

Innate Immune Dysfunction in HIV Infection: Effect of HIV Envelope-NK Cell Interactions^{1,2}

Shyam Kottlil,^{3*} Kyungmin Shin,* Julia O. Jackson,* Kristin N. Reitano,* Marie Angeline O'Shea,* Jun Yang,[†] Claire W. Hallahan,* Richard Lempicki,[†] James Arthos,* and Anthony S. Fauci*

We have previously described a number of NK cell dysfunctions in HIV-viremic individuals. In the present study, we performed DNA microarray analysis followed by phenotypic and functional characterization in an effort to investigate which HIV envelope glycoproteins (gp120) affect the physiologic functions of NK cells. Upon treatment of NK cells with HIV gp120, DNA microarray analyses indicated up-regulation of several categories of genes that are associated with apoptosis, suppression of both cellular proliferation and survival, as well as down-regulation of genes that play a vital role in cell proliferation, innate immune defense mechanism, and cell survival. Both subtypes of gp120 suppressed NK cell cytotoxicity, proliferation, and the ability to secrete IFN- γ . NK cells exposed to X4-subtype HIV gp120 showed a significant decrease in the levels of CC chemokines, while exposure to R5-subtype HIV gp120 had minimal effect. Extended exposure to HIV gp120 resulted in apoptosis of NK cells, further validating the microarray data. Our data demonstrate that exposure of NK cells to HIV envelope proteins results in profound cellular abnormalities at the level of gene expression as well as generic cell functions. These findings are likely to be a consequence of a direct HIV gp120-mediated effect on NK cells. Identification of specific surface receptors on NK cells that interact with HIV envelope proteins might explain how HIV is capable of circumventing innate immune defense mechanisms and establishing infection in susceptible individuals. *The Journal of Immunology*, 2006, 176: 1107–1114.

Natural killer cells are a critical component of the innate immune response to a variety of viruses, fungi, parasites, and bacteria (1, 2). They were originally identified functionally by their ability to lyse certain tumor cells without prior stimulation; however, they do not express clonally distributed Ag-specific receptors. Furthermore, it has been established that HLA class I molecules inhibit cytolytic activity of NK cells by their interaction with a series of cytotoxicity inhibitory receptors (inhibitory NK receptors (iNKR))⁴ (3, 4). Upon activation, NK cells release cytokines and chemokines that induce inflammatory responses, modulate hemopoiesis, control cell growth and function of monocytes and granulocytes, and influence the type of adaptive immune responses that follow. NK cells have both antiviral and antitumor activities and their ability to lyse targets does not depend on prior sensitization nor is their activity MHC-restricted (5). We have shown previously that HIV viremia affects the ability of NK

cells to produce CC chemokines and thereby suppress HIV replication *ex vivo* (6). Furthermore, NK cell-mediated redirected cytotoxicity assays have demonstrated the inability of NK cells from patients with viremia to lyse targets *in vitro* as a consequence of increased expression of iNKRs and a decreased expression of NK cytotoxicity receptors (NCR) (7). The underlying mechanisms by which HIV viremia influences NK cell receptor expression, and secretory and cytotoxic functions, are not fully delineated at present.

Several previous studies have demonstrated that HIV encodes for several structural proteins that interact with components of the human immune system, particularly CD4⁺ T cells (8–15). Interactions between CD4⁺ T cells and HIV envelope proteins (8–13, 15) have been shown to activate a large number of cellular genes associated with cellular activation and enhanced HIV replication. HIV envelope proteins have also been shown to enhance apoptosis in activated CD4⁺ T cells resulting in a reduced output of virions from such cells (16, 17). Several previous studies have addressed the effects of HIV gp120/gp160 or HIV peptides on NK cell cytotoxicity (18–21). These studies have demonstrated an inhibition of NK cell cytotoxicity upon exposure to HIV envelope proteins. However, these studies were performed using X4-subtype HIV envelope proteins or their derivatives. So far, no study has comprehensively addressed the effect of HIV envelope proteins derived from both R5- and X4-subtype primary isolates on generic NK cell functions. We used a DNA microarray and studied generic NK cell functions upon exposure to R5- and X4-subtype HIV envelope proteins derived from primary isolates. DNA microarray has been previously used to study the effect of HIV and/or HIV envelope proteins on profiles of gene expression in target cells (10, 22, 23). HIV gp120 transduces near-simultaneous signals through CD4 (14), which is absent on NK cells, and CCR5, a chemokine receptor (13), which is up-regulated on NK cells of viremic HIV-infected patients (24). To provide a comprehensive picture of the

*Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, and [†]Science Applications International Corporation-Frederick, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication May 26, 2005. Accepted for publication October 28, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The content of this publication does not necessarily reflect the views of policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organization imply endorsement by the U.S. Government.

