Low-Level Exposure to HIV Induces Virus-Specific T Cell Responses and Immune Activation in Exposed HIV-Seronegative Individuals

Clara Restrepo, Norma I. Rallón, Jorge del Romero, Carmen Rodríguez, Victoria Hernando, Mariola López,Alejandra Peris, Sara Lozano, José M. Sempere-Ortells, Vincent Soriano and José M. Benito

*J Immunol* published online 11 June 2010
http://www.jimmunol.org/content/early/2010/06/11/jimmunol.1000221
Low-Level Exposure to HIV Induces Virus-Specific T Cell Responses and Immune Activation in Exposed HIV-Seronegative Individuals

Clara Restrepo,* Norma I. Rallón,* Jorge del Romero,† Carmen Rodríguez,† Victoria Hernando,‡ Mariola López,* Alejandra Peris,* Sara Lozano,* José M. Sempere-Ortells,‡ Vincent Soriano,* and José M. Benito*

HIV-specific T cells response and T cell activation are frequently seen in exposed seronegative individuals (ESN). In this study, we report HIV-specific response and level of T cell activation in ESN partners of HIV-infected patients presenting low or undetectable levels of HIV-RNA. We evaluated 24 HIV-serodiscordant couples. ESN were classified into three categories of exposure to HIV (very low, low, and moderate-high), considering levels of HIV-RNA in their infected partner and frequency of sexual high-risk practices within the last 12 mo. HIV-specific T cell responses and activation levels in T cell subsets were evaluated by flow cytometry. We reported that 54% of ESN had detectable HIV-specific T cell responses, being the highest prevalence seen in the low exposure group (64%). Several T cell subsets were significantly increased in ESN when compared with controls: CD4+CD38+ (p = 0.02), CD4+HLA-DR+CD38+ (p = 0.02), CD4+CD45RA+CD27+HLA-DR+CD38+ (p = 0.002), CD8+CD45RA+CD27+CD38+HLA-DR+ (p = 0.02), and CD8+CD45RA+CD27+CD38+HLA-DR+ (p = 0.03). Activation of CD8+ T cells was increased in ESN with detectable HIV T cell responses compared with ESN lacking these responses (p = 0.04). Taken together, these results suggest that persistent but low sexual HIV exposure is able to induce virus-specific T cells response and immune activation in a high proportion of ESN, suggesting that virus exposure may occur even in conditions of maximal viral suppression in the HIV-infected partner. *The Journal of Immunology, 2010, 185: 000–000.

Some individuals remain HIV-seronegative despite repeated unprotected exposures to the virus (1). These exposed but seronegative (ESN) individuals include commercial sex workers (2), men having sex with men (3), injection drug users (4), hemophiliacs who received contaminated blood preparations (5), health care workers with accidental percutaneous exposure to infected blood (6), infants born to HIV-infected mothers (7), and individuals with HIV-infected heterosexual partners (8–11). The mechanisms of protection from infection in ESN individuals are largely undefined and most likely are multifactorial (1, 12). Host factors, including coreceptor susceptibility (13) and adaptive immunity (14), as well as viral factors, including replication level (15), have all been involved in this phenomenon.

The role of HIV-specific adaptive immunity in resistance to infection in ESN has attracted much attention. High expression levels of β-chemokines, as well as HIV-specific IFN-γ or IL-2–secreting T cells have been recognized in different cohorts of ESN individuals (16–21). However, others have not confirmed these findings acknowledging a lack of HIV-specific immune responses in ESN individuals (3). Detection of HIV-specific cytotoxic T lymphocytes in these individuals may simply be the consequence of exposure to the virus and not necessarily be associated with resistance to infection (20, 21). On the other hand, the recognition of significant mucosal and systemic cellular activation in ESN individuals clearly supports that viral exposure has occurred (22, 23).

