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*J Immunol* 2003; 171:5663-5667;
doi: 10.4049/jimmunol.171.11.5663
http://www.jimmunol.org/content/171/11/5663

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Cutting Edge: Increased NK Cell Activity in HIV-1-Exposed but Uninfected Vietnamese Intravascular Drug Users

Daniel Scott-Algara,2,* Lien X. Truong,† Pierre Versmisse, *, Annie David, *, Tram T. Luong,† Ngai V. Nguyen,‡ Ioannis Theodorou,§ Françoise Barre-Sinoussi, * and Gianfranco Pancino*

We addressed the role of innate immunity in the protection against HIV-1 infection by studying NK cell function in 37 Vietnamese intravascular drug users (IDUs), who appeared to remain HIV-1 uninfected despite many years of high-risk exposure (exposed uninfected, EU), 10 IDUs who underwent seroconversion and 28 unexposed blood donors. Main results were: NK cell lytic activities against both the NK-susceptible K562 cell line and the NK-resistant Daudi cell line were significantly augmented in EU IDUs compared with either controls or seroconverters before or after seroconversion; NK cells producing the cytokines IFN-γ and TNF-α and the β chemokines CCL3, CCLA, and CCL5 were also increased in the EU IDUs, either after in vitro activation or without stimulation. The finding of an enhanced NK cell function in EU IDUs, especially compared with IDUs who became HIV-1 infected, supports the hypothesis that NK cells contribute to the protection against HIV-1 infection. The Journal of Immunology, 2003, 171: 5663–5667.

Despite being repeatedly exposed to HIV-1 via sexual or systemic routes, some individuals remain uninfected. It is unclear whether this apparent resistance to HIV-1 infection is due to innate protection, adaptive immune response(s), or both. Acquired immune responses, such as HIV-specific CTL and induction of IgA Abs, have been detected in sexually exposed but apparently uninfected individuals such as commercial sex workers and the partners of HIV-1+ subjects (1, 2). However, not all exposed uninfected (EU)3 individuals mount these adaptive immune responses, and their role in protection against HIV-1 is not well understood. Innate immunity may also be involved in the resistance to HIV-1 infection and may be essential during the first encounters with HIV-1, as well as helping to expand subsequent adaptive immune responses. Some studies described an increased secretion of β chemokines or CD8+ cell-derived anti-HIV factor in EU individuals (3, 4).

However, the role played by innate immunity in the resistance to HIV-1 infection remains largely unknown. NK cells are a component of the innate immune system thought to play a key role in the control of pathogens through various mechanisms, including MHC-unrestricted cytotoxic activity, the secretion of antimicrobial or immunoregulatory cytokines, and Ab-dependent cell cytotoxicity (ADCC) (5, 6). Although the importance of NK cells in some viral infections is well documented, their role in the protection and control of HIV-1 infection is currently unclear (Ref. 6 and references therein). The contribution of NK cells to anti-HIV defenses can be inferred from studies showing alterations of NK cell function and number during HIV infection and progression to AIDS (7–9). Moreover, in vitro-activated NK cells secrete β chemokines and other cytokines that inhibit HIV infection (10) and this activity is affected by the level of viremia in HIV-infected patients (11). To evaluate the role of NK cells in the resistance against HIV-1, we studied NK cell function in a group of highly exposed seronegative intravascular drug users (IDUs) in Ho Chi Minh City, Vietnam (12). We chose this study group since factors, such as shared needle injections and multiple viral infections, found in Vietnamese IDUs (12) favor immune activation which may result in enhanced innate defenses, including NK cell responses, which in turn may interfere with the transmission of HIV. We compared NK cell cytotoxic and secretory activities in EU IDUs, unexposed blood donors, and IDUs who eventually underwent seroconversion.

