AIDS Rev 2002;4:165-176

Impact of Tuberculosis on HIV-1 Replication, Diversity, and Disease Progression

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Abstract

HIV and *Mycobacterium tuberculosis* not only co-circulate throughout the developing world but each has contributed to prevalence and mortality caused by the other. Several reports have described how HIV-1 increases the incidence of new *M. tuberculosis* infections, exacerbates the severity of tuberculosis (TB), and re-activates latent *M. tuberculosis*. However, the converse relationship is more difficult to understand considering TB can emerge in asymptomatic individuals and as an opportunistic infection during AIDS. Development of TB in HIV infected individuals with higher CD4 cell counts (>200/mm^3) appears to increase the rate of disease progression and mortality. Higher viral loads, increased HIV-1 diversity, and changes in cytokine/chemokine levels in HIV-infected individuals with TB appear to be related to a localized immune stimulation. Specifically, increased levels of TNFα and MCP-1, induced by TB, may activate HIV replication in lymphocytes, monocytes, and macrophages that are resident or have migrated to *M. tuberculosis* infected organs (e.g. pleura or lung). The HIV-1 found in blood following this TB-mediated burst in load and diversity appear to be phylogenetically-related to HIV-1 clones that have evolved independently in the lung or pleural compartments, now infected by *M. tuberculosis*.

Key words

HIV. Tuberculosis. Evolution. Genetic variability.

Public health perspective on TB and HIV-1

Acquired immunodeficiency syndrome (AIDS) is one of the most destructive infectious diseases in the world today. In December 2001, UNAIDS reported that AIDS was the world's fourth biggest killer and gaining ground on the leaders, tuberculosis (TB) and malaria. Of particular concern is the impact of HIV/AIDS in sub-Saharan Africa. In this region of the world, AIDS is the major cause of mortality with an estimated 2.3 million deaths in 2001 alone and three fourths of the 40 million HIV-1 infected people in the world [1]. According to the United Nations, HIV/AIDS is the biggest threat to the development of the African economy because this disease is common in young teachers, farmers, health-workers, civil servants, and young professionals.

The second leading cause of death in sub-Saharan Africa is TB. Unfortunately, these two

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diseases are not mutually exclusive, but tend to exacerbate the severity of each other. Incidence of TB increased between 1985 and 1992 in the United States as a result of several factors, but approximately 64% of these cases are attributable to HIV-1 infection[2]. Since 1993, TB control efforts have led to a steady decline in TB case rates[3]. In contrast to the United States, worldwide TB cases increased to 8.4 million in 1999, an increase of 5% from 1998[4]. HIV-1 associated increase in TB incidence is likely the result of the immunosuppression caused by HIV-1. Recent evidence suggests active TB may have adverse effects on HIV-1 disease by increasing HIV-1 replication both systemically[5-9] and at sites of infection[10-13]. These TB mediated affects may in turn lead to increased systemic heterogeneity and viral fitness.

This review will focus on the effects of TB on HIV-1 disease in co-infected individuals. We will compare HIV-1 dynamics, evolution, and fitness with immune regulation during HIV disease as well as discuss how TB affects these viral and host parameters leading to more rapid progression to AIDS. To place these themes into context, a general review of HIV-1 replication and disease is outlined below.

HIV-1 replication: an overview

HIV-1 belongs to the *Retroviridae* family of viruses, which carry two single stranded RNA molecules as the genetic material in the virion but require the synthesis of genomic DNA as an intermediate during the life cycle. Of the seven genuses in this family, HIV belongs to the lentivirus genus, which includes viruses that infect primates, sheep, goats, horses, cats, and cattle. Interestingly, many of the mammals that can contract a lentiviral-induced immunodeficiency can also be co-infected with the Mycobacterium (M.) genus. However, *M. avium* or *M. genavense* in cattle or cats co-infected with an onco- or lentiretrovirus (e.g. bovine leukemia virus or feline immunodeficiency virus) are only detected following immunodeficiency and as opportunistic infection[14,15]. In contrast, active *M. tuberculosis* infection is commonly diagnosed as a secondary infection in otherwise asymptomatic HIV-1 infected humans.

The HIV genome encodes three major genes: *env*, *gag*, and *pol*, flanked by both a 5' and a 3' long terminal repeat (LTR)[16]. The *env* gene encodes the *Env* gp160 glycoprotein, which is the precursor for the envelope protein surface unit (SU)-gp120 and transmembrane (TM)-gp41, found on the virion surface. The *gag* gene produces the viral structural protein p55-Gag, which is cleaved by protease (PR) to form the capsid (CA), matrix (MA), and nucleocapsid (NC) proteins. The *pol* gene is translated following ribosomal frameshift and encodes in addition to the gag proteins, the viral enzymes required for replication. These include PR, reverse transcriptase-RNaseH (RT), and integrase (IN). Lentiviral genomes such as that of HIV-1 also encode several regulatory and accessory proteins that are translated from multispliced or alternatively spliced mRNA transcripts[16].

