12(p40) levels in supernatants of HMDM infected with fast-growing environmental isolates 2521 and 1816 decreased consistently over 2 days, whereas infection with slow-growers 1817, 2895, and 2391 led to increased IL-12(p40) levels (P = .04 at 24 h, P = .02 at 48 h). TNF-α levels also were lower in supernatants of HMDM infected with fast-growing isolates compared with slow-growing isolates (P = .07 at 24 h, P = .01 at 48 h). There were no significant differences in TNF-α levels after infection with fast-growing environmental isolates compared with the homogeneously fast-growing AIDS isolates. However, AIDS-related MAC isolates did induce significantly higher IL-12(p40) levels than fast-growing environmental MAC isolates (P = .04 at 24 h, P = .03 at 48 h). In this respect, AIDS-related MAC isolates form a unique group, combining fast growth with a significant stimulation of IL-12(p40) secretion by the infected macrophage. Not a single environmental isolate presented this combination.

**Discussion**

Our results indicate fundamental differences between MAC isolates from the environment and from disseminated infection in AIDS patients. Environmental isolates that are able to multiply quickly inside the macrophage fail to induce an early secretion of IL-12(p40) by the infected cell. In contrast, AIDS-related MAC isolates, which uniformly reach high intracellular growth rates, do pose a remarkable IL-12(p40) stimulus upon the infected macrophage. In this respect, not 1 of the 7 tested environmental isolates resembled the AIDS isolates. This could be interpreted to mean that disseminated MAC infection in AIDS patients does not just promote the selection of fast-growing environmental isolates. Instead, there appears to be a selective pressure on the infecting bacteria to modulate their capacity to evoke cytokine secretion in the host cell. In earlier experiments, we demonstrated that phagocytosis can induce the expression of a specific gene (mig) by the ingested mycobacteria [12, 13]. This example shows that the phagosomal environment poses a significant stimulus for the adaptive mechanisms of the pathogen. Whereas mig is expressed early after phagocytosis, other, longer-lasting adaptive mechanisms may be effective in these mycobacterial isolates as well.

The antimycobacterial effect of TNF-α has been demonstrated before, although the exact mechanism remains unknown [5, 6]. Furney et al. [6] showed that fast-growing MAC isolates do not induce the secretion of TNF-α by murine macrophages, whereas slow-growing isolates do so [6]. Those observations are corroborated by our results. While the TNF-α–inducing isolates could not multiply within macrophages, isolates that failed to stimulate TNF-α secretion reached high growth rates.

IL-12 plays an important role in intracellular infection in general and in mycobacterial infection in particular. Neutralization of IL-12 in MAC-infected mice led to their inability to control the infection [4]. Frucht and Holland [14] describe a family with disseminated mycobacterial infection in the absence of HIV, who showed a defective IL-12 regulation leading to low IFN-γ production. Again, the virulent environmental MAC isolates we tested did not induce IL-12(p40) secretion. This could eventually enable the infecting isolate to multiply before a cellular immune response is established. The slow-growing isolates induced high levels of IL-12(p40), which, in a healthy host, would probably have led to the elimination of the bacteria.

The relatively high IL-12(p40) induction caused by fast-growing AIDS-related MAC isolates could paradoxically increase their virulence for HIV-coinfected individuals: Coinfection with HIV inhibits the monocyctic secretion of IL-12 [15]. In this context, Chougnet et al. [10] showed that the expression of p35 mRNA was more extensively reduced by HIV than was the expression of p40 mRNA. Also, the expression of p35 following stimulation with *Staphylococcus aureus* Cowan (SAC) was more reduced than that of p40. Ling et al. [8] showed that p40, either alone or as a homodimer (p40), is a potent antagonist of IL-12. While the p40 monomer only competitively binds to the IL-12 receptor without inducing any effects itself, the homodimer (p40) is a potent inhibitor of IL-12–induced T cell proliferation. Mattner et al. [9] demonstrated that (p40) protects mice from IL-12–dependent shock syndrome.

Taken together, these findings lead us to hypothesize that, at a certain stage of AIDS, the HIV-mediated suppression of p35 mRNA causes such an imbalance of the p70-to-p40 ratio that an IL-12(p40) stimulus, such as the one caused by our clinical MAC isolates, could lead to a critical surplus of p40. The (p40) homodimers could then act as IL-12 antagonists and thereby contribute to the fast progression of the disease and the poor prognosis of AIDS patients once they have acquired disseminated MAC infection.

In summary, the ability of our AIDS-related MAC isolates to multiply rapidly inside HMDM, to not induce the secretion of TNF-α by the infected cells, and to stimulate secretion of IL-12(p40) is not only unique to this group of isolates (compared with environmental ones) but might also play a role in the pathogenicity of disseminated MAC infection in AIDS patients and the poor prognosis indicated by the disease.

**References**