Most studies conducted in ESN individuals have examined heterogeneous populations, given the difficulties to recruit such individuals (2–6), have focused on HIV-specific immune responses, and have used cross-sectional designs. Thus, the interpretation of results in terms of a protective role of immune responses on resistance to HIV infection is often difficult. In contrast, most studies examining ESN individuals with stable sexual HIV-infected partners have selected only couples with an HIV-partner presenting high-level viremia (8, 9). In this regard, and given the cross-sectional design of these studies, the demonstration of HIV-specific immune responses cannot be considered as a proof of resistance to infection as it can only reflect exposure to the virus. Capture of viral particles or proteins by certain specialized cells rather than infection is necessary to elicit cell-mediated immune responses (24). To our knowledge, there are no studies examining the prevalence of detectable HIV-specific T cells response and systemic immune activation in ESN individuals split out according to the level of viral exposure.

Materials and Methods

Study population

Participants were recruited at Centro Sandoval, a sexually transmitted disease (STD) clinic located in Madrid, Spain. The study population consisted of ESN persons in stable relationship with their HIV-infected partner on regular follow-up. Criteria for inclusion were sexual partnership with an HIV-infected individual during at least 12 mo prior to the inclusion. The HIV...
status was regularly checked by routine HIV Ab testing using a commercial ELISA. The level of exposure to HIV in ESN subjects was regularly evaluated at each visit. Participants were examined for STDs and completed a structured questionnaire that included sexual behavior information, as well data concerning risk practices. For each risk practice modality, the number of protected (with condom), unprotected episodes, and breaking or slipping of condoms during sexual intercourse was recorded. With this information, ESN individuals were then classified into three levels of exposure to HIV according to plasma viremia in the HIV-infected partner and the frequency of high-risk practices within the last 12 mo: very low (use of condom during all sexual intercourses, regardless of plasma viremia in the HIV-infected partner), low (frequent unprotected sexual intercourses with HIV-infected partner with plasma HIV-RNA <50 copies/ml), and moderate-high exposure (frequent unprotected sexual intercourses with HIV-infected partner with plasma HIV-RNA >50 copies/ml). Twenty HIV-seronegative persons with no risk behavior for acquiring HIV infection were identified and recruited as healthy controls (HCs). Written informed consent was given by all subjects before enrollment and the study protocol was approved by the hospital ethical committee.

Sample collection and preparation

PBMCs were isolated from blood collected in EDTA anticoagulant by density gradient centrifugation (Ficoll-Hypaque, Sigma-Aldrich, St. Louis, MO) and were frozen (90% FBS and 10% DMSO) in liquid nitrogen until use. Viability of thawed samples was always >85%.

HIV peptides

A panel of 769 overlapping peptides spanning the whole HIV genome was used in the stimulation assays. Peptides were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (www.aidsreagent.org). They were 15 aa long overlapping by 11 aa and were distributed in 11 different pools (one for Nef, two for Gag, three for Pol, three for Env, two for Reg). Each of them contained between 61 and 83 individual peptides, depending on the total number of peptides spanning each protein. Peptides were dissolved in DMSO and the concentration of each individual peptide in the pools was 100 µg/ml.

Intracellular staining of β-chemokines and cytokines

Production of macrophage inflammatory protein 1β (MIP-1β), and IFN-γ by CD4+ and CD8+ T cells was simultaneously examined in response to HIV overlapping peptide pools using multiparameter flow cytometry. In brief, 1 × 10^6 PBMCs were cultured in complete medium (RPMI 1640, 10% FCS, l-glutamine and antibiotics; R10) and stimulated with the different pools of overlapping peptides (final concentration of each peptide in the culture, 2 µg/ml) during 6 h at 37°C with 5% CO₂.

Control conditions included stimulation with medium alone as negative control, and with PMA (50 ng/ml) plus ionomycin (1 µM) as positive control. Brefeldin A was added 5 h before the end of culture. Thereafter, cells were harvested, washed with PBS and incubated with anti-CD3-PE, anti-CD27-PE (Becton-Dickinson, San Jose, CA) and anti-CD4 and anti-CD8-PECy7 (Becton-Dickinson). Cells were then incubated with anti-MIP-1β-FITC (R&D Systems, Minneapolis, MN), and anti–IFN-γ-PE (BD Biosciences). Gating was performed on CD3+CD8− or CD3−CD4+ cells and a minimum of 50,000 of the cells was obtained for further analysis using an FCS® flow cytometer (Beckman Coulter, Fullerton, CA) for 30 min at 4°C. The cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) and were then incubated with anti-MIP-1β-FTC (R&D Systems, Minneapolis, MN), and anti–IFN-γ-PE (BD Biosciences). Gating was performed on CD3+CD8− or CD3−CD4+ cells and a minimum of 50,000 of the cells was obtained for further analysis using an FCS® flow cytometer (Beckman Coulter, Fullerton, CA). On the basis of IFN-γ and MIP-1β expression, three unique functional subsets of CD4+ and CD8+ T cells were defined: IFN-γ−MIP-1β−, IFN-γ+MIP-1β−, and IFN-γ+ MIP-1β+ cells.