HIV-1 entry into the host cell is mediated by the viral envelope glycoproteins (gp120/gp41). Extracellular subunit, gp120 interacts with the CD4 cell receptor, causing a conformational change necessary for interaction with a seven-transmembrane G protein-coupled protein chemokine receptor (CCR5 or CXCR4)[17,18]. Following this interaction the gp41 forms a prehairpin intermediate, which inserts into the cellular membrane[18]. This structure causes membrane apposition leading to fusion of the membranes, and release of the HIV-1 core into the cell. Surprisingly, these interactions remain conserved functions in all HIV-1 even though the *env* gene of many HN-1 isolate share less than 70% amino acid sequence identity. The primary coreceptors for HIV-1 are CCR5 and CXCR4, other chemokine receptors such as CCR3, CCR2, CCR7, and CCR8 can be utilized at lower efficiency[17]. HIV-1 isolates that utilize CCR5 for entry do not induce syncitia formation (NSI), are transmitted between donor and recipient and persist throughout asymptomatic disease. NSI HIV-1 isolates typically replicate slower than syncytium inducing (SI) CXCR4-tropic isolates, i.e. found late in HIV-1 disease.

Reverse transcription occurs shortly after entry (reviewed in[19]) and involves the transcription of two genomic RNA templates into double stranded DNA. As described below, retroviral reverse transcription is a error prone process due to lack of a proof-reading or exonuclease activity. Approximately, one to ten nucleotide substitutions are introduced into the 10,000 base pair genome during synthesis of HIV-1 DNA from the RNA genome. Following reverse transcription, HIV-1 proviral DNA is then stably integrated into the host cell DNA genome. Transcription of HIV-1 mRNA from the LTR is catalyzed by the host cell RNA polymerase II but is initially regulated by the 5' LTR [20]. The U3 region of the LTR has several cis-acting sites for transcriptional activation, including two to three nuclear factor κB (NFκB) sites[21], a major activator of HIV-1 transcription. These nuclear factors are often induced by host cytokines such as tumor necrosis factor alpha (TNFα)[22] and interleukin-1 (IL-1)[23]. As discussed below, many opportunistic/secondary infectious including *M. tuberculosis* increase TNFα expression, which in turn activates NFκB, increases HIV-1 transcription, and may lead to greater viral loads in HIV-1/TB individuals. In the absence of HIV-1 Tat, HIV-1 mRNA transcription is aborted approximately 100 nucleic acids downstream of initiation. Following binding to the Tat activating region (TAR, a 59 bp RNA leader sequence)[24], Tat recruits cellular factors that stabilize RNA pol II and increase the production of full length HIV-1 transcripts.

Natural course of HIV infection and HIV-specific immune response

There are several ways of transmitting HIV-1, including IV drug use, homosexual transmission, and heterosexual transmission. SIV models for heterosexual transmission have indicated that HIV-1 penetrates the vaginal mucosa within an hour after transmission. Langerhans cells have dendritic processes that penetrate the vaginal squamous epithelium [25] and are likely to be the first susceptible cell exposed to the virus. These cells may transport the virus to the nearest lymph node from 30 minutes to 24 hours post infection[26]. In the lymph nodes, the virus spreads to CD4 T lymphocytes[27,29]. Peak viremia in the blood is typically found concurrent or following peak viremia in lymph nodes[28,30].

Primary HIV-1 infection is followed by a subclinical incubation period, acute retroviral syndrome, a clinically latent period, clinical apparent disease, AIDS, and ultimately death. After infection, the subclinical incubation can last from 1 to 8 weeks[31-35] but is typically followed by acute retroviral syndrome, which is characterized by high titers of the HIV virus, marked decline in CD4 cell counts, and widespread dissemination of the virus[34-36]. Control of primary infection typically occurs at about 3 weeks after the onset of symptoms, and is characterized by an absence of neutralizing antibody, an HIV-specific cytotoxic T-lymphocyte response (CTL), a lowering of the viral loads, and a return of CD4 cell counts to normal levels[37]. Viral loads are lowered to a steady state level called the virologic setpoint. Higher setpoints are indicative of more rapid disease progression, while lower setpoints are associated with better prognosis[38].

Infected individuals depend on a robust immune response to help contain HIV. The course of HIV-1 infection is variable, with some individuals dying as early as 1-2 years after infection, while others have survived for over 20 years without antiretroviral therapy. As discussed below, development of TB is one factor that can lead to rapid disease progression and may be due in part to chronic immune stimulation. Of utmost importance for all HIV-1 infected individuals is the state of the CTL response, which has been shown to be particularly important in the resolution of the acute retroviral syndrome[37]. Higher HIV-specific CTL activity typically corresponds to lower plasma viral load[39,40] but is absent in patients with rapid progression to AIDS[41] and vigorous in long term progressors[39,42]. Unfortunately, in most individuals, HIV viremia is not controlled and progression to AIDS is inevitable. The failure of the CTL response to control the virus may be a result of the infection and deletion of HIV-1-specific helper CD4 cells early in HIV-1 infection[43].

Infection of CD4 lymphocytes is detrimental to the immune response because CD4 cells are the main regulators of cell-mediated immunity. In patients with progressive disease, HIV-1 specific CD4 cell proliferative responses are weak or non-detectable. In addition to dysregulating the CD4 T-cell function, gradual depletion of the CD4 positive T-cells in HIV-1-infected individuals leads to nearly complete destruction of the adaptive immune system. Direct infection and killing of CD4 T cells partly accounts for the HIV-1 associated T cell depletion. In addition, there is CD4 cell depletion through immune activation. As the CD4 T-cell counts decline, the patient becomes increasingly susceptible to secondary infections. AIDS defining infections include candidiasis, cryptococcosis, cytomegalovirus, Kaposi's sarcoma, *M. avium*, and *Pneumocystis carinii* pneumonia, and are often diagnosed when CD4 T-cell counts fall below 200 cells/mm. In addition to these infections, HIV-1 disease can lead to increased susceptibility to infection by *M. tuberculosis*, which will be discussed in more detail later.

