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Mycobacterium tuberculosis-Induced CXCR4 and Chemokine Expression Leads to Preferential X4 HIV-1 Replication in Human Macrophages

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Opportunistic infections such as pulmonary tuberculosis (TB) increase local HIV-1 replication and mutation. As AIDS progresses, alteration of the HIV-1 gp120 V3 sequence is associated with a shift in viral coreceptor use from CCR5 (CD195) to CXCR4 (CD184). To better understand the effect of HIV/TB coinfection, we screened transcripts from bronchoalveolar lavage cells with high density cDNA arrays and found that CXCR4 mRNA is increased in patients with TB. Surprisingly, CXCR4 was predominantly expressed on alveolar macrophages (AM). Mycobacterium tuberculosis infection of macrophages in vitro increased CXCR4 surface expression, whereas amelioration of disease reduced CXCR4 expression in vivo. Bronchoalveolar lavage fluid from TB patients had elevated levels of CCL4 (macrophage inflammatory protein-1α), whereas amelioration of disease reduced CXCR4 expression in vivo. Bronchoalveolar lavage fluid from TB patients had elevated levels of CCL4 (macrophage inflammatory protein-1α), CCL5 (RANTES), and CX3CL1 (fractalkine), but not CXCL12 (stromal-derived factor-1α). We found that M. tuberculosis infection of macrophages in vitro increased viral entry and RT of CXCR4, but not of CCR5, using HIV-1. Lastly, HIV-1 derived from the lung contains CD14, suggesting that they were produced in AM. Our results demonstrate that TB produces a permissive environment for replication of CXCR4-using virus by increasing CXCR4 expression in AM and for suppression of CCR5-using HIV-1 by increasing CC chemokine expression. These changes explain in part why TB accelerates the course of AIDS. CXCR4 inhibitors are a rational therapeutic approach in HIV/TB coinfection.


Patients with coinfection of HIV and pulmonary tuberculosis (TB) present a great clinical problem. Worldwide, 9% of the 8.3 million annual new cases of TB are in HIV-infected individuals (1). Managing coinfected patients in the developing world is difficult due to the expense and interaction of the complex drug treatments required for each infection. Coinfection with HIV markedly increases the risk of progressing to active TB and limits the ability of the host to control infection (2). The presence of a large group of HIV-1-infected individuals is exacerbating the epidemic of TB. TB also accelerates the course of AIDS. This may be due to increased HIV-1 replication and mutation in the lung produced by TB (3, 4). Understanding the mechanisms underly the synergistic interaction between HIV and TB may lead to improved strategies for the control of both infections (5).

HIV-1 strains have a tropism for immune cells based on CD4 and chemokine receptor use. Early in the course of HIV-1 infection, the virus predominately uses CCR5 as a coreceptor, whereas late in the course the CXCR4 coreceptor is used more frequently (6). HIV-1 strains isolated during primary and latent infections were initially called macrophage tropic because they grew in macrophages, but not in CD4+ T cell lines. They are now classified as R5 strains because they use CCR5, a CC chemokine receptor, as their coreceptor. During disease progression, HIV-1 mutation leads to the ability to replicate in CD4+ T cell lines. These T cell-tropic HIV-1 strains are now called X4 strains because they use CXCR4 as their coreceptor (7, 8). X4 strains are associated with a sharp decline in the number of peripheral CD4+ T cells (9).

In monocyte-derived macrophages (MDM) prepared from PBMC, only dual-tropic (R5X4) strains can replicate (10). In the CNS, syncytium-inducing (SI) X4 virus can replicate in macrophages (11). In TB, X4 virus is obtained from the lung, whereas R5 virus is found in the pleural space, suggesting compartment-specific support replication of X4 or R5 virus (5, 12).

