Low-Level CD4+ T Cell Activation in HIV-Exposed Seronegative Subjects: Influence of Gender and Condom Use

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Immune activation has been suggested to increase susceptibility to human immunodeficiency virus type 1 (HIV-1) transmission, while at the same time it could be deemed essential for mounting an effective antiviral immune response. In this study, we compared levels of T cell activation between exposed seronegative (ESN) partners in HIV-1 discordant couples and HIV-unexposed control subjects in Dakar, Senegal. ESN subjects showed lower levels of CD38 expression on CD4+ T cells than did control subjects. However, this was found to be associated with concurrent differences in the use of condoms: ESN subjects reported a higher degree of condom use than did control subjects, which correlated inversely with CD38 expression. In addition, we observed markedly higher levels of T cell activation in women compared with men, irrespective of sexual behavior. These findings question the relevance of low-level CD4+ T cell activation in resistance to HIV-1 infection and underscore the need to take gender and sexual behavior characteristics of high-risk populations into account when analyzing correlates of protective immunity.

Rare individuals remain persistently human immunodeficiency virus (HIV)–seronegative despite multiple high-risk exposures to the virus (HIV-exposed seronegative [ESN] subjects) [1, 2]. These individuals belong to various risk groups such as commercial sex workers, healthcare workers, hemophilia patients who received HIV-contaminated blood products, intravenous drug users, promiscuous homosexual men, and subjects with stable HIV-infected partners (ie, HIV-discordant couples). Clearly, sexual transmission of HIV is influenced by characteristics of the infecting virus, as well as of the HIV-infected index partner, such as the viral load [3]. In several other cases, however, protection against HIV infection was found to be associated with factors related to the ESN partner, which include but are not limited to genetic predisposition (eg, CCR5-Δ32 mutation, particular HLA alleles), intrinsic cellular defense (eg, β-chemokines, defensins, APOBEC3G), and innate or adaptive immune responses (eg, natural killer cell activity, HIV-specific immunoglobulin A, and HIV-specific T cells) [1, 2]. Better understanding of how ESN subjects resist HIV infection may be crucial for the development of future therapies or vaccines.

Immune activation has been suggested to be critical in susceptibility to HIV type 1 (HIV-1) transmission [4]. In vitro, HIV-1 requires activated T cells for a productive infection [5], and subjects with an activated immune system show increased in vitro susceptibility to HIV and higher in vivo replication [6, 7]. The widespread HIV-1 epidemic in Africa has been linked to higher numbers of activated CD4+ T cells in the peripheral blood and genital tract of African subjects.
[8–10], possibly as a consequence of a higher prevalence of parasitic infections [11]. In agreement with this, researchers in a number of studies observed low levels of immune activation in ESN subjects [12–15]. However, at the same time immune activation could be deemed essential for mounting effective host immune responses to invading pathogens like HIV, and in line with this a number of studies reported increased levels of immune activation in ESN populations [16–19].

Immune activation likely depends on individual predisposition, as well as on environmental factors. We hypothesized that temporal changes in environmental exposure through unprotected sexual contacts could help explain the discrepancy observed in previous studies. Therefore, in this study we compared levels of T cell activation in ESN partners in HIV-discordant couples with those of unexposed control subjects, while taking into account the level of unprotected sexual exposure and the occurrence of sexually transmitted infections (STIs). In line with previous studies, we observed lower levels of CD4+ T cell activation in ESN subjects than in control subjects; however, this appeared to be associated with a higher degree of condom use among ESN subjects.

MATERIALS AND METHODS

Study population. Twenty-six HIV-1 discordant and 13 HIV-seronegative heterosexual couples were enrolled. The couples were recruited at the Department of Infectious Diseases at the Centre Hospitalier Universitaire Fann, Dakar, Senegal. Included couples were married for at least 6 months. Blood samples and standard questionnaires with information on sociodemographics and sexual behavior were collected. Husbands and wives were interviewed separately by a trained social assistant. Male condom provision and sexual risk reduction counseling were provided during every visit. The study was approved by the Internal Review Board of the Institute of Tropical Medicine (Antwerp, Belgium) and by the Ethical Committees of the Senegalese Ministry of Health (Dakar, Senegal) and the University Hospital of Antwerp (Belgium). All study subjects gave written informed consent before enrollment.