Typically in HIV infection, the majority of target cells rapidly produce progeny virions. However, a small subset of HIV-1 infected cells are not activated or egress from activated to memory T cells. This cell population does not actively produce virus, but still forms a pool of latently infected cells that are not recognized by the immune response[44]. Latently infected cells include resting memory CD4 T cells[45], resting *naïve* CD4 T cells[46], monocytes/macrophages[47], and the CD4 positive subset of NK cells[48] can all be activated to produce HIV-1. These cells are also distributed throughout the body resulting in various anatomical reservoirs and a physical compartmentalization of HIV-1-infected cells within these organs. These latent reservoirs such as the lung appear to exacerbate HIV-1 disease when activated by a localized infection/stimulus such as TB (see below).

Etiology and clinical features of TB

To establish infection, *M. tuberculosis* must be inhaled, carried to the pulmonary alveoli[49], and phagocytized by resident alveolar macrophages[50] (Fig. 1). The initial defense by the host is characterized by inflitration of polymorphonuclear leukocytes (PMNs) and monocytes into the granuloma[51]. These cells in turn produce β chemokines (e.g. RANTES, MIP-1α, MCP-1) for the recruitment of T cells as well as cytokines (e.g. IL-1, IL-12) for the activation of T cells reactive to *M. tuberculosis* antigen (Fig. 1). T cells will then secrete IL-2 for further T-cell activation and IFN-γ for macrophage activation. These factors are important for granuloma formation and containment of the bacteria [52]. In addition to these cells, gamma-delta T cells also provide protective immunity by lysing infected monocytes and macrophages[53].

After *M. tuberculosis* primary infection, most individuals develop an immune response rigorous enough to eliminate or contain the bacteria. IL-12

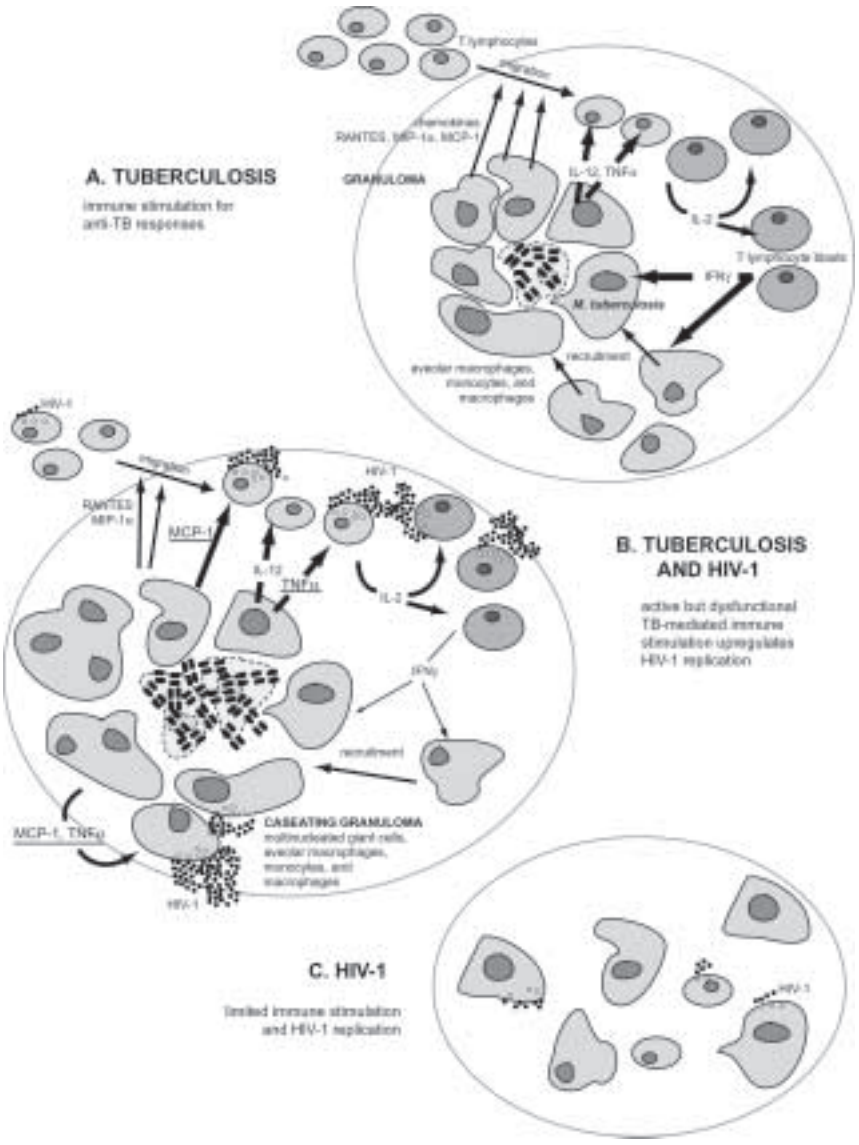

Figure 1. ***Differences in the pathogenesis of tuberculosis and HIV-1 in the lung.***
A schematic representation of the lung microenvironment following infection with HIV-1 and/or M. tuberculosis is described in panels A (TB), B (TB and HIV-1) and C (HIV-1). The thickness of the line represents the relative production of chemokines/ cytokines in the lung following HIV and/or M. tuberculosis infection. The cytokines/chemokines underlined in panel B (i.e. MCP-1 and TNFα.) have been shown to up-regulate HIV-1 replication and production.

secretion drives the Th1 T-cell response, which increases the secretion of macrophage activating IFN-γ[51] (Fig. 1). The hallmark of the immune response is the development of tuberculous granulomas, which are formed by the differentiation of activated macrophages to form multinucleate giant cells[54]. Failure to limit the infection by these cells results in liquification of the caseous granuloma center, allowing for extracellular bacterial replication and cavity formation[55]. Approximately 5% of infected individuals fail to limit infection and develop primary TB within 3 years of infection, while another 5% develop reactivation TB after the 3 year period[56].