Viral entry can be inhibited by the chemokine ligands of CCR5 or CXCR4. The CC chemokines (macrophage inflammatory protein-1α (MIP-1α, CCL3), MIP-1β (CCL4), and RANTES (CCL5)) inhibit R5 strains, but not X4 strains (13–18). Conversely, the CXC chemokine stromal-derived factor-1α (SDF-1α; CXCL12) inhibits X4, but not R5, replication (19). TB is likely to induce CC chemokines by multiple pathways. In vitro, Mycobacterium tuberculosis (M. tb.) heat shock protein 70 induces CC chemokines in macrophages via direct stimulation of CD40 (20). Alternately, stimulation of CD40 by CD40 ligand (CD154) induces CC chemokines (21, 22). CD40 may be important for the
control of chemokine expression in the lung, as alveolar macrophages (AM) strongly express CD40 on the cell surface during TB (23). CC chemokines are induced in bronchoalveolar lavage (BAL) fluid from TB patients and are found in the supernatant from AM infected by M. tb. in vitro (24, 25). Moreover, stimulation of CD40 can increase CXCR4-dependent infection of MDM by HIV-1 (26). Fractalkine (FKN; CX3CCL1) is a CX3C chemokine that is induced in HIV-1 infection and other inflammatory diseases (27, 28). It is also induced by CD40 ligand (29). FKN recruits CXCR4-expressing monocytes using its receptor CX3CR1, which also can serve as a coreceptor for HIV-1 (30–32). Polymorphisms of CX3CR1 are associated with more rapid AIDS progression, and elevated levels of FKN in the brain are associated with HIV-1-associated dementia (27, 33).

Tuberculosis provides an excellent model for understanding the mechanisms by which opportunistic infections accelerate the course of AIDS. We detected X4 strains in BAL fluid from involved lung segments of AIDS patients, but not from lung segments of AIDS patients without TB (5). This observation was difficult to reconcile with the findings that AM are the major source of HIV-1 in the lung, and these macrophages express CCR5 (23, 34). In this study we demonstrate that after M. tb. infection, macrophages up-regulate CXCR4, permitting X4 viral entry, and also up-regulate CC chemokines, inhibiting R5 viral entry. This combination of events will select for the production of X4 virus during TB and may in part explain why this opportunistic infection accelerates the course of AIDS.

Materials and Methods

Study population

We performed BAL on 32 patients, including 23 with active TB, four with HIV–I/II-TB coinfection, two with HIV-1 and atypical mycobacterial infections, and three with HIV-1 without lung disease. We also performed BAL on five normal volunteers. The TB patients had unilateral segmental infiltrates. We collected plasma from 10 normal volunteers and 25 patients with mycobacterium diseases. The BAL protocol was approved by the human subjects review committees of New York University School of Medicine and Bellevue Hospital Center and was performed as previously described (23, 35).

Bacterial strains, viral strains, cell culture, and cytokines

M. tb. strain TN913, a clinical isolate, and H37Rα were grown as previously described (35, 36). HIV-1, HIV-1 (X4 strain) and HIV-1ΔL (R5 strain) were obtained from K. Martin (University of California, Los Angeles). AD-10 cells were infected with the indicated strain of M. tb. at a multiplicity of infection (MOI) of 1, as previously described (38), and were harvested 3 days later. The CFU of the inoculum and lysates from harvested cultures was determined as described previously (5). Only reverse transcriptase-dependent reaction products were sequenced with an ABI PRISM autosequencer using a reverse primer. The genotype of viruses in BAL fluids from coinfected patients was determined as described previously (5). Only reverse transcriptase-dependent reaction products were sequenced, thereby eliminating the possibility of cross-contamination by HIV-1 cDNA recovered from in vitro experiments. For anti-CD14-captured viruses, nested PCR products were subcloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). The PCR products were sequenced with an ABI PRISM autosequencer using a BigDye-Dexoy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequences were deposited in GenBank under accession numbersAY289008 to AY289017.