Subjects and Methods

Subjects

Thirty-seven individuals who had been in high risk of exposure to HIV-1 were recruited from a population of seronegative subjects identified between 1996 and 1998 among IDUs treated in the Binh Trieu Detoxification Centre in Ho Chi Minh City (12). The prevalence of risk factors and of other infections was similar in these seronegative IDUs and in the HIV-infected IDUs attending this center (12). Subjects were followed up for between 3 and 5 years at the Binh Trieu Hospital. During follow-up visits, patients underwent a clinical examination, gave a blood sample, and were tested negative for HIV by ELISA test (Genescreen; Bio-Rad, Hercules, CA) and PCR on PBMC DNA (13). Analysis of the CCR5 polymorphism did not reveal the presence of the variants known

1 Abbreviations used in this paper: EU, exposed uninfected; ADCC, AB-dependent cell cytotoxicity; IDU, intravascular drug user; LU30, lytic units 30; HTLV, human T cell leukemia virus; KIR, killer cell Ig-like receptor.

2 This work was supported by grants from the Agence National de Recherche sur le Sida, France, and the Institut Pasteur (Programmes Transversaux de Recherche).

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0022-1767/03/$02.00

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to confer resistance to HIV1 (Δ32, m303, and 893(−)) in the EU IDUs (Ref. 14 and data not shown). Risk reduction counseling, as well as psychological and medical care, was provided to all participants. Although most of them eventually stopped either IDU or sharing needles over time, and despite counseling, HIV-negative IDUs continued to report high-risk behaviors, such as needle sharing and/or frequenting public drug injectors (shooting galleries), during the first years (1–3 years) after recruitment. Ten IDUs who underwent seroconversion and from whom PBMC samples had been collected 6–18 mo before the detection of HIV-1 seroconversion were also studied. Finally, 28 healthy subjects were recruited from the Blood Donor Bank in Ho Chi Minh City as a control group. The characteristics of the study groups are presented in Table I. No differences were observed between EU IDUs and seroconverters, except for the human T cell leukemia virus (HTLV) seroprevalence (p = 0.047). Informed consent was obtained from each participant at the time of recruitment. This study was approved by the Vietnamese Ethics Committee of the Binh Trieu Hospital in Ho Chi Minh City. Free diagnosis, medical follow-up, and treatment for opportunistic infections were offered to HIV-infected IDUs.

**Effectors**

Blood samples were collected from each subject. PBMC were recovered by centrifugation on Ficoll-Hypaque gradients and cryopreserved in 90% FCS/10% DMSO. PBMC were thawed and cultured overnight in complete culture medium (RPMI 1640, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine) before being used in the NK assays.

**Target cells**

Target cells for cytoxic assays were K562, an erythroleukemia cell line, and Daudi, a B cell line derived from a Burkitt lymphoma. Uninfected or HIV-1-infected allogenic PHA-activated CD4+ cells, isolated from the PBMC of a Vietnamese donor, were also used as targets in NK cytototoxicity and ADCC assays. CD4+ cells were infected with the Vietnamese primary isolate HIV-1 132W. NK assays were performed on day 13 postinfection, when 10–20% of the cells were HIV-1 positive by FACS analysis.

**NK cell cytotoxicity assay**

Cytotoxicity assays were conducted using a standard 51Cr release assay as described previously (15). All experiments were done in triplicate. The percentage of cytotoxicity was calculated as follows: (cpm experimental – cpm spontaneous)/(cpm maximum – cpm spontaneous) × 100. The spontaneous release of 51Cr was taken as the radioactivity in the supernatant of the target cells alone, whereas the maximum release was taken as the radioactivity after target cell lysis with HCl. We also calculated the number of effectors cells per million PBMC or NK cells required to achieve 30% net lysis (lytic units 30 or LU30), where net lysis is equal to the experimental cpm – spontaneous cpm.

**ADCC**

ADCC assays were performed using HIV-1 infected or uninfected allogenic CD4+ cells as target cells and PBMC as effector cells in the presence of autologous plasma at two different dilutions (1/10 and 1/100). Lysed of target cells was measured by a standard 51Cr release method.