HIV infection alters the course of TB

In patients coinfected with HIV-1, the course of *M. tuberculosis* infection is very different. In HIV-1 infected individuals, the risk of developing active TB is 20 times greater[57] due to increased risk of progressive disease from new *M. tuberculosis* infection[58]. In addition, HIV-infected individuals have a higher rate of latent TB reactivation[59] as well as a higher frequency of extrapulmonary TB (e.g. pleural TB)[60, 61]. Thus, the clinical course of TB disease is often shortened from years to a few months[62]. The pathological features of TB in HIV-1 infected patients vary depending on the degree of

immunosuppression in the individuals. Patients with better immune function tend to have caseating granulomas with mature epithelioid cells and multinucleated giant cells[63]. As CD4 cell count declines, there are more diffuse lesions with tissue necrosis, few epithelioid cells, and many *M. tuberculosis* bacilli[63]. In terminally ill patients, the lungs are characterized by fibrous and calcified TB lesions in addition to active lesions containing *M. tuberculosis* bacilli[64].

Immunological studies indicate that HIV-1 positive patients with tuberculosis have an ineffective immune response in the lung, which is characterized at the site of infection by fewer total lymphocytes and smaller proportions of CD4+ lymphocytes[65]. There are also decreased interferon-γ mRNA levels[65], which would lead to decreased production of interferon-γ, a potent activator of macrophages. When peripheral blood mononuclear cells (PBMC) isolated from HIV-1 infected individuals with TB are exposed to *M. tuberculosis in vitro*, the cells produce less interferon-γ, but similar amounts of Th2 cytokines as compared to HIV-1 negative individuals with TB[66]. This indicates that HIV-1 infected individuals are less efficient at activating *M. tuberculosis* infected macrophages, but are still able to mount a humoral response. These immune characteristics likely provide a weaker granulomous immune response, which allow for the more frequent progression to active TB. Pathogenesis of pulmonary TB in relation to HIV-1 is schematically illustrated in figure 1.

TB affects HIV disease progression

In addition to HIV-1 affecting the progression of TB, TB facilitates HIV-1 disease progression. A retrospective study indicates that concurrent TB is associated with a more rapid progression to AIDS and increased mortality[67]. Isoniazid prophylaxis in PPD+ individuals decreases not only TB, but also decreases HIV-related disease[68]. In a prospective study, active TB was associated with increased one and two year mortalities when the CD4 cell counts were above 200 cells/μl[69]. One contribution of TB to HIV-1 disease progression is the increased viral loads, which is most significant at higher CD4 cell counts (greater than 500 cells/μl), suggesting that TB increases HIV-1 replication *in vivo*[5,7]. TB is also associated with a reversible increase in HIV-1 replication in activated cells as demonstrated by the incorporation of HLA-DR into virions[70]. In TB-involved lung segments of patients with HIV-1, viral replication is much higher as compared to the replication in lobes without TB infection[10, 71].

M. tuberculosis also enhances HIV-1 replication *in vitro* by activating transcription and enhancing viral entry. *M. tuberculosis* mediated HIV-1 transcriptional activation has been demonstrated in the monocytic cell line U1[72]. *M. tuberculosis*-mediated stimulation of HIV-1 replication appears to be related to the increased levels of specific cytokines (e.g. TNF-α) and chemokines (e.g. MCP-1 but not RANTES or MIP-1α) during *M. tuberculosis* infection and activation of host immune response[12,73-75]. Addition of TNF-α antibody and mutation of NFκB binding sites in the HIV-1 LTR inhibited the M. tuberculosis-induced activation of HIV-1 transcription and replication. Although β-chemokines have been associated with inhibition of HIV-1 entry, β-chemokines can also bind to chemokine receptors or gylcosylamino glycans and induce a cell signaling cascade that activates HIV-1 replication. Interestingly, MCP-1, which is increased by TB, does not bind to CCR5 or inhibit NSI/R5 HIV-1 entry but is a potent activator of HIV-1 replication (Fig. 1). Finally, M. tuberculosis directly increases HIV replication and virus production in coinfected blood monocytes[76] and unpurified mononuclear cells[5]. This may be related to a TB mediated increase in cell susceptibility to HIV-1 infection. Monocytes from TB patients tend to be more easily infected by HIV-1 as compared to monocytes from healthy donors[9]. Infection of blood monocytes with *M. tuberculosis* also makes the transmission of HIV-1 to T cells more efficient[76].