Immunological virus capture assay targeting discriminatory host proteins

To determine whether the viruses in BAL fluid derived from alveolar macrophages or alveolar lymphocytes, we conducted an immunological capture assay using anti-CD14 (for macrophage-derived viruses), anti-CD26 (for lymphocyte-derived viruses), and nonspecific serum (for negative control) (42). Filtered BAL fluids from HIV-1- and M. tb.-coinfected patients were ultracentrifuged, precipitated with specific Abs, and then incubated with agarose-protein A/G beads. Captured viruses were directly extracted by TRIReagent. Real-time quantitative RT-PCR was performed for the captured viruses as described above.

Determination of viral genotyp

The genotype of viruses in BAL fluids from coinfected patients was determined as described previously (5). Only reverse transcriptase-dependent reaction products were analyzed, thereby eliminating the possibility of cross-contamination by HIV-1 cDNA recovered from in vitro entry experiments. For anti-CD14-captured viruses, nested PCR products were subcloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). The PCR products were sequenced with an ABI PRISM autosequencer using a BigDye-Dexoy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequences were deposited in GenBank under accession numbersAY289008 to AY289017.

Biostatistics

Data are expressed as the mean ± SEM for experiments performed in triplicate or more. Comparisons between groups used Student’s t test, with p < 0.05 chosen as significant. For comparison of confocal intensities
between M. tb. and those with M. tb., Mann-Whitney’s U test was used.

Results
CXCR4 expression is increased during M. tb. infection in vivo
To better understand the effect of M. tb. infection on HIV replication in the lung, we screened BAL cell mRNA using genomic filters containing 4058 genes. CXCR4 mRNA was increased 2-fold in BAL cells from the lobes of TB patients compared with subjects with no lung disease (Fig. 1A; p = 0.03). As CXCR4 mRNA could be derived from alveolar macrophages or alveolar lymphocytes, FACS analyses were performed on BAL cells from lung segments with mycobacterial infections. As shown in Fig. 1B, alveolar macrophages from a TB patient expressed more CXCR4 than alveolar lymphocytes. Alveolar macrophages also expressed higher levels of CXCR4 relative to CCR5 (Fig. 1, B and C). Similar to our previous report (40), alveolar macrophages were, on the average, 4 times more prevalent than alveolar lymphocytes in BAL fluid. Therefore, 90% of the total CXCR4 was found on the surface of alveolar macrophages in the mycobacteria-infected lung (Fig. 1C). The level of CXCR4 on alveolar macrophages as well as alveolar lymphocytes decreased with clinical improvement.

Expression of CC and CX3C chemokines is induced in macrophages during M. tb. infection in vivo
We next examined the expression of CC chemokines, which could inhibit infection by R5 HIV-1 strains. The expression of mRNA was increased in BAL cells from involved lung segments compared with those from no lung diseases (RANTES, 1.31 ± 3.33 (p < 0.03); MIP-1α, 0.60 ± 1.83 (p < 0.04); MIP-1α, 0.31 ± 0.66 (p < 0.04); CCL2 (monocyte chemotactic protein-1), 0.22 vs 1.06 (p < 0.02)). The increase in CC chemokine mRNA was associated with increased BAL chemokine concentration. BAL fluid from involved lung segments of patients with M. tb. contained high levels of RANTES and MIP-1β (Fig. 2A; p < 0.05 and p < 0.002, respectively). In contrast, SDF-1α was not increased (Fig. 2A; p > 0.2). FKN and its receptor CX3CRI mRNA were significantly increased in TB patients compared with individuals who had clear chest radiographs with or without HIV-1 infection (Fig. 2B; p = 0.01 and p = 0.02, respectively). BAL fluid from TB patients also had high levels of FKN protein, demonstrating the functional effect of the elevated mRNA (Fig. 2B; p = 0.03). Similar induction of CC and CX3C chemokine transcription, mRNA, and protein were obtained by infection of cultured macrophages infected with M. tb. in vitro (data not shown). This suggests that the chemokine response seen in vivo is a manifestation of the innate immune response. As CD40 ligand can induce CC and CX3C chemokines, we measured plasma-soluble CD40 ligand in patients with mycobacterium infection with or without HIV-1 infection. The level of soluble CD40 ligand was significantly increased in patients with mycobacterial pneumonia (Fig. 2C; p = 0.0004 and p = 0.0002, respectively).

