Decreased Immune Activation in Resistance to HIV-1 Infection Is Associated with an Elevated Frequency of CD4+CD25+FOXP3+ Regulatory T Cells

Catherine M. Card,1 Paul J. McLaren,1,4 Charles Wachihi,4 Joshua Kimani,4 Francis A. Plummer,1,2,3 and Keith R. Fowke1,2,4

Departments of 1Medical Microbiology and 2Community Health Sciences, University of Manitoba, and 3Public Health Agency of Canada, Winnipeg, Canada; 4Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya

Human immunodeficiency virus (HIV)–resistant commercial sex workers provide a unique opportunity to study correlates of protection associated with natural resistance to HIV infection. Emerging data from studies of these individuals and other uninfected individuals who have been exposed to HIV suggest that low levels of immune activation may contribute to protection against infection. In the present study, HIV-resistant individuals were shown to have reduced frequencies of T cells expressing the activation marker CD69. They were also found to have elevated frequencies of regulatory T (Treg) cells, compared with HIV-negative control individuals. By controlling levels of T cell activation, Treg cells may contribute to HIV resistance by minimizing the pool of cells susceptible to infection.

Heterogeneity in susceptibility to HIV-1 infection has been demonstrated in several high-risk populations, including commercial sex workers, discordant couples, and uninfected infants born to HIV-positive mothers [1, 2]. Various mechanisms have been put forth to explain the protection observed in these rare individuals, but none are sufficient to explain all cases of resistance to HIV-1 infection. Elucidating the biological characteristics underlying protection against HIV will provide useful information on the protective mechanisms that may be harnessed for the development of new treatments and vaccine design.

Cohorts of commercial sex workers who are persistently exposed to yet remain uninfected with HIV are one of the best models of resistance to infection. We have followed HIV-resistant women from the Majengo commercial sex worker cohort in Nairobi, Kenya, for >20 years [3]. Studies of these women have provided evidence for a mechanism of HIV resistance involving differential regulation of the immune response, perhaps with a genetic basis. In addition, HIV-specific T cell responses have been characterized in HIV-resistant participants in the Majengo cohort and others [1, 2].

Nonhuman primate models have shown that a proportion of resting CD4+ T cells are susceptible to simian immunodeficiency virus infection yet are only capable of low levels of virus production. Infection of activated CD4+ T cells is required for dissemination of infection and seroconversion [4, 5]. Emerging data from the Majengo cohort and others [6–8] suggest that low levels of immune activation may play a role in HIV resistance. However, a mechanism by which low levels of activation are maintained has not been identified.

Regulatory T (Treg) cells have been implicated in the suppression of T cell activation and proliferation and cytokine production. These cells play a central role in preventing self-directed immune responses [9] and controlling inflammation induced by chronic pathogens [10, 11]. Despite a lack of research on the role of Treg cells in HIV resistance, one study examined these cells in neonates exposed to but uninfected with HIV. High levels of Treg cells and low levels of T cell activation were observed in cord blood samples obtained from HIV-exposed but -uninfected neonates, along with HIV-specific CD8+ T cell responses. CD8+ T cell responses were augmented on depletion of Treg cells but were detectable in their presence. In this context, Treg cells may help to prevent vertical transmission of HIV-1 by maintaining a low level of activation, while allowing polyfunctional T cell responses to clear any infected cells [12].

The present study sought to compare Treg cell frequencies and T cell activation levels between HIV-resistant women, HIV-negative women newly enrolled in the study, and HIV-positive women from the Majengo cohort in Nairobi, Kenya. Our data
support a role for low levels of immune activation in individuals with HIV resistance and suggest that T_{reg} cells play a role in maintaining this low activation state.