² This work was presented by S. Kottlil, K. Shin, M. A. O'Shea, J. Yang, C. W. Hallahan, R. Lempicki, J. Arthos, and A. S. Fauci at the 10th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 2, 2002.

³ Address correspondence and reprint requests to Dr. Shyam Kottlil, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N204, 9000 Rockville Pike, Bethesda, MD 20892. E-mail address: Skottlil@niaid.nih.gov

⁴ Abbreviations used in this paper: iNKR, inhibitory NK receptor; NCR, NK cytotoxicity receptor; SAM, significant analysis of microarray; PI, propidium iodide; CBA, cytometric bead array.

complex cascades of gene expression events induced by HIV gp120 on NK cells, we treated freshly isolated NK cells from seronegative donors with HIV envelope proteins derived from R5- and X4-subtype virus and measured changes in the levels of gene expression over time. In addition, we used standard functional NK cell assays to study the effect of R5- and X4-subtype HIV gp120 on generic NK cell functions including cytotoxicity and secretion of cytokines and chemokines.

Materials and Methods

Study subjects

Ten HIV seronegative donors were subjected to leukapheresis to obtain PBMCs. Two volunteers who were previously screened as being homozygous for the CCR5 Δ 32 mutation were also subjected to leukapheresis to obtain PBMCs. All donors signed informed consents approved by the Institutional Review Board (IRB) at the Warren Grant Magnusson Clinical Center (Bethesda, MD).

Isolation of NK cells and culture conditions

NK cells were isolated from PBMCs using column-based cell separation techniques (StemCell Technologies) as previously described (6). The NK cell enrichment mixture contained Abs to CD3, CD4, CD14, CD19, CD66b, and glycophorin A. The resulting purity of NK cells was generally >95%. NK cells were treated with monomeric R5-(TH 14-12) and X4-subtype (92Ug12-9) envelope proteins at a final concentration of 200 nM for 60 min at 37°C, washed and resuspended in complete medium containing 20 U of IL-2 (Roche Diagnostics). The concentrations of each HIV envelope used were similar to that detected in the serum of AIDS patients (25). Untreated NK cells were used as controls.

DNA microarray

Highly purified NK cells from HIV-uninfected normal volunteers were cultured in the presence of R5- and X4-subtype HIV gp120 for 60 min at 37°C, washed in PBS with 1% FCS, and total RNA was extracted using TRIzol (Invitrogen Life Technologies). Control samples were prepared in the same manner without exposure to the HIV gp120. DNA microarray analysis (26) was performed using Affymetrix Human Genome U133A oligonucleotide arrays according to protocols specified by the manufacturer. A significant analysis of microarray (SAM) algorithm was used to determine the genes that were significantly differentially regulated after extensive prefiltering processes (26).

NK cell cytotoxicity assay

Freshly isolated NK cells were tested for cytotoxicity against K-562 targets. K-562 cells were suspended in complete medium and labeled with the green fluorescent cell linker PKH67-GL (Sigma-Aldrich). The stained target cells were washed three times in complete medium and 2×10^5 cells were added to 12 \times 75 mm round-bottom polystyrene tubes (Falcon) in duplicate. NK (effectors) cells were then added to the above target cells to yield E:T ratios of 30:1, 20:1, and 10:1. Control tubes containing target cells alone were also assayed. Effector and target cells were mixed by gentle tapping, centrifuged at 25°C for 4 min at 300 rpm ($25 \times g$), and incubated at 37°C for 3 h. After incubation, 10 μ l of propidium iodide (PI; BD Pharmingen) was added to each tube 10–15 min before data acquisition. A total of 100,000 total events were collected per sample using FAC-SCalibur (BD Biosciences). Target cells were gated by side scatter and fluorescence (FL-1). PI uptake was determined within the gated cells. The percent NK-specific lysis was calculated using the formula: NK-specific lysis = ((percent PI staining of sample – percent PI staining of negative control)/(100 – percent PI staining of negative control)) \times 100.