Laboratory methods for HIV and STI diagnosis. Whole blood was drawn into edetic acid tubes (Becton Dickinson). Plasma was separated from cells by centrifugation, separated into aliquots, and stored at −80°C. HIV status was evaluated in plasma by current serological testing combining enzyme-linked immunosorbent assays (ELISAs) and Western blotting. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by gradient centrifugation, stored at −80°C overnight, and then transferred to liquid nitrogen. The HIV-negative status of ESN and control subjects was confirmed by HIV-1 and HIV-2 diagnostic polymerase chain reaction on genomic DNA extracted from PBMCs. Vaginal and cervical samples were collected at each visit after a physical examination including examination of the vulva, vagina, and cervix. The presence of yeast (*Candida albicans*) and *Trichomonas vaginalis* was detected by light microscopy on a saline wet preparation. Bacterial vaginosis was evaluated by microscopy of a vaginal Gram stain. Herpes simplex virus type 2 (HSV-2) infection was detected by ELISA (Kalon). The screening for syphilis was done by *Treponema pallidum* hemagglutination assay (TPHA; Fuji-rebio) and the rapid plasma reagin (RPR) test (Becton Dickinson). A positive diagnosis of syphilis was made if both TPHA and RPR test results were positive.

T cell subsets and activation markers. Phenotypic analysis of peripheral blood lymphocytes was performed on fresh whole blood within 4 h of collection. Percentages of CD4+ and CD8+ T cells and the levels of CD4+ and CD8+ T cell activation were assessed by flow cytometry using the following monoclonal antibody combinations: anti-CD3-FITC, anti-CD4-PE, and anti-CD8-PerCP; anti-CD3-FITC, anti-CD38-PE, and anti-CD8-PerCP; and anti-CD3-FITC, anti-HLA-DR-PE, and anti-CD8-PerCP (all from Becton Dickinson). Samples were analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson). For the activation markers CD38 and HLA-DR, both percentages of positive cells and median fluorescence intensity levels were analyzed within CD4+ and CD8+ T cell subsets.

Statistical analysis. Differences in continuous and categorical variables between groups were analyzed with nonparametric Mann-Whitney *U* test and Fisher exact test, respectively. Correlation analyses were performed with the nonparametric Spearman rank correlation. The level of significance for all statistical tests was set at *P* < .05. Statistical analyses were performed with GraphPad Prism (version 5.01) and SPSS (version 15.0) software.

RESULTS

Study population. A total of 26 ESN subjects in 26 HIV-1 discordant couples were compared with 26 HIV-unexposed control subjects in 13 HIV-negative couples (Table 1). Thirteen ESN subjects (50%) and 13 control subjects (50%) were men. Male ESN subjects were slightly older than male control subjects, whereas female ESN subjects were slightly younger than female control subjects, although these differences were not statistically significant. No statistically significant differences were found in CD4+ and CD8+ T cell counts between ESN and control subjects.

Expression of activation markers among ESN and control subjects. To investigate whether immune activation plays a role in host susceptibility to HIV-1 infection, we compared the expression of activation markers on CD4+ and CD8+ T cells between ESN and control subjects (Table 2). ESN subjects showed significantly lower percentages of CD38 expression on CD4+ T cells than did control subjects (*P* = .019; Table 2 and
Table 1. Characteristics of HIV-Exposed Seronegative (ESN) Subjects and HIV-Unexposed Control Subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>Male subjects</th>
<th>Female subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESN subjects</td>
<td>Control subjects</td>
<td>ESN subjects</td>
</tr>
<tr>
<td></td>
<td>(n = 26)</td>
<td>(n = 26)</td>
<td>(n = 13)</td>
</tr>
<tr>
<td>Age, years</td>
<td>41 (30–46)</td>
<td>42 (34–42)</td>
<td>46 (41–56)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>13 (50)</td>
<td>13 (50)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>CD4+ T cell counts</td>
<td>736 (599–928)</td>
<td>879 (709–1119)</td>
<td>672 (517–921)</td>
</tr>
<tr>
<td>CD8+ T cell counts</td>
<td>502 (280–580)</td>
<td>481 (332–804)</td>
<td>352 (246–553)</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) values or n (%), if indicated. HIV, human immunodeficiency virus.