**Subjects, materials, and methods.** The study participants were members of the Majengo commercial sex worker cohort in Nairobi, Kenya. HIV infection status was determined by serologic testing and confirmed by HIV-1 polymerase chain reaction (PCR) analysis. Fresh blood samples were collected from HIV-infected (n = 45), HIV-resistant (n = 23), and newly enrolled HIV-uninfected controls (n = 34). Study subjects were classified as HIV resistant if they were HIV negative at the time of enrollment into the cohort, remained HIV negative for 7 years of follow-up, and continued sex work during this time. HIV-uninfected women who were enrolled in the cohort for <7 years were classified as HIV negative. The women in the HIV-negative population are exposed to the same virus strains and other immunologic challenges as are HIV-resistant women, but the vast majority of them will experience seroconversion within a few years of initiation of sex work.

Written informed consent was obtained from all study participants. The study was approved by ethics review boards from the University of Manitoba and the Kenyatta National Hospital.

Freshly isolated peripheral blood mononuclear cells (PBMCs) were immunophenotyped using multicolor flow cytometry, to determine levels of T cell activation and T_{reg} cell frequency. To determine levels of T cell activation, PBMCs were stained with anti-CD3 AmCyan, anti-CD4 AlexaFluor700, anti-CD8 Pacific Blue, anti-CD69 fluorescein isothiocyanate, anti–HLA-DR APCCy7, and anti-CD38 allophycocyanin (APC) (BD Biosciences). T_{reg} cell immunophenotyping was performed by staining with anti-CD3 AmCyan, anti-CD4 AlexaFluor700, anti-CD8 Pacific Blue, anti-CD25 phycoerythrin (BD Biosciences), and anti–forkhead box P3 (FOXP3) APC (eBioscience). FOXP3 staining was performed using the FOXP3 staining set (eBioscience) according to the manufacturer’s instructions. Data were acquired on a LSRII flow cytometer (BD Biosciences) and were analyzed using FACSDiva software (version 5.0.1; BD Biosciences).

Comparisons of immune parameters between groups were performed using the nonparametric Mann-Whitney U test. Differences were considered to be statistically significant if P < .05. Statistical analyses were performed using GraphPad Prism software (version 4.0).

**Results.** A total of 102 participants were enrolled in the present study. The median follow-up times since enrollment in the cohort were 16 years for HIV-resistant, 2.6 years for HIV-negative, and 4 years for HIV-positive participants. The median number of self-reported years of sex work were 21 years for HIV-resistant, 7 years for HIV-negative, and 10 years for HIV-positive participants. The HIV-resistant women were significantly older than both the HIV-negative (P < .001) and the HIV-positive (P < .001) controls (median age, 49, 36, and 35 years, respectively). This is to be expected, because most participants are HIV infected at the time of entry into the cohort, and, of those who are seronegative, the majority become infected within a few years. HIV-resistant individuals are healthy and can remain in sex work and, therefore, are older. However, we recognize age as a possible confounding variable of our data on T_{reg} cell frequency. We therefore analyzed T_{reg} cell frequency as a function of age within each study group. No correlation was found between age and T_{reg} cell frequency in any of the groups studied (data not shown), indicating that observed differences are a function of HIV infection status, rather than age.

To determine frequencies of activated CD4^+ and CD8^+ T cells, expression of CD69, HLA-DR, and CD38 was measured by flow cytometry (figure 1A). CD4^+ and CD8^+ T cells expressing CD69 were found at a lower frequency in HIV-resistant women than in HIV-negative controls (P < .05 and P < .01, respectively) (figure 1B and 1C). HIV-positive individuals had a greater frequency of CD4^+CD69^+ T cells than did HIV-negative individuals (P < .05) (figure 1B). No significant differences in expression of CD69 were observed between HIV-positive and HIV-negative individuals. The number of cells expressing the chronic activation markers HLA-DR and CD38 were found to be higher in both the CD4^+ and CD8^+ T cell subsets in HIV-positive individuals (P < .001) (figure 1G) than in either HIV-negative or HIV-resistant individuals.