NK cell proliferation assay

Freshly isolated NK cells were cultured in the presence or absence of HIV envelope proteins with 20 IU of IL-2 as described above. After a 1-h incubation with the HIV envelopes, cells were washed and resuspended in complete RPMI 1640 supplemented with IL-2 (20 IU/ml). After 72 h of incubation, NK cell proliferation was measured by [3 H]thymidine uptake during an additional 16 h of incubation. Negative controls for proliferation included NK cells cultured in the absence of IL-2.

Cytokine ELISA of culture supernatants

Culture supernatants from NK cells (cultured under similar conditions as described above) were also tested for levels of IFN- γ , RANTES, MIP-1 α , and MIP-1 β secretion by ELISA (R&D Systems).

NK cell apoptosis assay

Freshly isolated NK cells were cultured with IL-2 (20 IU/ml) for 48 h and incubated with or without R5- and X4-subtype HIV gp120 for an extended period of 18 h with complete RPMI 1640 medium and IL-2 (20 IU/ml) and the level of apoptosis was measured using the Annexin V staining kit (R&D Systems). Cell lysates were prepared from NK cells cultured as described above with or without incubation with the R5- and X4-HIV envelope for an extended period of 18 h with complete RPMI 1640 supplemented by IL-2 (20 IU/ml). The concentration of activated caspase-3 was determined by using a Cytometric Bead Array (CBA) Human Apoptosis kit Assay (BD Biosciences) following manufacturer's protocol.

Statistical analysis

The means of the treatment groups were compared by ANOVA with blocking on donor. The Tukey method for multiple comparisons of means was used. Means and SEs are reported. The method of SAM was used for DNA microarray analysis.

Results

DNA microarray analyses of freshly isolated NK cells in the presence or absence of R5- or X4-subtype HIV envelope proteins

To delineate the molecular mechanism(s) by which NK cell function is impaired upon interaction with HIV envelope proteins, we performed DNA microarray analyses using total RNA isolated from fresh NK cells from four HIV seronegative donors in the presence or absence of R5- or X4-subtype HIV envelopes. Using Affymetrix human genome U133 A oligonucleotide arrays consisting of probes encompassing over 33,000 genes and a SAM algorithm (26), we identified 335 differentially expressed genes (Fig. 1). The corresponding genes and samples from the seronegative donors were grouped by using K-means and hierarchical clustering, respectively (Fig. 1). The hierarchical analyses classified the genes into four distinct categories or clusters (Fig. 1). Of these, cluster I consisted of 29 genes that were up-regulated by exposure to the R5-subtype HIV envelope, but were down-regulated by exposure to the X4-subtype HIV envelope when compared with the gene expression profiles of untreated NK cells. Cluster II was represented by a total of 26 genes that were up-regulated by the X4-subtype HIV envelope but were down-regulated by exposure to the R5-subtype HIV envelope when compared with the gene expression profiles of untreated NK cells. Cluster III consisted of 113 genes that were up-regulated upon exposure to both R5- and X4-subtype HIV envelope, when compared with the gene expression profiles of untreated NK cells. Cluster IV consisted of 167 genes that were down-regulated by both R5- and X4-subtype HIV envelopes when compared with the gene expression profiles of untreated NK cells.

Hierarchical clustering analyses indicated that there was a striking similarity in the transcriptional profile of genes that were differentially expressed by NK cells treated with either R5- or X4-subtype HIV envelopes (Fig. 1; clusters III and IV 280 of a total 335 genes). Statistical analyses identified genes that were significantly associated with programmed cell death and regulators of apoptosis ($p < 0.01$) as the top two functional categories from cluster III relative to the frequency of all annotated genes on the DNA microarrays. Statistical analyses also identified genes that were significantly associated with response to stress, pathogens and inflammation ($p < 0.001$) and cell growth and proliferation ($p < 0.001$) as the top functional categories reflected in cluster IV relative to the frequency of all annotated genes on the DNA microarrays. These results strongly suggest that interaction with HIV envelopes, either R5- or X4-subtype, resulted in expression of genes involved in inhibition of cellular proliferation and innate immune defenses together with genes promoting programmed cell death.