**Flow cytometry analysis of cytokine production by NK cells**

PBMC from EU IDUs, seroconverters, or control individuals were cultured overnight alone or with K562 cells (ratio 50:1) in 2 μg/ml brefeldin A (Sigma-Aldrich, Paris, France) at 37°C in 9% CO2. PBMC were stained for membrane markers (CD3, CD8, CD56, and CD16, Beckman Coulter, Paris, France) and then permeabilized and stained for IFN-γ, TNF-α, CCL3, CCL4, and CCL5 with appropriate Abs (BD PharMingen, Le Pont de Claix, France). NK cells were defined as the CD3+CD56+CD16+ population. PBMC stimulated with PMA/ionomycin (10 and 500 ng/ml, respectively; Sigma-Aldrich) were used as positive controls for cytokine production, whereas negative controls were incubated with irrelevant isotype-matched Abs.

**Statistical analysis**

Statistical analyses were conducted using the Student’s t test, Fisher’s exact test, and the nonparametric Mann-Whitney U test. The level of significance of each test was adjusted for multiple testing using the Bonferroni correction. Since we had three comparisons for each test, the significance threshold was p < 0.017.

**Results**

**NK cytotoxic activity is higher in EU IDUs than in unexposed controls or seroconverters**

The percentage of NK cells among PBMC did not differ between EU IDUs and the unexposed controls (17.5 ± 8.4% and 23.5 ± 5.6%, respectively, p = 0.2). The percentage of NK cells were also similar in EU IDUs (17.5 ± 8.4%) and in seroconverters both before (18.2 ± 10.8%) and after (14.8 ± 9.4%) seroconversion.

NK cell lytic activity against the K562 cell line was significantly higher in EU IDUs than in unexposed controls (p < 0.001 and <0.01 with E:T ratios of 50:1 and 25:1, respectively; Fig. 1). The expression of NK cytolytic activity in lytic units per PBMC or per NK cell confirmed the difference between the EU IDU and control groups: 11.21 vs 1.45 LU30 (p < 0.001) per PBMC and 69.77 vs 6.93 LU30 (p < 0.001) per NK cell, respectively. NK cell lytic activity was also higher in EU IDUs than in unexposed controls when the NK cell-resistant Daudi cell line was used as a target (p < 0.01 with an E:T ratio of 50:1; Fig. 1).

No significant NK cell activity against allogenic HIV-infected CD4+ cells was detected in PBMC from the EU IDUs or from the controls (results not shown).

If NK cells play a role in natural resistance against HIV in EU IDUs, then individuals who undergo seroconversion might not display similar elevated NK cell activities before infection. To test this hypothesis, we studied 10 IDUs who underwent seroconversion during the study. NK cell activity was analyzed in PBMC collected 6–18 mo before the detection of seroconversion. In six cases, PBMC collected after the detection of the seroconversion were also available. Before seroconversion, the NK cytotoxic activity against the K562 cell line in the seroconverters was comparable to that found in unexposed controls and lower than in EU IDUs (p < 0.001 with an E:T ratio of 50:1; Fig. 1). After seroconversion, levels of NK cytolytic activity

**Table I. Characteristics of the study population**

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age (years)a</th>
<th>Drug Use (years)b</th>
<th>Injections/Day (range)</th>
<th>Needle Sharing/Shooting Galleries (%)</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU IDU (37)</td>
<td>M, 34/F, 3</td>
<td>46 ± 5.4</td>
<td>24.6 (13–31)</td>
<td>3.3 (1–4)</td>
<td>100</td>
<td>89 (8) 100 (8) 720.7 ± 282.8 704.9 ± 240.2</td>
</tr>
<tr>
<td>Seroconvertersa (10)</td>
<td>M, 8/F, 2</td>
<td>47 ± 4.4</td>
<td>25.3 (12–35)</td>
<td>2.9 (1–4)</td>
<td>100</td>
<td>80 (7) 100 (5) 734.5 ± 284.6 937.9 ± 540.2</td>
</tr>
<tr>
<td>Controls (28)</td>
<td>M, 26/F, 2</td>
<td>40 ± 7.7</td>
<td>—</td>
<td>—</td>
<td>0 (0) 0 (0) ND ND ND</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± SD

b Data on the duration of IDUs were available for 35 EU IDUs and 7 seroconverters. Means and ranges were calculated on these data. For the remaining 2 EU IDUs and 3 seroconverters, IDU duration of more than 15 years was estimated based on patient history.

a HBV, Hepatitis B virus; HCV, hepatitis C virus; M, male; F, female; ND, not done.

b EU IDUs vs seroconverters: p = 0.047.

c Before seroconversion.
were even lower still (Fig. 1). Both before and after seroconversion, the seroconverters showed little or no cytololysis activity against the Daudi cell line, similarly to the unexposed controls (Fig. 1).