With the reduced levels of cellular activation observed in HIV-resistant women kept in mind, we next investigated whether T_{reg} cell frequency differed between our study populations. Conflicting data from previous reports of T_{reg} cell frequency in HIV infection and other diseases may be a result of inadequate phenotypic identification of T_{reg} cells. We therefore used a strict phenotypic definition involving coexpression of CD3, CD4, CD25, and FOXP3 to identify T_{reg} cells (figure 2A).

The majority of T_{reg} cell studies define T_{reg} cell frequency as a percentage of CD4^+ T cells. CD4^+ T cells are progressively lost in HIV disease, so expression of T_{reg} cells as a percentage of CD4^+ T cells may not accurately represent the T_{reg} cell population in HIV-positive subjects. Therefore, we examined the frequency of T_{reg} cells in each group both as a percentage of CD4^+ T cells and as a percentage of CD3^+ T cells. This approach has previously been used to quantify T_{reg} cells in HIV-positive subjects [13].

We found that T_{reg} cell frequency was significantly elevated in HIV-resistant women, compared with HIV-negative controls. This was evident when T_{reg} cells were expressed both as a percentage of CD4^+ T cells (P = .01) (figure 2B) and as a percentage of CD3^+ T cells (P < .01) (figure 2C). When T_{reg} cells were expressed as a percentage of CD3^+ T cells, HIV-positive subjects were shown to have lower T_{reg} cell frequencies than both HIV-resistant (P < .001) (figure 2C) and HIV-negative (P < .001) (figure 2C) controls.

**Discussion.** Previous data from the Majengo commercial sex worker cohort suggest a role for low T cell activation in pro-
tection against HIV infection. Specifically, a recent genomic study showed that CD4+ T cells from HIV-resistant women had lower levels of generalized gene expression than did those from HIV-negative controls. Among the suppressed genes were some required for cellular activation and HIV-1 replication (P.J.M. and K.R.F., unpublished data). In addition, several studies emerging in the literature support a role for low levels of immune activation in HIV resistance. In a cohort of highly exposed but HIV-seronegative men who have sex with men in Amsterdam, The Netherlands, protection was associated with low levels of CD4+ T cell activation, as measured by expression of HLA-DR, CD38, and CD70 [6]. HIV-exposed but -seronegative sex workers in Abidjan, Côte d’Ivoire, expressed low levels of CD69 and had decreased secretion of effector molecules after allostimulation, compared with controls [7]. In the Central African Republic, exposed but uninfected partners of HIV-infected in-

![Figure 1. Expression of activation markers CD69, HLA-DR, and CD38 on CD4+ and CD8+ T cells in HIV-negative, HIV-resistant, and HIV-positive women. A, Representative flow cytometry staining of CD4+ and CD8+ T cells for CD69, HLA-DR, and CD38 expression. HIV-resistant women have significantly fewer CD4+ (B) and CD8+ (E) T cells expressing the activation marker CD69. HIV-positive women have significantly higher frequencies of CD4+ and CD8+ T cells expressing HLA-DR (C, F) and CD38 (D, G), compared with HIV-negative or HIV-resistant women. Box plots denote the median frequencies of positive cells and respective SDs. Whiskers denote the range of frequencies of positive cells. Statistical comparisons were made using Mann-Whitney U test. APC, allophycocyanin; FITC, fluorescein isothiocyanate; HIV-, HIV negative; HIV+, HIV positive; HIV-R, HIV resistant.](image-url)
Individuals had lower levels of CD4+ T cell activation, as measured by expression of HLA-DR and chemokine receptor 5. Furthermore, unstimulated PBMCs isolated from these individuals showed reduced in vitro susceptibility to HIV-1 infection, an effect that was overcome by mitogenic stimulation [8].