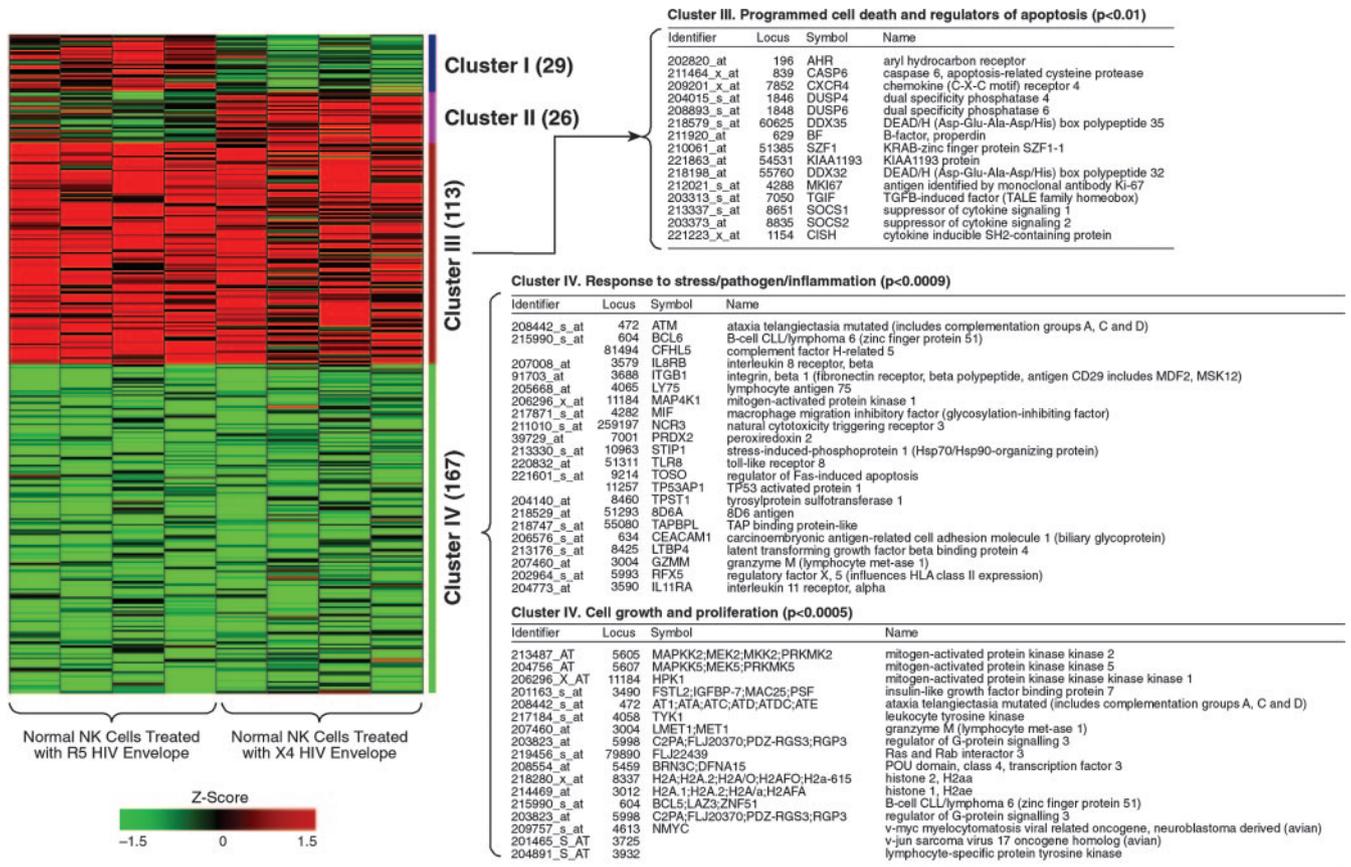


FIGURE 1. Clustering of differentially expressed genes in fresh NK cells of HIV-negative individuals after exposure to HIV R5- or X4-subtype HIV envelopes. Transcription profiles of freshly isolated NK cells from four uninfected donors were examined before and after exposure to HIV R5- and X4-subtype HIV envelope proteins using high-density DNA microarrays. Levels of gene expression were assayed using Affymetrix Human Genome U133A chips, and 335 differentially expressed genes were identified. Genes were grouped using K-means clustering, and samples were grouped by hierarchical clustering. Cluster III indicates a set of 113 genes that were up-regulated in NK cells after exposure with both R5- and X4-subtype HIV envelopes relative to the untreated NK cells from the same donor. Cluster IV indicates a set of 167 genes that were down-regulated in NK cells after exposure to both R5 and X4 HIV envelopes relative to the untreated NK cells from the same donor. Statistical analyses of the genes that were up-regulated in cluster III determined that the top functional categories, programmed cell death and promoters of apoptosis, were overrepresented in this list of genes relative to all annotated genes on the chip. Similarly, statistical analyses of the genes that were down-regulated in cluster IV determined that the top functional categories, response to stress, pathogen and inflammation and cell growth and proliferation, were overrepresented in this list of genes relative to all annotated genes on the chip. Differences in relative levels of gene expression (Z-score) are indicated in color, where red indicates up-regulation and green indicates down-regulation relative to that of corresponding gene expression in untreated controls. Some genes are listed twice because certain probes recognize multiple regions of a single gene.