Increased CXCR4 is a direct effect of infection by M. tb.
To determine whether increased CXCR4 expression in BAL cells from TB patients was indeed caused by M. tb., we infected THP-1 macrophages in vitro. This model system produced results similar to those obtained previously by infection of human AM (35, 36, 38). CXCR4 mRNA increased 2.0-fold after infection with M. tb. (Fig. 3A; p < 0.05), whereas CXCR4 on the cell surface increased 1.8-fold (Fig. 3B; p < 0.03). Nuclear run-on experiments showed that M. tb. infection activated CXCR4 transcription 1.7-fold (p < 0.02), thus demonstrating that transcriptional induction was the basis for increased CXCR4 mRNA expression (data not shown). Confocal microscopy of M. tb.-infected MDM produced results similar to those of THP-1 macrophages. As shown in Fig. 3C, CXCR4 fluorescence intensity on uninfected MDM (mean, ~20 U) was near background levels (mean, <10 U). Twenty-four hours after infection, the mean CXCR4 intensity increased 2-fold, to ~40 U. More importantly, colocalization of CXCR4 and M. tb. by confocal microscopy (Fig. 3D) showed that cells that displayed high levels of CXCR4 (red fluorescence) also displayed high levels of M. tb. (green fluorescence). When 100 individual MDM were analyzed, a significant correlation was found between the level of CXCR4 expression and M. tb. infection (Fig. 3E; r² = 0.40; p < 0.001).

M. tb. infection increases HIV-1 X4 virus entry in macrophages
We performed an assay for viral entry into M. tb.-infected macrophages to test whether the elevated CXCR4 expression increased...
X4 viral entry. We also determined whether R5 virus entry would be inhibited by the secreted CC chemokines. This assay included quantitative PCR without the addition of exogenous reverse transcriptase so that any increase in viral genomic DNA would be due to endogenous virion reverse transcriptase activity that synthesized cDNA after viral entry. The assay was performed 24 h after HIV-1 infection, a time sufficient for only one round of viral replication in macrophages (43). SDF-1α was used to inhibit X4 HIV-1 entry, RANTES to inhibit R5 entry, and AZT to inhibit viral genomic DNA synthesis by the endogenous reverse transcriptase. These controls gave a baseline level of HIV-1 genomic DNA produced by partially replicated retrovirus in viral stocks. HIV-1 genomic DNA was normalized to the amount present in the cells treated with both AZT and either SDF-1α or RANTES.

In four independent experiments, MDM infected by M. tb. were permissive for synthesis of X4 HIV-1_{\text{IIIB}} genomic DNA, whereas uninfected MDM were not (Fig. 4A; p < 0.05). SDF-1α inhibited production of X4 HIV-1_{\text{IIIB}} genomic DNA in M. tb.-infected MDM (p < 0.05). A markedly different result occurred when an R5 strain was used in these experiments. MDM did support an increase in R5 HIV-1 genomic DNA in the absence of M. tb. infection (Fig. 4B), and this was inhibited by RANTES. In two of three experiments, however, the amount of R5 genomic DNA decreased after M. tb. infection, and RANTES did not affect the level of R5 genomic DNA in M. tb.-infected MDM. These data support the conclusion that M. tb. infection produces a permissive environment for entry of X4 HIV-1.