For each of these unique functional subsets, all values obtained in 20 HCs after stimulation with the 11 different pools of HIV peptides were pooled together (after background subtraction) to obtain a total of 220 values (11 different stimulations in 20 different individuals), and the 99th percentile of the data distribution was chosen as the cutoff for a positive response. In this way, six different cutoffs were generated (three for CD4+ T cells and three for CD8+ T cells).

Maturation stage and degree of T cell activation

Levels of different subsets of CD4+ and CD8+ T cells, and immune activation (using CD38 and HLA-DR as surrogate markers) were evaluated on PBMCs. CD45RA and CD27 were used to define the maturation stage of CD4+ and CD8+ T cells. Accordingly, four different subsets were defined: naive (CD45RA+CD27−), central memory (CM) (CD45RA−CD27+), effector memory (EM) (CD45RA−CD27−), and effector (CD45RA+CD27−) cells. CD38 and HLA-DR were simultaneously analyzed on each of these subsets, using five-color flow cytometry. For this purpose, the next combinations of mAbs were used: anti-CD4-PECy7, anti-CD8-PECy7, anti-HLA-DR-PCy5, anti-CD38-FITC, anti-CD45RA-PEC (Beckman-Coulter), and anti-CD27-PE (Becton-Dickinson, San Jose, CA). The 250,000 PBMCs were incubated for 30 min at 4°C with two combinations of fluorochrome-labeled Abs (CD4/CD27/CD45RA/HLA-DR/CD38 and CD8/CD27/CD45RA/HLA-DR/CD38). A minimum of 5000 CD4 or CD8+ cells per sample were examined.

Statistical analysis

Median and interquartile range (IQR) were used to describe each of the continuous variables analyzed. Data analysis and comparisons for the different parameters were made using the Kruskal-Wallis, Mann-Whitney, or Wilcoxon nonparametric tests as appropriate. For all analyses, the level of significance was set at p < 0.05. Statistical analysis was performed using the SPSS statistical package v 15.0 software (SPSS, Chicago, IL).

Results

Study population

Table I summarizes the main characteristics of the 24 HIV-serodiscordant couples enrolled in the study. At enrollment, median (IQR) CD4 count in HIV-infected partners was 492 (211–705) cells/µl, and the median length of HIV infection was 108 (42–207) months. Overall, 63% (15/24) of HIV-infected partners had undetectable plasma viremia, ranging in the remaining nine patients from 68 to 121,792 HIV-RNA copies/ml. At the time of enrollment in the study, all ESN individuals were HIV-negative using a commercial ELISA. Ten (40%) ESN individuals were male and the median number of sexual intercourses was eight (4–12) per month. Twelve (50%) ESN individuals reported never using condoms, seven (29%) reported sporadic use, four (16%) frequent use, and only one ESN individual reported always using a condom. With this information and the viral load of the HIV-infected partner, ESN individuals were classified into three levels of exposure to HIV: 1 (4%) with very low, 14 (58%) with low, and 9 (38%) with moderate-high level of HIV exposure.

HIV-specific T cells response

HIV-specific T cells response in ESN and HIV-infected subjects was considered to be positive when values for IFN-γ and/or MIP-1β-producing T cells were above different cutoffs for each functional subset, based on values obtained in 20 HCs. The six cutoff values obtained were as follows: 0.03% for CD4+IFN-γ−MIP-1β−, 0.02% for CD4+IFN-γ−MIP-1β−, 0.08% for CD4+IFN-γ−MIP-1β−, 0.04% for CD8+IFN-γ−MIP-1β−, 0.03% for CD8+IFN-γ−MIP-1β−, and 0.14% for CD8+IFN-γ−MIP-1β−. Representative dot plots of IFN-γ and/or MIP-1β–production are depicted in Fig. 1.