Nonspecific antigenic stimulation of the immune system can also affect HIV-1 replication and heterogeneity. For example, tetanus vaccination of HIV-1 infected individuals leads to an increase in viral load and a transient shift in the distribution and composition of the viral quasispecies[77]. However, TB causes chronic immune activation[13], affecting both viral replication and heterogeneity for more prolonged periods of time. TB increases both the replication and heterogeneity of HIV-1 at the site of *M. tuberculosis* infection[6,10]. This may lead to rapid evolution of HIV-1 quasispecies in the lung, which could affect the systemic heterogeneity (Fig. 2). The remainder of this review will focus on the changes in intrapatient HIV-1 diversity resulting from co-infection with *M. tuberculosis*.

Genetic diversity of HIV-1

As described earlier, HIV-1 reverse transcriptase is an error prone polymerase leading to an *in vivo* error rate of about 3.4×10^{-5} mutations per bp per cycle[78]. In addition to mutations generated by base pair substitutions, recombination events are very frequent during reverse transcription, i.e. about 2 events per replication cycle[79]. High HIV turnover (10^{10} viral particles per day)[80,81] coupled with this high mutation rate can generate every single and combination of double point mutations in the 10,000 bp genome every day.

A high mutation rate is ultimately responsible for divergence of HIV-1 within an infected individual and among human hosts. HIV-1 has evolved into three groups: group M (for main), group O (for outlier), and group N (non-M, non-O)[82]. The group M sequences are 39 to 49% divergent from group O[83,84], while group N is equidistant from both groups M and O[85]. Group M HIV-1 infects the vast

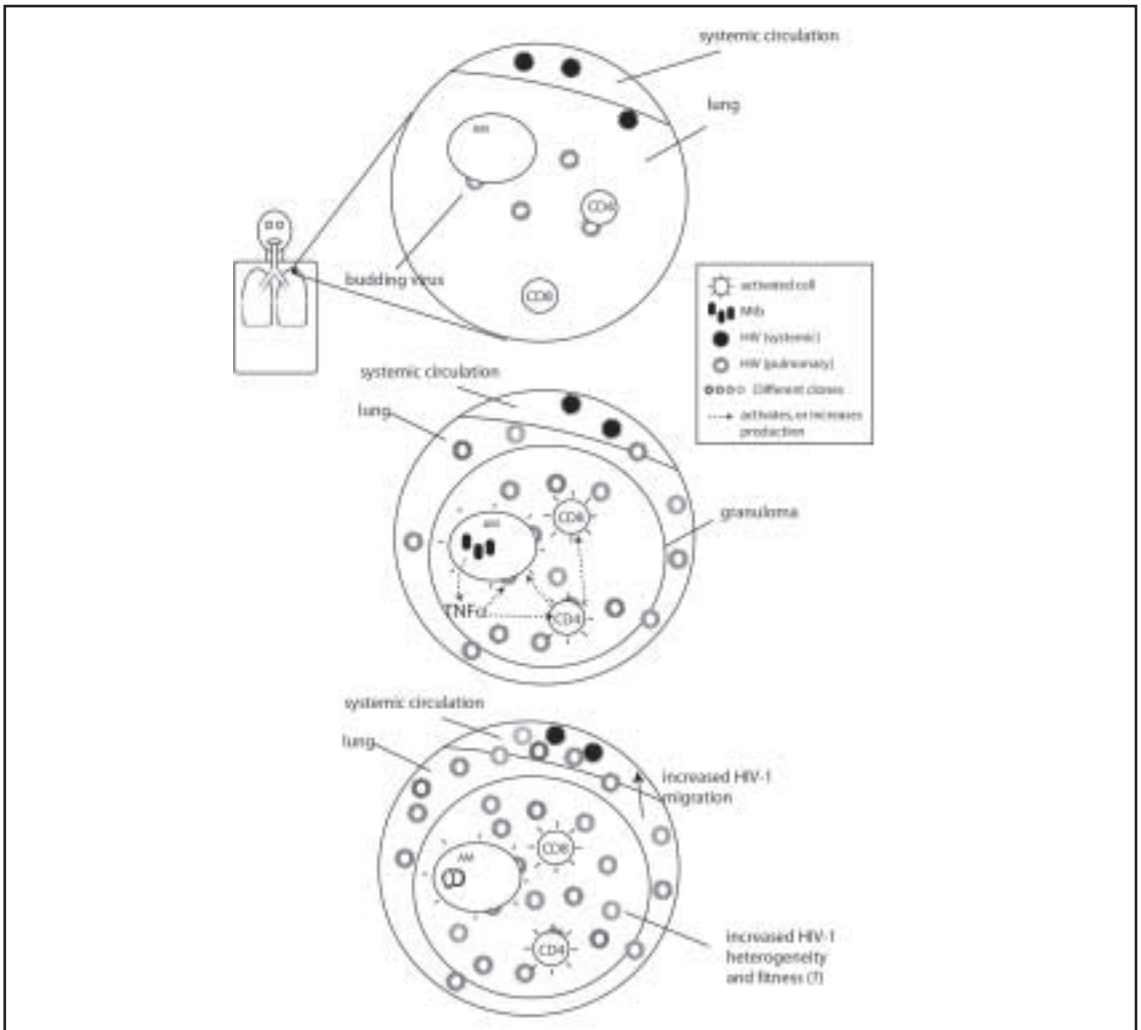

Figure 2. ***Heterogeneity of lung-derived HIV-1 isolates and migration to the blood upon TB-mediated stimulation.*** *HIV-1 replication is induced in the presence of M. tuberculosis infection in the lung. Increased HIV-1 levels in the lung appear to "spill-over" or migrate to blood or systemic circulation resulting in a burst in HIV-1 diversity and load.*