The genes in cluster I and II represent a family of genes that are associated with the regulation of immune responses. One of these, which was found to be up-regulated upon exposure to the R5 envelope, is the gene that encodes TGF- β , an important cytokine that has been shown to inhibit NK cell function by down-regulating the surface expression of NCRs (27). This cytokine probably plays an important role in the HIV-NK cell interaction in vitro that could explain, at least in part, defective NK cell function in vivo (6, 7, 24, 28).

R5- and X4-subtype HIV envelope proteins suppress the ability of NK cells to lyse susceptible target cells and to proliferate in the presence of IL-2

To validate the findings from DNA microarray analyses, we performed several NK cell functional assays including cytotoxicity against K-562 (Fig. 2A) and the ability of NK cells to proliferate in response to IL-2 (Fig. 2B) in the presence or absence of R5- and X4-subtype HIV envelopes. The mean cytotoxicity against K-562 cells by freshly isolated untreated NK cells ($30.8 \pm 2.9\%$) was significantly higher than that of NK cells exposed to R5- ($16.5 \pm 1.4\%$) and X4-subtype ($11.8 \pm 1.2\%$) HIV envelopes ($p < 0.001$; Fig. 2A).

DNA microarray analyses have shown a significant down-regulation of genes in NK cells that facilitate cell proliferation. We performed in vitro assays to test the ability of NK cells to proliferate in response to IL-2 in the presence or absence of R5- and X4-subtype HIV envelopes. When proliferation of NK cells in response to IL-2 was tested using a [3 H]thymidine incorporation assay, untreated NK cells (43970 ± 2824 cpm) proliferated significantly better than those exposed to R5- (13762 ± 1683 cpm) and X4-subtype (13658 ± 1234 cpm) HIV envelopes ($p < 0.001$; Fig. 2B).

Presence of CCR5 on the cell surface is required for the maximal suppressive effect of R5 envelope on NK cells

Cytotoxicity and proliferation assays were performed using purified NK cells isolated from those patients with homozygous CCR5 $\Delta 32$ deletion mutations with or without exposure to R5 and X4 HIV envelopes (Fig. 2, C and D). NK cells isolated from individuals who are homozygous for CCR5 $\Delta 32$ mutations had only slightly lower levels of cytotoxicity against K562 and slightly impaired ability to proliferate in the presence of IL-2 whereas they