Table 2. Expression of Activation Markers on CD4+ and CD8+ T Cells among HIV-Exposed Seronegative (ESN) Subjects and HIV-Unexposed Control Subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ESN subjects (n = 26)</th>
<th>Control subjects (n = 26)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38, %</td>
<td>44.1 (33.6–67.8)</td>
<td>52.6 (44.6–66.5)</td>
<td>.019</td>
</tr>
<tr>
<td>CD38, MFI</td>
<td>21.6 (15.7–47.8)</td>
<td>31.6 (24.9–76.3)</td>
<td>.023</td>
</tr>
<tr>
<td>HLA-DR, %</td>
<td>14.9 (10.8–19.4)</td>
<td>14.4 (11.3–17.1)</td>
<td>.687</td>
</tr>
<tr>
<td>HLA-DR, MFI</td>
<td>7.3 (4.1–8.5)</td>
<td>6.4 (5.2–8.4)</td>
<td>.869</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38, %</td>
<td>50.8 (24.5–53.2)</td>
<td>47.5 (28.0–64.7)</td>
<td>.194</td>
</tr>
<tr>
<td>CD38, MFI</td>
<td>19.1 (12.1–35.8)</td>
<td>24.7 (14.2–48.7)</td>
<td>.297</td>
</tr>
<tr>
<td>HLA-DR, %</td>
<td>36.5 (27.4–47.4)</td>
<td>30.7 (27.5–41.8)</td>
<td>.319</td>
</tr>
<tr>
<td>HLA-DR, MFI</td>
<td>12.0 (8.7–18.3)</td>
<td>11.2 (8.6–16.0)</td>
<td>.589</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) values. P values below .05 are in bold. HIV, human immunodeficiency virus; HLA-DR, human leukocyte antigen DR; MFI, median fluorescence intensity.

Figure 1A). Similar conclusions were reached when median fluorescence intensity (MFI) levels of CD38 expression were analyzed. Differences were present for both male and female subjects, although they were most pronounced for the male subjects. No differences were found in CD38 expression on CD4+ and CD8+ T cells between ESN and control subjects. Interestingly, within both ESN and control groups, male subjects showed markedly lower levels of CD38 expression on both CD4+ and CD8+ T cells than did female subjects (ESN, P = .026 and .004 for CD4+ and CD8+ T cells, respectively; controls: P = .011 and .069 for CD4+ and CD8+ T cells, respectively; Table 2 and Figure 1B). (Similar differences were found for MFI levels of CD38 expression; data are not shown.) No differences were found in HLA-DR expression on CD4+ and CD8+ T cell subsets between ESN and control subjects, or between male and female subjects.

Expression of activation markers in relation to STI in ESN and control subjects. Next, we evaluated whether the differences found in CD38 expression levels on CD4+ T cells between ESN and control subjects might be associated with differences in the occurrence of STI (Figure 2). Ten of 25 tested ESN subjects were seropositive for HSV-2 compared with 4 of 26 control subjects (40% vs 15%; P = .064); 4 of 11 tested female ESN subjects were positive for Gardnerella vaginalis infection compared with 5 of 12 tested female control subjects (36% vs 42%; P > .99). Prevalences of syphilis, C. albicans, and T. vaginalis infection were too low to allow relevant comparisons between ESN and control subjects (data not shown). No differences were found in CD38 expression on CD4+ T cells according to HSV-2 serostatus among ESN subjects (P = .318; Figure 2A). Interestingly, however, control subjects with HSV-2 showed significantly higher percentages of CD38 expression on CD4+ T cells than did those control subjects without HSV-2 infection (P = .016; Figure 2A). No differences were found in CD38 expression regarding presence or absence of G. vaginalis infection among female subjects in either group (Figure 2B). Overall, differences in immune activation between ESN and control subjects could not be attributed to differences in the prevalence of STI. Similar conclusions were reached when MFI levels of CD38 expression were analyzed (data not shown).

Association between immune activation and sexual exposure among ESN and control subjects. Next, we sought to analyze whether the differences in immune activation could be associated with the levels of sexual exposure among ESN and control subjects. An overview of sexual exposure characteristics
Figure 1. Expression of CD38 on CD4+ T cells of exposed seronegative (ESN) subjects and controls. (A) Comparison of ESN subjects (n = 26) and control subject (n = 26) groups. (B) Comparison of ESN subjects (n = 26) and control subjects (n = 26) stratified by sex. Horizontal bars represent median values. Differences in CD38 expression were analyzed with Mann-Whitney U-tests.