majority in the worldwide epidemic, and is divided into at least 11 phylogenetic subtypes (A through K)[86]. In addition, there are at least 12 circulating recombinant forms (CRF) of the virus that result from intersubtype recombination events. Subtypes typically share 75-90% sequence identity in the *env* gene. Although different subtypes now circulate, all have emigrated from the African continent. Central Africa appears to be the HIV-1 epicenter and may be the site of the original zoonotic jumps. This region is also endemic for TB. Subtype A, C, and D predominate in Uganda, but the incidence of TB appears to be unrelated to subtype[6]. This, however, does not imply that the impact of TB on HIV-1 disease progression will be the same in infections with different subtypes. Surprisingly, much of the research on TB-HIV-1 interaction has involved cohorts of subtype B infected individuals or subtype B HIV-1 laboratory strains for *in vitro* experiments. Subtype B is rarely found in Africa and TB endemic areas but is responsible for most HIV-1 infections in the developed world.

Relationship between HIV diversity and disease progression

Diversity in the human population originally stems from heterogeneity within individual host infections. Despite marked HIV-1 heterogeneity within a donor, initial infection is characterized by the transfer of a relatively homogeneous HIV-1 population[87] during sexual[88], mother to infant[89], and parental transmissions[88,90]. R5 HIV-1 predominate in newly infected individuals[91,92], even though donors may harbor both X4 and R5 viruses[92,93], suggesting that there is selection for R5 and against X4 viruses during HIV transmission.

The diversity and divergence from the founder strain in typical progressors can be divided into three phases during disease progression[94]. Both divergence and diversity increase linearly during the first and longest phase of disease progression. Diversity eventually reaches a plateau or declines, but HIV-1 sequences continue to diverge from the founder strain. Divergence will

eventually stabilize as diversity decreases. The second phase is associated with the appearance of X4 viruses, which typically peak prior to the third phase of intrapatient HIV-1 evolution. Finally, viral diversity is stable or continues to decrease during the third phase and is associated with a drop in CD4 T cell counts to under 200 cell/μl and reappearance of R5 HIV-1 isolates[94].

The vast majority of studies investigating HIV-1 heterogeneity within an infected individual or the human population have focused on the *env* gene. HIV-1 *Env* glycoproteins are involved in host cell entry, immune evasion, and act as a target for virus neutralization. The gp120 coding domain of *env* has been divided into alternating constant and variable regions, referred to as C1 through C5 and V1 through V5, respectively[95]. The variable regions lie mostly within regions encoding disulfide-constrained, surface-exposed loops[96]. Of particular interest is the *env* V3 region. This region is important for both CTL immune recognition[97], antibody mediated neutralization[98] as well as co-receptor usage (CCR5 or CXCR4)[99].

The nature of mutations in *env* provides insight into *in vivo* selection within the host[100]. The average ratio of synonymous (ds) to nonsynonymous (dn) substitutions is indicative of changes in primary amino acid sequence and has been used to describe viral evolution. ds/dn ratios much greater than one suggest negative selection, ratios less than one indicate positive selection, and values close to one indicate genetic drift[101]. HIV-1 isolates evolving with lower *env* ds/dn ratios tend to be under greater immune selective pressure. For instance, long term progressors, who have a strong anti-HIV-1 immune response[102], have been found to have lower ds/dn ratios as compared to typical progressors[103,104]. Although the values of ds/dn ratios are consistently lower in slower progressors, studies describing the association between disease progression and increase in heterogeneity have been inconsistent[103,104].

Ability of the immune system to contain virus may be related to an antigen diversity threshold, as suggested by Nowak, et al.[105]. When the viral diversity exceeds a threshold, the immune system can no longer contain the infection, and virus replicates without immune mediated hindrance[105]. This model suggests that the diversity of the virus is the cause of the immune failure, and not simply a consequence of the immune response. Studies that support this model indicate that rapid progressors have a more rapid increase in diversity[104,106]. However, other studies are inconsistent with this model and show that slow progressors may have a more rapid increase in *env* diversity[107-109]. The continuous virus adaptation model suggests that the immune system causes the virus to constantly evolve and adapt to the immune system[110]. Adaptation allows the virus to continuously replicate, which leads to the gradual depletion of CD4 T cells, and ultimately the development of AIDS. As described below, we have evidence that the onset of TB can lead to a burst in HIV-1 diversity and an irreversible increase in rate of disease progression.

Compartmentalization of HIV in the lung and divergent intrapatient evolution

Another important aspect of the evolution of HIV-1 is the anatomical compartmentalization of the virus. Phylogenetically distinct viral sub-populations have been found in the kidney, brain, blood, genital tract, and lung in HIV-1 patients. Explanations for the observed compartmentalization include physical separation of tissue compartments, selective migration of infected cells, distinct target cells, and selective pressure within compartments[111]. Understanding how different compartments contribute to the systemic quasispecies can give insights into the pathogenesis of HIV-1. In addition, *M. tuberculosis* establishes infection and a granuloma in the same tissues involved in HIV-1 compartmentalization (Figs. 1 and 2).