**HIV-1 in BAL fluid is derived from alveolar macrophages**

The HIV-1 envelope carries plasma membrane proteins expressed by its cellular source. We used immunoprecipitation experiments to identify the source of HIV-1 in the lung during TB (23, 34). Lymphocyte-derived virus should contain CD26, whereas macrophage/macrophage-derived virus should contain CD14. Ab to CD14 precipitated >75% of the HIV-1 detected in BAL fluid from two of three TB patients (Fig. 5A, BAL fluids 1 and 2) and 33% of the HIV-1 virus in a third patient (Fig. 5A, BAL fluid 3). When HIV-1_{\text{BAL}} cultured in PBMC was used as a positive control, 95% of the viral total was precipitated by anti-CD14 antibody. These data support the conclusion that macrophages are a major source of HIV-1 replication during TB.

The predicted amino acid sequence of the gp120 V3 loop was determined for HIV-1 that was immunoprecipitated by anti-CD14 to investigate the tropism of macrophage-derived virus. A net positive charge ≥6 strongly suggests CXCR4 use and is a marker of X4 HIV-1 (44–46). We compared the sequence of input virus (present in BAL fluid) and that of immunocaptured virus. In each case the CD14-containing virus had a more positive charge than the input virus. In all instances available for study, the macrophage-derived virus had a charge of ≥6. The high net positive charge in the gp120 V3 loop of HIV immunocaptured by CD14 suggests an X4 phenotype for macrophage-derived HIV-1. In addition, the CD14-captured virus of BAL fluid 2 is predicted to be an S1 phenotype.

**Discussion**

In this study we have observed that TB is associated with high level expression of CXCR4 on AM, which allows for X4 HIV-1 entry into AM, and with high level chemokine expression, which inhibits R5 viral entry. This environment probably produces a selective pressure for X4 viral replication in the lung during TB and may contribute to the acceleration of AIDS produced by this opportunistic infection. Clinical data suggest that patterns of expression of chemokines and their receptors affect viral pathogenesis. Homozygous for deletion of CCR5 produces resistance to HIV-1 infection, and even heterozygous individuals have slow disease progression (47, 48). Patients with a single base pair substitution of CCR2, another CC chemokine receptor, have slower disease progression (49, 50). Alternately, polymorphisms in the promoters of RANTES and the FKN receptor, CX3CR1, are associated with rapid progression of HIV disease (33, 51). These observations emphasize the importance of understanding the interactions between chemokines and their receptors in both primary HIV infection and HIV disease progression.

We observed high levels of MIP-1β, RANTES, FKN mRNA, and protein in the BAL of HIV-1-positive and HIV-1-negative
patients with TB. The increase in CC chemokines may explain the low level of R5 HIV-1. The increase in FKN may explain the abundance of CXCR4 high expressing macrophages in the BAL fluid (31). As CD40 ligand induces chemokines in macrophages, the increased systemic levels of CD40 ligand may be part of a feedback loop that amplifies chemokine production during TB (21, 29).

An assay of viral entry into macrophages was used to assess the functional effects of high level CC chemokine and CXCR4 expression produced by M. tb infection. We used a strategy of quantitative PCR without exogenous RT to score the conversion of viral RNA to cDNA by endogenous HIV-1 RT. Infection of MDM with M. tb increased X4 and reduced R5 viral cDNA production. This observation supports the conclusion that the increased CXCR4 expression produced by M. tb infection enhanced X4 viral entry, whereas the high levels of chemokines inhibited R5 viral entry. In this assay no part of the viral life cycle distal to cDNA synthesis is required, so no long terminal repeat (LTR) promoter activity is needed. Our current observations that M. tb infection of macrophages in vitro enhances viral entry is compatible with our previous data demonstrating that M. tb infection represses HIV-1 LTR activity and inhibits viral replication (35). Viral entry and LTR promoter activity are separate stages of the viral life cycle; both are necessary for viral replication, but neither is sufficient. M. tb infection of macrophages in vitro inhibits viral replication, even though there is more virus entering the cell. In vivo, inhibition of the LTR in macrophages is overcome by lymphocyte contact, lead-