To analyze ADCC in the study population, we tested the ability of PBMC from EU IDUs ($n = 27$) and unexposed controls ($n = 15$) to lyse allogenic CD$^+$ cells infected or not with HIV-1 in the presence of autologous plasma. No ADCC was observed when PBMC from controls were used as effector cells (1.35 ± 2.96% of uninfected CD$^+$ cells and 2.89 ± 3.46% of infected CD$^+$ cells were lysed in the presence of a 1/10 dilution of plasma). However, ADCC against allogenic CD$^+$ cells was observed in 3 of the 27 EU IDUs tested. Similar percentages of infected and uninfected allogenic CD$^+$ cells were lysed by the PBMC from two of these three EU IDUs (13.5 and 13.6% of uninfected and 11.2 and 14.7% of infected CD$^+$ cells were lysed in the presence of a 1/10 dilution of plasma, respectively). In the third case, HIV-1-infected but not uninfected CD$^+$ cells were lysed (2.7% of uninfected and 7.9% of infected CD$^+$ cells were lysed at 1/10 dilution of plasma, respectively).

Secretion of cytokines and chemokines by NK cells is higher in EU IDUs

We evaluated the production of IFN-γ, TNF-α, and the β chemokines CCL3, CCL4, and CCL5 by intracellular staining of NK cells. Fig. 2 shows the results for each cytokine and chemokine studied, expressed as the percentage of cytokine-stained cells among total NK cells. In the absence of K562 cell stimulation, the proportions of stained NK cells from both unexposed controls and seroconverters before and after seroconversion were low or at background levels for all of the cytokines and chemokines (Fig. 2, left panels). In contrast, higher proportions of NK cells from EU IDUs stained positive for every cytokine and chemokine (Fig. 2, left panels). After K562 cell stimulation, more cytokine/chemokine-positive NK cells were also found in EU IDUs than in unexposed controls. However, after the correction for multiple comparisons was applied, statistical significance ($p < 0.017$) was reached only for CCL3 and CCL5 because of the dispersion of values (Fig. 2B, right panels). In comparison with seroconverters (both before and after seroconversion), EU IDUs had significantly more stained NK cells for all cytokines and β chemokines after K562 cell stimulation (Fig. 2, right panels).

Blood samples from EU IDUs used in this study were obtained when they still practiced high-risk behaviors, except for eight individuals who reported behavioral changes that reduced the risk at the time of blood sampling.
activities did not differ between these eight individuals and the other EU IDUs tested (data not shown).

Because the HTLV Ab was more frequently detected in EU IDUs than in seroconverters (Table I), we stratified NK cytotoxic and secretory activities according to HTLV status. No association was found with any variable (data not shown).

**Discussion**

We show that NK cell cytolytic activity is higher in Vietnamese EU IDUs than in healthy unexposed individuals or in IDUs who eventually underwent seroconversion. The percentage of NK cells producing IFN-γ, TNF-α, and β chemokines after in vitro activation was also higher in the EU IDUs than in the unexposed controls or the seroconverters. Notably, both NK cytolytic activity against NK-resistant Daudi cells and NK secretory activity in the absence of previous in vitro activation were also higher in EU IDUs than in the controls and seroconverters. These last results suggest that NK cell activity in vivo may also be increased in EU IDUs. The increased production of cytokines and chemokines by activated NK cells in EU IDUs may play a role in anti-HIV-1 defense. Both IFN-γ and TNF-α modulate HIV-1 infection in vitro and can suppress viral replication in certain conditions (16). β Chemokines inhibit R5 HIV-1 virus infection by competition and/or by down-regulating the surface expression of the CCR5 coreceptor (17). Supernatants from NK cells stimulated by cytokines or following CD16 cross-linking efficiently suppress HIV-1 replication in PBMC and T cells, partly due to β chemokine secretion (10, 11).