Figure 2. Expression of CD38 on CD4+ T cells in relation to herpes simplex virus type 2 (HSV-2) and Gardnerella vaginalis infection. (A) Comparison of exposed seronegative (ESN) subjects (n = 25) and control subjects (n = 26) stratified for HSV-2 infection. (B) Comparison of female ESN subjects (n = 11) and controls (n = 12) stratified for G. vaginalis infection. Horizontal bars represent median values. Differences in CD38 expression were analyzed with Mann-Whitney U tests. G. v, Gardnerella vaginalis.
Male subjects
Female subjects

Table 3. Sexual Behavior Characteristics of HIV-Exposed Seronegative (ESN) Subjects and HIV-Unexposed Control Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th></th>
<th>Male subjects</th>
<th></th>
<th>Female subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESN subjects</td>
<td>Control subjects</td>
<td>ESN subjects</td>
<td>Control subjects</td>
<td>ESN subjects</td>
</tr>
<tr>
<td>Duration of relation, years</td>
<td>8 (6–18)</td>
<td>10 (5–15)</td>
<td>.826</td>
<td>7 (5–13)</td>
<td>10 (5–15)</td>
</tr>
<tr>
<td>Sexual contacts per month, no.</td>
<td>4 (3–8)</td>
<td>6 (3–8)</td>
<td>.524</td>
<td>5 (3–8)</td>
<td>6 (3–8)</td>
</tr>
<tr>
<td>Condom use, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Always</td>
<td>17 (65)</td>
<td>0 (0)</td>
<td>.001</td>
<td>11 (85)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Often</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Occasionally</td>
<td>5 (19)</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Never</td>
<td>3 (12)</td>
<td>26 (100)</td>
<td></td>
<td>2 (15)</td>
<td>13 (100)</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) values unless otherwise indicated. \( P \) values below .05 are in bold. HIV, human immunodeficiency virus.

DISCUSSION

Immune activation has been suggested to increase susceptibility to HIV transmission; at the same time, however, it could be deemed essential for mounting an effective host immune response to invading pathogens like HIV [4]. Indeed, researchers in previous studies observed HIV-specific CD4\(^+\) T cell and cytotoxic T lymphocyte responses in ESN subjects, as well as evidence of increased natural killer cell activity [1,2], all of which could be expected to contribute to some kind of localized or systemic immune activation. Such an ambiguous situation appears to reflect findings in previous studies of ESN subjects: decreased as well as increased levels of immune activation in comparison with low-risk control subjects [12–19]. We hypothesized here that part of this controversy could be explained by variability in exposure to environmental factors such as STI and the frequency of unprotected sexual contacts.

We found that, in comparison with low-risk control subjects, ESN subjects in our cohort of Senegalese HIV-discordant couples showed lower levels of CD38 expression on CD4\(^+\) T cells. This finding is in agreement with previous studies that showed low-level immune activation in populations of ESN subjects, including ESN gay men from the Amsterdam cohort [12], ESN subjects in HIV-discordant couples from the Central African Republic [13], and ESN female sex workers from Kenya [15]. However, in our study this was found to be associated with concurrent differences in the use of condoms. Indeed, a substantial proportion of ESN subjects (65%) reported always using condoms with their HIV-1–infected partners compared with none of the control subjects, and these ESN subjects preferentially showed the decrease in CD4\(^+\) T cell activation. Interestingly, differences in immune activation between ESN and control subjects were most pronounced for male subjects, which is consistent with the higher degree of condom use noted among male than among female ESN subjects (85% vs 46% reporting always using condoms). In addition, ESN subjects reported slightly lower numbers of sexual contacts with their stable partners than did control subjects. Together, this raises the possibility that the lower levels of CD4\(^+\) T cell activation in ESN subjects have resulted from a decrease in unprotected sexual exposure in this population. Clearly, this decrease in immune activation among ESN subjects must have resulted from a decrease in exposure to genital secretions in general rather than to HIV specifically, given that control subjects included in our study were all at low risk for HIV exposure.

In fact, a decrease in unprotected sexual exposure among ESN subjects may not be unexpected given their higher risk perception for HIV infection [20,21]. In addition, most study sites provide safe-sex counseling to ESN subjects, which likely reinforces these effects [22–24]. In that respect, we hypothesize that some or all of the previous studies that observed low-level CD4\(^+\) T cell activation among ESN subjects may have underestimated the use of condoms in their populations [12,13,15]. Unfortunately, there are limited data available on condom use or numbers of unprotected sexual contacts in previous studies. Neither Koning et al [12] nor Card et al [15] specified the frequency of condom use. Begaud et al [13] observed a markedly lower frequency of condom use by ESN subjects than we did in our study (16% vs 88% of ESN subjects reporting some degree of condom use), but they did not investigate the association between condom use and immune activation. Our findings could also imply that ESN populations with increased levels of immune activation may be characterized by higher levels of unprotected sexual exposure than their respective control populations, but again, due to limited data availability, this is difficult to confirm. Prior unprotected sexual intercourse was an enrollment criterion in the study by Suy et al [19], but no association between condom use and immune activation was shown. Interestingly, ESN female sex workers from our previous study in Abidjan, Côte d’Ivoire, were characterized by high numbers of sexual contacts (median value of 20 sexual contacts per week), together with a relatively low frequency of condom use (37% reporting always using condoms) [17]. This could indeed explain their increased level of immune activation in comparison with female blood donor control subjects, which
are expected to experience a much lower level of sexual exposure [17]. This was confirmed by a subsequent study that showed higher levels of mitogen-stimulated T cell activation in this population [25]. However, whether increased levels of immune activation could be a general feature of highly exposed female sex workers could not be verified because Card et al [15] did not include a non–sex worker control group.