Detectable HIV-specific CD4+ and/or CD8+ T cells response were observed in 13 of 24 (54%) ESN individuals and in 18 of 24 (75%) HIV-infected partners. Of 13 ESN individuals presenting detectable T cells response, 23% (3/13) had only CD4+ response, 23% (3/13) only CD8+ response, and 54% (7/13) presented both responses.

When ESN individuals were stratified according to the level of HIV exposure, the highest prevalence of detectable T cells response was seen in the low-level exposure group (64%; 8/14) compared with the moderate-high group (33%; 3/9) although the difference was not statistically significant. Moreover, the proportion of ESN presenting both CD4+ and CD8+ T cells response was higher in those with low exposure (63%; 5/8) compared with those with moderate-high exposure (33%; 1/3). Surprisingly, the single ESN individual belonging to the very-low exposure group also displayed detectable T cells response (Fig. 2).

The intensity of global T cell response was generally low (<1% of T cells) in the majority (62%) of ESN individuals, although this was not the case for five individuals presenting responses that ranged from 1% to as much as 4%. Overall, CD8+ response was
### Table I. Clinical and epidemiological characteristics of HIV-serodiscordant study couples

<table>
<thead>
<tr>
<th>HIV-ESN Individuals</th>
<th>HIV-Infected Partners</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Couple No.</strong></td>
<td><strong>Gender</strong></td>
</tr>
<tr>
<td>1</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
</tr>
</tbody>
</table>

**Footnotes:**

- During the 12 mo prior to the inclusion.
- Anal and/or genital intercourse.
- Very low: used condoms during all sexual intercourse, regardless viremia in the HIV-infected partner; low, frequent unprotected sexual intercourse with HIV-infected partner with plasma HIV-RNA <50 copies/ml; Moderate-high, frequent unprotected sexual intercourse with HIV-infected partner with plasma HIV-RNA ≥50 copies/ml.
- Median (IQR) of the values during the 12 mo prior to inclusion, except those cases where only a single determination was available.
- F, frequently; N, never; S, sporadic; A, always; NA, not available.
greater than CD4+ response in most ESN individuals. A similar profile was seen in HIV-infected partners (Fig. 2).

Profile of CD4+ and CD8+ T cells response according to HIV proteins

The proportion of individuals who showed T cells response varied according to targeted protein. In ESN individuals, Pol and Env were the proteins most frequently targeted by CD4+ T cells (22% and 26% of individuals, respectively), and Nef the most frequently recognized by CD8+ T cells (26% of individuals). In HIV-infected subjects, the most frequently recognized HIV proteins by CD4+ T cells were Gag and Nef (28% and 24% of subjects, respectively) and Gag and Pol by CD8+ T cells (52% and 48% of subjects, respectively) (data not shown).

Intensity of T cells response to each individual HIV protein was low in both ESN individuals and HIV-infected partners, being CD8+ response higher than CD4+ response, especially in ESN individuals (data not shown). Lastly, there were no significant differences in the breadth of CD4+ and CD8+ T cells response (defined as the number of peptide pools with detectable response per individual) when comparing ESN and HIV-infected partners. Median breadth for CD4+ and CD8+ T cells response in ESN individuals were 3 (1–4) and 1 (1–7) respectively, whereas in HIV-infected partners these figures were 2 (1–3) and 3 (1–7) respectively (data not shown).

Functional profile of CD4+ and CD8+ T cells response

To assess the functional profile of HIV-specific T cells response, the contribution of each unique functional subset (IFN-γ+MIP-1β−, IFN-γ+MIP-1β+, and IFN-γ−MIP-1β−cells) to the global HIV-specific CD4+ and CD8+ T cells response was calculated. Moreover, the contribution of monofunctional subsets (single-cytokine–producing subsets: IFN-γ−MIP-1β− or IFN-γ−MIP-1β+, and bifunctional subsets (double-cytokine–producing subset: IFN-γ−MIP-1β+), as well as the contribution of all subsets producing one particular cytokine (IFN-γ+ or MIP-1β+) was further analyzed.