Although we did not observe any significant lytic activity directed against HIV-1-infected CD4⁺ cells, according to previous reports (18), we cannot exclude the possibility that NK cell cytotoxicity plays a role in vivo. Indeed, high lytic activity against the NK-resistant Daudi cell line was detected in EU IDUs, but not in the control or seroconverter groups (Fig. 1). Daudi cell lysis might be mediated by the activation of NK receptors, such as NKG2D receptor, which recognize self-molecules expressed by stressed cells such as virus-infected cells (19). In addition, we detected ADCC against uninfected allogenic CD4⁺ cells in 2 of 27 EU IDUs. This activity might be due to the presence of alloantibodies elicited by allogenic stimulation in these individuals. In a third EU IDU, HIV-specific ADCC was observed, yet HIV-1-specific Abs were not detected by ELISAs performed each year during the 4-year follow-up period (results not shown). Thus, it is unclear whether ADCC in this individual was due to HIV-cross-reactive Abs that are not detectable by HIV-ELISA or due to Abs directed against epitopes expressed on infected cells. Although it is difficult to evaluate the relevance of ADCC in the protection against HIV-1 on the basis of our current data, its presence in some EU IDUs is interesting and warrants further studies.

Among IDUs, behaviors that reduce the risk of transmission may account for the lack of infection in some cases. However, the EU IDUs studied here practiced high-risk behaviors for many years. It is likely that different mechanisms related either to immune responses and/or to genetic mechanisms contribute to the resistance to infection in EU individuals (1–4, 13, 20). Our results suggest a potential role of enhanced NK cell activity in the resistance against HIV-1 infection in Vietnamese EU IDUs. The number and proportion of NK cells were similar in all four of our study groups, indicating that the differences in NK cell activities are related to an increased NK functional activity in EU IDUs compared with either unexposed individuals or seroconverters rather than to an expansion of NK cells. A study on Italian HIV-1-serodiscordant couples (21) did not correlate NK cell activity to the resistance against HIV infection. However, in this study, environmental conditions and exposure criteria (≥1 year of unprotected sexual intercourse) were very different from those of the Vietnamese population studied here. In Vietnamese IDUs, the immune system could be activated due to the multiple infections that affect this population (12). Exposure to viruses stimulates NK activity in vivo and in vitro (5). Furthermore, allogenic stimulation following the injection of allogenic cells during needle sharing may also activate NK cells. It is possible that allogenic stimulation provides some protection against HIV-1 infection, possibly by inducing the production of HIV-1-specific inhibitory factors (22). Nevertheless, although EU and seroconverters IDUs had similar drug use habits, seroconverters exhibited lower NK cell cytolytic and secretory activities before seroconversion than did EU IDUs. Therefore, our results reveal an association between NK cell function and resistance to HIV-1 infection. In IDUs, NK activity may be differently affected by environmental parameters. A decreased NK activity has been associated with parenteral use of heroin (23). EU IDUs may present a genetic background that favors NK cell activation through the expression of a particular pattern of NK activator or inhibitory receptors or of their ligands (24). The expression of the HLA-Bw4 Ag, a ligand for the killer cell Ig-like receptor (KIR) receptor on NK cells, has been associated with the control of HIV-1 load and progression to AIDS (25). A combination of the activating KIR allele, KIR3DS1, and the HLA-B Bw4–80Ile allele has been associated with delayed progression to AIDS in HIV-1-infected individuals (26). The study of the repertoire of NK cell receptors and their ligands in Vietnamese EU IDUs will provide further information about the mechanisms of resistance to HIV-1 infection associated with the increase in NK cell activity.

**Acknowledgments**

We thank all of the physicians, family practitioners, and medical staff from the Binh Trieu Hospital who took part in this study. We also thank Dr. C. Leclerc and Dr. B. Bassin for critically reading our manuscript and for useful discussions, Dr. Arnaud Fontanet for advice on statistical analysis, and Dr. J.-Y. Fellaz for assistance with the regional database.

**References**