We also analyzed levels of CD4+ T cell activation in ESN and control subjects in relation to the occurrence of STI in both groups. A higher proportion of ESN subjects tested positive for HSV-2 than did control subjects; however, there were no differences in CD4+ T cell activation between ESN subjects with HSV-2 infection and those without. Neither did we see any influence of the other STI on CD4+ T cell activation, although some of these analyses were impaired because of too few positive cases. Nevertheless, these data suggest that the observed differences in CD4+ T cell activation between ESN and control subjects did not result from differences in STI, which is in agreement with findings in the study by Koning et al [12], who also found low levels of T cell activation in ESN subjects to be independent of STI. It is well established that HSV-2 infection is associated with an increase in HIV-1 acquisition [26], and it was recently suggested that high numbers of CCR5-bearing and activated CD4+ T cells found in the genital tract of HSV-2–infected subjects are responsible for this [27, 28]. In that respect, it is remarkable that HSV-2–infected ESN subjects did not show an increase in CD4+ T cell activation, given that we did see such an increase among the 4 HSV-2 infected control subjects. Although this could reflect differences in immune function between peripheral blood and mucosal T cells, it could also point toward a specific capacity of ESN subjects to actively maintain a low-grade CD4 T cell activation state, irrespective of the degree of condom use.

Interestingly, we observed significantly higher levels of CD38 expression on CD4+ as well as on CD8+ T cells in women compared with men, and this was true for both ESN and control groups. It is known that functional immune subsets may be different between men and women, and marked sex-based differences in susceptibility to infectious and autoimmune diseases have been described [29]. Differences between men and women have also been reported for HIV-1 disease progression, with women experiencing an increased risk of developing AIDS compared with men for the same level of viral replication [30,31]. An explanation for such a difference was recently provided by a study that showed higher levels of CD38 expression on CD8+ T cells in women compared with men as a result of higher levels of interferon-α production by female plasmacytoid dendritic cells in response to HIV-1–encoded Toll-like receptor ligands [32]. Our data are in line with these findings and suggest that higher levels of T cell activation in women are already present before HIV-1 acquisition, thus also providing an explanation for the

Figure. 3. Expression of CD38 on CD4+ T cells in relation to sexual behavior characteristics. (A) Comparison of exposed seronegative (ESN) subjects (n = 26) and control subjects (n = 26) stratified for the duration of the sexual relationship. (B) Comparison of ESN subjects (n = 26) and control subjects (n = 26) stratified for the number of sexual contacts per month. (C) Comparison of ESN subjects (n = 26) and controls (n = 26) stratified for the frequency of condom use. Horizontal bars represent median values. Differences in CD38 expression were analyzed with Mann-Whitney U tests.
relatively increased HIV-1 susceptibility of women compared with men [33]. Another intriguing gender-related finding of this study was the substantially higher proportion of male compared with female ESN subjects reporting always using condoms with their HIV-1 infected partners (85% vs 46%). This confirms the known difficulties of African women to negotiate condom use during marital sex [34]. Importantly, because ESN and control groups consisted of equal proportions of male and female subjects (ie, 50% in both cases), these immunological and behavioral sex-based differences have not affected our analyses in any way.

In summary, we found that ESN subjects in our cohort of Senegalese HIV-1 discordant couples are characterized by low levels of CD4+ T cell activation but that this is associated with an increase in safe sex behavior in this population. In addition, we observed marked differences in CD4+ and CD8+ T cell activation between men and women irrespective of sexual behavior. These findings question the relevance of low-level CD4+ T cell activation in resistance to HIV-1 infection and underscore the need to take gender and sexual behavior characteristics of high-risk populations into account when analyzing correlates of protective immunity. Prospective studies of ESN populations will be required to further our understanding of the role played by immune activation in susceptibility to HIV-1 infection.

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References