CD4+ T cells response were mainly mediated by IFN-γ+MIP-1β− cells in both ESN and HIV-infected partners, with much less contribution of IFN-γ−MIP-1β+ and IFN-γ−MIP-1β− cells. As a consequence, CD4+ T cells response were predominantly monofunctional and dominated by IFN-γ–producing cells in both groups, without significant differences between them (Fig. 3).

The functional profile of HIV-specific CD8+ T cells was more heterogeneous. All three different T cell subsets contributed to CD8+ T cells response, although the contribution of IFN-γ−MIP-1β− cells was lower in ESN than in HIV-infected partners (median contribution: 0% [0–24] and 31% [6–69], respectively, \( p = 0.08 \)). As for CD4+ response, CD8+ response was dominated by monofunctional cells in both groups. However, in contrast to CD4+ response, both IFN-γ–producing and MIP-1β–producing cells contributed in a significant extent to CD8+ response, although HIV-infected partners displayed a slightly higher contribution of MIP-1β than IFN-γ–producing cells; whereas, in ESN, the contribution of MIP-1β and IFN-γ–producing cells was more balanced (Fig. 3).

T cell immunophenotype and activation levels

Expression of HLA-DR and/or CD38 activation markers in different T cell subsets was examined in 18 serodiscordant couples and
As expected, different immune alterations were observed in HIV-infected patients compared with HCs, including lower levels of naive CD8$^+$ T cells ($p = 0.03$), as well as increased activation levels in CM ($p = 0.03$) and EM ($p = 0.017$) CD8$^+$ T cell subsets (data not shown).

There were also significant differences in the proportion of activated CD4$^+$ and CD8$^+$ T lymphocytes when comparing ESN individuals and HCs. ESN individuals had higher percentages of CD4$^+$CD38$^+$ (p = 0.006), CD4$^+$HLA-DR$^+$CD38$^+$ (p = 0.02), and CD4$^+$CD45RA$^+$CD27$^+$HLA-DR$^+$CD38$^+$ (p = 0.002) T cells compared with HCs. In contrast, there were no significant differences between ESN and HIV-infected partners regarding CD38 expression on CD4$^+$ T cells (Fig. 4).

With respect to CD8$^+$ T cell subsets, ESN showed a significant increase of CD8$^+$CD45RA$^+$CD27$^+$HLA-DR$^+$CD38$^+$ (p = 0.02) and CD8$^+$CD45RA$^+$CD27$^+$HLA-DR$^+$CD38$^+$ (p = 0.03) T cells compared with HCs; whereas, no differences were recognized in these T cell subsets comparing ESN and HIV-infected partners (Fig. 4).

**FIGURE 2.** Global HIV-specific CD4$^+$ (A) and CD8$^+$ (B) T cell responses in ESN subjects and their HIV-infected partners (HIV$^+$). Couples are grouped according to level of exposure to HIV in ESN individuals.

The presence of HIV-specific T cells response and systemic immune activation were investigated in a group of HIV-seronegative individuals having stable heterosexual relationship with HIV-infected patients. Nearly two thirds of infected partners were on antiretroviral therapy and had undetectable plasma viremia. This cohort of ESN differed from most others, whose HIV-infected partners had high-level viremia (8, 9, 17, 25). To our knowledge, this is the first study conducted in HIV-serodiscordant couples examining the prevalence of detectable HIV-specific T cells response and systemic immune activation levels in ESN individuals according to HIV-specific T cells response

To examine the potential association between HIV-specific T cells response and T cell subsets and their activation level, ESN individuals were split out according to the presence or absence of detectable HIV-specific T cells response. Of the 18 ESN individuals for whom information on T cell subsets and activation levels were available, 10 individuals had detectable T cells response. Regarding activation markers, there were significantly higher levels of CD8$^+$CD45RA$^+$CD27$^+$HLA-DR$^+$CD38$^+$ (p = 0.03), CM HLA-DR$^+$CD38$^+$ (p = 0.03), EM HLA-DR$^+$CD38$^+$ (p = 0.08), and naive HLA-DR$^+$CD38$^+$ (p = 0.05) CD8$^+$ T cells (Fig. 5). Most of these abnormalities also differed significantly when comparing ESN individuals with detectable HIV-specific T cells response and HIV-unexposed controls. Furthermore, the percentage of CD8$^+$ T cells expressing HLA-DR and/or CD38 did not differ between ESN individuals without detectable HIV-specific T cells response and HIV-unexposed controls.