These studies prompted us to investigate levels of cellular activation in the Majengo cohort. CD69 has been shown to be up-regulated in the early stages of the cell cycle, signifying transcriptional activation and increased cellular susceptibility to HIV infection [14]. We found fewer CD69-expressing CD4+ and CD8+ T cells in HIV-resistant individuals than in HIV-negative controls, indicating reduced cellular activation. HIV-positive individuals had a greater frequency of CD4+CD69+ T cells than did HIV-resistant individuals, and a similar trend was observed in the CD8+ T cell subset. Differences in T cells expressing CD69 were not observed between HIV-positive and HIV-negative individuals. This is to be expected, because expression of CD69 does not characterize progressive HIV disease. In contrast, the chronic activation markers HLA-DR and CD38 were strongly up-regulated in HIV-positive individuals, compared with HIV-resistant and HIV-negative individuals. Levels of HLA-DR and CD38 expression were similar between HIV-resistant and HIV-negative individuals. However, these markers are up-regulated late in the cell cycle and may not be informative for discriminating between quiescent cells that are resistant to infection and recently activated cells that become susceptible to HIV infection.

Because of the capacity of Treg cells to suppress cellular activation, we suspected that these cells may play a role in maintaining the reduced cellular activation observed in HIV-resistant women. We found higher levels of Treg cells in HIV-resistant women relative to HIV-negative controls. In addition, HIV-positive women had depleted frequencies of Treg cells when expressed as a percentage of total CD3+ T cells. These data are in agreement with other studies suggesting that, during progressive HIV disease, Treg cell levels decrease in the periphery. This loss of Treg cells may be partially due to compartmentalization in sites of viral replication, and it may drive hyperactivation of the immune system during progressive HIV disease. Indeed, HIV-driven Treg cell depletion has been associated with elevated levels of CD4+ and CD8+ T cell activation [11]. However, there is conflicting evidence regarding the effects of HIV infection on frequency of Treg cells. Some studies observed an increase in peripheral Treg cells after infection [11]. Conflicting results may arise from differences in the methods of Treg cell identification used or in the populations studied. Despite the beneficial effects of Treg cells, their role after HIV infection is complex. They have been implicated in the suppression of HIV-specific T cell re-

Figure 2. CD4+CD25+FOXP3+ regulatory T (Treg) cell frequencies in HIV-negative, HIV-resistant, and HIV-positive women. A, Representative flow cytometry gating strategy for identification of CD4+CD25+FOXP3+ Treg cells in peripheral blood. HIV-resistant women have elevated frequencies of Treg cells, compared with HIV-negative women, when Treg cells are expressed as a percentage of CD4+ T cells (B) or total CD3+ T cells (C). HIV-positive women have depleted levels of Treg cells when Treg cells are expressed as a percentage of total CD3+ T cells (C). Box plots denote median frequencies of positive cells and respective SDs. Whiskers denote the range of frequencies of positive cells. Statistical comparisons were made using the Mann-Whitney U test. HIV−, HIV negative; HIV+, positive; HIV-R, HIV resistant.
responses such that $T_{reg}$ cell depletion enhances cytokine production and proliferation by T cells in vitro [11].

The results of the present study led us to hypothesize that a higher proportion of $T_{reg}$ cells mediates the observed suppression of CD4$^+$ and CD8$^+$ T cell activation in HIV-resistant women. $T_{reg}$ cells have been shown to interact directly with target T cells, resulting in transcriptional repression [15]. By reducing the cellular activation and gene transcription required for HIV-1 replication, the pool of CD4$^+$ T cells susceptible to HIV is reduced. Some target cells likely become infected; however, in the environment of low cellular activation and limited availability of substrates for viral replication, HIV-specific T cell responses may be sufficient to clear infection and interfere with efficient dissemination of the virus. This study provides evidence that decreased immune activation driven by an elevated frequency of $T_{reg}$ cells plays a role in HIV resistance. Future studies will address this hypothesis by evaluating the effects of $T_{reg}$ cell depletion on cellular activation and in vitro susceptibility to HIV-1 infection.

Acknowledgments

We would like to thank Blake Ball for critical review of the manuscript and Lyle McKinnon for technical assistance.

References