Several studies have indicated that HIV-1 in the lung may evolve separately from blood isolates. Phylogenetic analysis of *env* DNA in the blood and lung shows separate clustering of isolates in each compartment[112-114]. *In vivo* analysis of coreceptor utilization of the V3 regions of primary HIV-1 isolates derived from the lung and blood indicates that lung strains are restricted to using CCR5, whereas blood strains may use CCR5 and/or CXCR4[115]. It has also been shown that HIV strains from bronchoalveolar lavage cells, but not from peripheral blood cells, contain V3 domain nucleotide sequences with a greater degree of homogeneity in the C-terminal region and a highly conserved, negatively charged amino acid motif[113]. This indicates that strains infecting alveolar macrophages may have evolved further from the founder strain than those infecting blood monocytes. Longitudinal phylogenetic analysis has shown the lung quasispecies evolve separately from the blood, and harbor a very diverse population of HIV-1 quasispecies. This compartmental evolution may also be a continual HIV-1 reservoir.

Unlike other anatomical HIV-1 compartments, the lung is characterized by recurrent exposures to antigen through air and blood exposure. Alveolar macrophages have been implicated in preventing uncontrolled inflammation in the lung[116]. For example, the presence of alveolar macrophages blocks nitric oxide-synthase inhibitor in T cells, which temporarily blocks T cell proliferation[117]. The immunosuppression likely results in suppression of HIV-1 replication in the lung, which may be suppressed by surfactant[118], and type-1 interferon[119,120]. However, lung infection is a leading cause of morbidity and mortality during HIV-1 disease [118]. Pulmonary infections, such as *P. carinnii*, *M. tuberculosis*, *M. avium*, or *Aspergillus*,

lead to immune activation in the lung, which lead to the increased pulmonary HIV-1 replication upon coinfection[121]. Worldwide, *M. tuberculosis* is the most common HIV-1 coinfection[122]. As a result, we have used TB as a model for the effects of opportunistic infections on HIV-1 disease progression.

Impact of TB on HIV heterogeneity

Secondary infections such as TB can affect HIV-1 evolution and compartmentalization (Fig. 2). Nakata, et al.[10] studied the effects of pulmonary TB on HIV-1 replication and heterogeneity in 11 cases of pulmonary TB. Consistent with *in vitro* experiments[71,76], HIV-1 replication and heterogeneity was higher in TB-involved lung segments than the uninvolved segments. In a related study, tetanus inoculation led to no consistent change in HIV-1 viral heterogeneity[77]. However, tetanus inoculation models an acute immune activation, whereas TB models a more chronic immune stimulation, resulting in more persistent effects on HIV-1. In a majority of cases, the actual diagnosis and subsequent treatment of TB occurs on average two months after active TB infection has been initiated. As described above, TB is associated with increased systemic viral replication[7] and heterogeneity[6], decreased CD4 cell counts, a more rapid progression to AIDS, and increased mortality[67]. *In vitro, M. tuberculosis* infection or PPD stimulation of primary cell populations results in potent activation of HIV-1 replication and may explain the increase in HIV-1 viral loads following the diagnosis of TB [5].

Increased systemic HIV-1 heterogeneity was observed in patients with active pulmonary TB[6]. In a CD4 matched cohort, the mutation frequency of HIV-1 quasispecies in HIV-1-infected adults with TB (HIV/TB) patients was at least 2- to 3-fold greater than in HIV-1 patients without TB. It was hypothesized that this increase in systemic viral heterogeneity may be due to stimulation of HIV-1 replication at sites of *M. tuberculosis* infection (e.g. lung and/or pleural space) that could lead to a significant migration of genetically distinct lung-derived HIV-1 quasispecies into the blood (Fig. 2). This hypothesis was supported by a greater frequency of distinct HIV-1 quasispecies lineages in the blood of HIV/TB patients as compared to HIV-1 patients.

To test this hypothesis, pleural TB was used to model effects of TB on intrapatient HIV-1 evolution. Pleural TB is found in 28 to 38% of HIV-1-infected patients with TB[123,124] and is diagnosed by culturing *M. tuberculosis* from the pleural fluid, and/or histological examination of pleural biopsies. An extensive phylogenetic analysis was performed on the HIV-1 *env* quasispecies from all four compartments, i.e. cells and fluid from the pleural space and blood of HIV-1-infected patients with pleural TB[11]. Despite increased viral loads in the pleural space, there was only a slight trend for increased heterogeneity in the pleural space as compared to the blood. However, phylogenetic separation of the blood quasispecies from the pleural quasispecies in several patients was suggestive of compartmentalization and divergent evolution in pleural effusions. Also, there were substantial migration events between compartments in all of the patients[11]. Upon removing obvious migrants from the heterogeneity analysis, the pleural compartments contained a more heterogeneous HIV-1 population than that found in the blood (Fig. 2). In addition, the majority of these migrants tended to be viral particles that relocated from the pleural space to the blood. Thus, this migration from the pleura to the blood coupled with the higher viral loads in the pleura support the hypothesis that pleural (or lung) quasispecies are contributing to the increase in systemic HIV-1 heterogeneity observed in the presence of local *M. tuberculosis* infection[11] (Fig. 2).

Previous studies suggest a compartmentalization of HIV-1 quasispecies in the brain[125-127], cerebrospinal fluid[128,129], spleen[125], lymph node[125,127], lung, and semen[130,131]. In each of these studies, there is compartmentalization between the blood and the respective organ, with varying amounts of cross talk between compartments. However, it is not unusual to find lack of compartmentalization in these various organs. For instance, Delwart, et al.[131], have shown that some patients have clear communication between the blood and semen, whereas other patients have more defined compartments. These studies indicate that TB-mediated compartmentalization of HIV-1 in the pleural space is as defined as compartmentalization in any other organ. In addition, this model of pleural TB is quite suitable to explore HIV-1 migration between an *M. tuberculosis* infected organ and the blood (Fig. 2).