Regarding CD4$^+$ T cells, overall there were no significant differences between ESN subjects with and without HIV-specific T cells response for any of the T cell subset analyzed. However, higher percentages of CD38 expression on total CD4$^+$ T cells (p = 0.03) as well as a significant increase in CD45RA$^+$CD27$^+$HLA-DR$^+$CD38$^+$ (p = 0.01), CD4$^+$ T cells were seen comparing ESN subjects with detectable HIV-specific T cell responses and HIV-unexposed.

**Discussion**

The prevalence of HIV-specific T cells response and systemic immune activation were investigated in a group of HIV-seronegative individuals having stable heterosexual relationship with HIV-infected patients. Nearly two thirds of infected partners were on antiretroviral therapy and had undetectable plasma viremia. This cohort of ESN differed from most others, whose HIV-infected partners had high-level viremia (8, 9, 17, 25). To our knowledge, this is the first study conducted in HIV-serodiscordant couples examining the prevalence of detectable HIV-specific T cells response and systemic immune activation levels in ESN individuals according to HIV-specific T cells response.
immune activation in HIV-seronegative individuals with repeated exposures to very-low HIV inocula. Interestingly, despite low HIV exposure, a relatively high prevalence of HIV-specific T cells response was seen in this cohort of ESN individuals. Similarly, high prevalence has been found in previous studies using different cohorts of ESN individuals (4, 8, 26), although this has not always been the case (9). More intriguing was the fact that ESN individuals with infected partners having undetectable plasma viremia exhibited more frequently HIV-specific immune responses than ESN individuals with viremic partners. Altogether, these results suggest that low but persistent exposure to HIV appears to be sufficient to elicit virus-specific immune responses.

In addition, some methodological issues may have contributed to explain the relatively high prevalence of HIV-specific immune responses seen in this study. Firstly, a panel of overlapping peptides spanning the whole HIV genome was used in the stimulation assays, in contrast to other studies which used only a few viral proteins (8, 9, 21) or peptide panels restricted to few HLA I alleles (3, 27). Secondly, we measured two different cytokines to assess immune responses, thus increasing the probability of finding a positive response. IFN-γ and MIP-1β were chosen based on prior studies showing that they are the most accurate for assessing HIV-specific T cells response in infected patients (28).

The methodological approach used in this study enabled us to analyze not only the level but also the breadth and the functional profile of HIV-specific T cells response. Although the breadth of response was similar in ESN individuals and their respective HIV-infected partners, the most frequently targeted viral proteins differed between them. ESN individuals had CD4+ T cells response mainly against Pol and Env proteins; whereas, Nef was the most frequently recognized by CD8+ T cells. In contrast, most CD4+ T cells response in HIV-infected patients was directed against Gag and Nef, being Gag and Pol the most frequently recognized proteins by CD8+ T cells. These findings are in agreement with prior observations of different HIV epitope recognition in ESN and HIV-infected individuals (21, 26). However, because we did not perform HLA

---

**FIGURE 3.** Pie chart graphs showing the functional profile of HIV-specific CD4+ (A) and HIV-specific CD8+ (B) T cells response in HIV-ESN individuals (ESN) and HIV-infected partners (HIV+). Data show the percentage of contribution of different functional T cell subsets to the global response. (1) The contribution of each unique functional subset, (2) the contribution of monofunctional and bifunctional subsets, and (3) the contribution of subsets producing one particular cytokine are shown.

**FIGURE 4.** Activation levels in T cell subsets. CD4+ and CD8+ cells in HIV-unexposed controls (HCs), HIV-ESN (ESN), and HIV-infected (HIV+) subjects. Statistical differences were tested using the nonparametric Mann-Whitney U test, and the level of significance was set at p < 0.05.
tying, the different profile of targeted viral proteins could also be due to differences in HLA haplotypes comparing ESN and HIV-infected partners.