FIGURE 3. Expression of CXCR4 after M. tb infection in vitro. A, Relative CXCR4 mRNA abundance in THP-1 macrophages measured by cDNA gene filters. Results are the mean ± SEM of triplicate experiments. B, Expression of CXCR4 on THP-1 macrophages. Cells were labeled with allophycocyanin-anti-CXCR4. The mean allophycocyanin intensity on CXCR4 cells was weighted by the percentage of CXCR4+ cells in each sample. The data shown are the mean ± SEM of three experiments. C, CXCR4 expression on MDM with or without M. tb infection. Mean CXCR4 fluorescence intensity was 2-fold higher with M. tb infection (p < 0.001). D, Images of CXCR4 and M. tb on MDM detected by immunofluorescence microscopy. E, Correlation of CXCR4 intensity and M. tb intensity on infected MDM.

FIGURE 4. HIV-1 Genomic DNA production measured by the quantitative PCR method. A (X4 strain). Results shown are the relative HIV-1 ratio normalized to the result obtained with added AZT and SDF-1α in four independent experiments. The yield from infected cells (+TB) was increased 2.4-fold compared with that from uninfected cells (−TB; p < 0.05, by two-tailed t test); however, SDF-1α competitively inhibited the increased score with M. tb (+TB). B (R5 strain). Results shown are the relative HIV-1 ratio normalized to the result obtained with added AZT and RANTES in three independent experiments. The yield from infected cells (+TB) was decreased 7.0-fold compared with that from uninfected cells (−TB). RANTES competitively reduced the yield in the absence of M. tb. The long bars indicate the median for each group.
As the increase in viral genomic DNA observed requires multiple steps, including viral binding, fusion, entry, and RT, it is not possible to precisely define which step(s) is altered by M. tb. infection. However, it is likely that changes in CXCR4 expression contribute to the observed changes, as addition of SDF-1 significantly inhibited X4 viral entry into M. tb.-infected MDM.

During TB there is a marked increase in HIV replication in the macrophages from radiographically involved lung segments (5). The observation that a large proportion of HIV-1 derived from the lung during TB has CD14 in its envelope supports the conclusion that AM are the major source of viral replication. Interaction of gp120 with CXCR4 is probably due to electrostatic interactions between the positively charged coreceptor binding site of the V3 loop in gp120 and the negatively charged extracellular domains of CXCR4 (45, 46). All CD14-captured viruses had V3 loops with higher positive charge than the loops of input or CD26-captured viruses. We also found a putative SI phenotype in one of the three macrophage-derived HIV-1 viruses in the BAL fluid (5). The biological phenotype and coreceptor usage of HIV in the alveolar space seem to be different from those in the pleural space, where the nonsyncytium-inducing CCR5-tropic phenotype predominates (12, 52, 53). Taken together these data suggest that virus derived from AM during TB has an X4 phenotype.

The changes in CXCR4 expression in macrophages are due to the innate immune response to M. tb. infection. Isolated macrophages infected in vitro increase CXCR4 transcription, mRNA, and cell surface expression. There is a good correlation between the amount of M. tb. Ag expressed in the macrophage and the level of CXCR4, suggesting that paracrine factors are not responsible for induction of CXCR4 surface expression. Further, infection of macrophages in vitro induces MIP-1β and RANTES transcription, mRNA, and protein. Therefore, the innate immune response to M. tb. probably inhibits R5 viral entry. Together these effects of M. tb. infection probably provide a selective pressure that leads to a change in viral tropism from R5 to X4 virus. Our findings support the use of competitive inhibition for CXCR4 to prevent HIV-1 entry in AM, especially during active TB. Recently, the HIV-1 fusion inhibitor enfuvirtide (Fuzeon, T-20) was approved by the
Food and Drug Administration. Other types of CXCR4 competitive inhibitors are also in clinical trial. We observed that aerosol IFNγ treatment reduces the HIV-1 viral load in BAL fluid of patients coinfected with HIV-1 and M. tb (54). If these competitive inhibitors were administered by aerosol inhalation, their efficacy might be increased in the coinfected patients.

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References