Does increased HIV-1 heterogeneity mediated by TB have any significance? Relating HIV-1 diversity to viral fitness and disease progression

It is quite evident that TB increases systemic or blood HIV-1 heterogeneity through a “spill-over” of the divergent HIV-1 population from TB-affected organs. As discussed earlier, HIV-1 quasispecies will evolve from the founder or infecting isolate as well as increase in heterogeneity during the course of disease. Emergence of TB during this phase is associated with a burst in HIV-1 diversity. In the absence of TB, intrapatient HIV-1 heterogeneity will slowly increase with time and then reach an inflection point prior to development of AIDS[94]. If HIV-1 diversity is a causative factor of disease, increased HIV-1 heterogeneity due to TB may decrease the time to AIDS. HIV-1 diversity and divergence from the founder decreases or stabilizes after this inflection point. A decrease in diversity also appears to coincide with a switch in

HIV-1 phenotype, i.e. from non-syncytium inducing (NSI) HIV-1 isolates utilizing the CCR5 coreceptor (R5) for host cell entry to CXCR4 (X4)-tropic, syncytium inducing (SI) isolates[94]. Host factors involved in this genotypic and phenotypic bottleneck are poorly understood but may be due to depletion of susceptible cell populations or destruction of the host immune system.

Although the impact of HIV-1 heterogeneity on disease is not entirely clear, specific factors that augment HIV-1 diversity or distribution of HIV-1 clones in various tissue compartments appear to decrease the time to AIDS. Immune activation due to other stimulants or antigens often has a direct affect on viral replication, load, and evolution. For example, tetanus inoculations have been shown to temporarily increase HIV-1 viral loads and the composition of the quasispecies[77]. Even with effective treatment, active TB results in transient increases in viral load, sustained increases in HIV-1 heterogeneity, and higher mortality in HIV-infected individuals. Sustained increases in HIV-1 heterogeneity in HIV-1/TB-infected individuals appear to be due to migration of divergent HIV-1 quasispecies into the blood from TB-affected, HIV-infected organs, e.g. lung or pleura[11]. This burst in the circulating quasispecies population may be responsible for the rapid progression to AIDS observed in HIV-infected individuals contracting TB.

Increases in HIV-1 quasipecies diversity and fitness during the course of disease have now been observed in two independent studies[94,132]. However, a direct relationship between increasing heterogeneity and fitness of an RNA virus has only been characterized *in vitro* models utilizing foot and mouth disease virus (FMDV)[133,134] and vesicular stomatitis virus (VSV)[135,136]. Domingo, Holland, and colleagues have shown that exponential increases in population size and diversity will lead to continual increases in viral fitness or replication efficiency (Red Queen hypothesis)[137]. Any changes in selection pressure or a decrease in population size will invoke Muller's Ratchet, i.e. the accumulation of escape/modulating mutations that are deleterious to the virus and lead to loss in fitness. In HIV-1 infections, a decrease in viral fitness may be due to an active and effective HIV-1-specific immune response and a continual selection of escape mutants. Certainly, antiretroviral treatment does select for resistant HIV-1 variants with reduced fitness as compared to wild type[138-140]. In the absence of therapy, strength of the immune pressure may even be measured by a net loss or limited fitness gains due to these continual bottlenecks, i.e. evidence of Muller's ratchet. Alternatively, the immune response may impose minimal pressure resulting in a net increase in HIV-1 replication efficiency during disease progression.

Recently we have compared HIV-1 evolution and fitness to disease progression in the absence and presence of ARV treatment. Detailed sequence analyses of the average as well as clones in the *env* quasispecies suggest that HIV-1 continues to diverge and diversify during disease progression. In both untreated and treated HIV-infected patients, variations in quasispecies diversity appeared to correspond to respective changes in *ex vivo* HIV-1 fitness. A decrease in the quasispecies diversity was observed after the initiation of ARV therapy, but this was also associated a drop in relative HIV-1 fitness. Overall, a positive and direct correlation was evident between quasispecies diversity and HIV-1 fitness (or replication efficiency in PBMC) whereas a negative correlation was observed when comparing *ex vivo* fitness to CD4 cell counts, i.e. a determinant of disease progression. These data provide the first *in vivo* support for the Red Queen hypothesis that is increases in HIV-1 fitness may be related to increasing viral loads and quasispecies diversity.

In conclusion, pulmonary TB may facilitate the progression of HIV-1 disease by increasing viral replication and through immune activation. Direct activation of viral replication occurs through *M. tuberculosis* induction of TNF-α that in turn activates NFκB and HIV-1 transcription. This TB-mediated HIV-1 replication leads to increased HIV-1 viral loads and heterogeneity at the site of infection. The increased quantity and diversity of the HIV-1 quasispecies allow for the evolution of more fit variants, some of which exit the lung and enter the systemic population (Fig. 2). The result is a more fit viral population, which may facilitate disease progression.

Acknowledgements

E.J.A. was supported by NIAID/NIH grants (AI49170 and AI 43645-02). Z.T. and M.E.Q-M were supported by the NHLBI/NIH grants (HL51636-06 and K01-HL67610-01, respectively). Additional support was provided by the CWRU Center for AIDS Research Grant (AI36219) and Tuberculosis Research Unit Grant (A1-45244) from NIAID/NIH. We thank Drs. Henry Boom and Richard Silver, Case Western Reserve University for their insightful comments.

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