The cross-sectional design of the study did not allow us to ascertain if the existence of HIV-specific T cells response had a protective role against HIV infection. This question can only be addressed in longitudinal studies. However, in view of the findings in HIV-infected long-term nonprogressors in whom a polyfunctional response is associated with the spontaneous control of viral replication (28), the finding of a T cell response mainly mediated by monofunctional cells in our cohort of ESN individuals argues against a potential protective role for this response in HIV transmission.

The results of this study have important clinical implications, because they constitute a proof-of-concept of the existence of a real exposure to the virus even in HIV-seronegative individuals admitting sexual intercourses with HIV-infected patients having complete viral suppression under antiretroviral therapy. In favor of this hypothesis was also the recognition of increased T cell activation in ESN subjects, resembling what happens in HIV-infected patients.

Prior studies in HIV-serodiscordant couples have suggested that altered T cell subsets distribution and activation in ESN subjects may result from HIV exposure and, accordingly, that activation of CD4+ T cells by itself is not sufficient to favor HIV infection (9, 22). Alternatively, increased CD8+ T cell activation in ESN subjects might reflect an intrinsic characteristic of these individuals, rather than an immunologic response to the virus (29). In contrast, other studies have reported low level of immune activation in ESN subjects, which might contribute to low HIV susceptibility or resistance to infection (30, 31). Thus, high levels of activated CD4+ T cells at the site of HIV exposure would increase HIV transmission efficiency; whereas, low levels of activated CD4+ T cells might be associated with a persistent HIV-seronegative status despite high-risk sexual behavior (30).

ESN individuals with detectable HIV-specific T cells response showed a reduced number of naive and increased number of effector CD8+ T cells, along with high-level activation of several CD8+ T cell subsets, in comparison with ESN lacking HIV-specific T cells response. This association between the existence of virus-specific responses and immune activation are clearly in favor of a real virus exposure, and less in support of resistance to infection. Assuming that the immune abnormalities seen in ESN persons are a direct consequence of virus exposure, it would be interesting to know for how long they may persist after cessation of high-risk practices. The only single ESN individual in our cohort that reported using condoms in all sexual intercourses had detectable HIV-specific T cells response. Interestingly, his partner had been pregnant 2 y earlier after natural conception. Thus, one might consider that in this ESN subject, T cells response were most likely elicited time ago and at least have persisted for 2 y in the absence of high-risk sexual practices. This finding is consistent with two previous studies showing long-lasting HIV-specific T cells response in ESN individuals after interruption of high-risk behavior (8, 32). Altogether, these results suggest that continuous exposure to HIV is not required for maintaining an HIV-specific immunity in ESN individuals. Clearly, further studies are warranted to confirm this assumption. A longitudinal follow-up of ESN individuals in our cohort is being carried out to address this issue.

An important epidemiological implication of our results is that virus exposure may occur even in ESN individuals having HIV-infected partners with undetectable plasma viremia. The probability of sexual transmission is known to be dependent of plasma viral load (15), and based on this observation it is presumed that HIV-infected patients under antiretroviral therapy having undetectable viremia are rarely infectious and cannot transmit HIV through sexual contact (33–35). The lack of transmissibility of HIV from aviremic patients, however, does not mean that a residual number of viral particles, including defective virions, or viral proteins could not be present in semen or cervicovaginal fluids (36–39), being able to elicit HIV-specific immune responses.

In summary, the results of this study suggest that low but persistent HIV exposure is able to induce virus-specific T cells response and other immune abnormalities in a high proportion of persistently HIV-seronegative individuals. These observations support that real virus exposure occurs, even in conditions of maximal viral suppression under antiretroviral therapy in the infected partner. Given that our ESN cohort remains seronegative despite the existence of real exposure to the virus, our data suggest that viral
The authors have no financial conflicts of interest.

Acknowledgments

We thank the group of ESN individuals and their HIV-infected partners, whose cooperation has made this study possible.

Disclosures

The authors have no financial conflicts of interest.

References