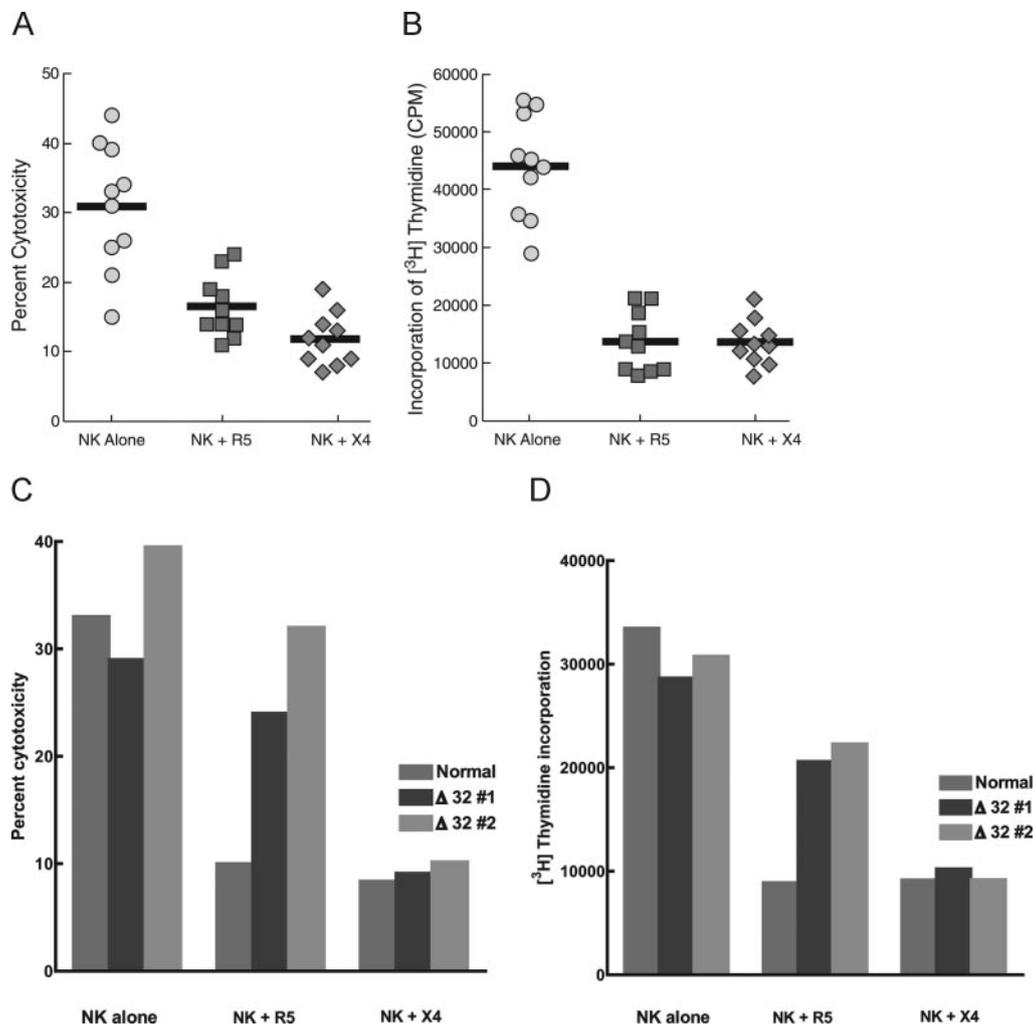


FIGURE 2. R5- and X4-subtype HIV envelopes suppress the ability of NK cells to proliferate and lyse susceptible targets. *A*, Cytotoxicity of freshly isolated NK cells was measured against K 562 targets before and after exposure to HIV R5- and X4-subtype envelopes as described in *Materials and Methods*. The mean cytotoxicity for untreated NK cells was significantly higher than that of NK cells exposed to R5- and X4-subtype HIV envelopes ($p < 0.001$). *B*, Proliferation of NK cells in the presence of IL-2 was measured before and after exposure to HIV R5- and X4-subtype HIV envelopes as described in *Materials and Methods*. Untreated NK cells proliferated better than those exposed to R5- and X4-subtype HIV envelopes ($p < 0.001$). *C*, Cytotoxicity of freshly isolated NK cells isolated from individuals who are homozygous for the CCR5 $\Delta 32$ mutation was measured against K 562 targets before and after exposure to HIV R5- and X4-subtype envelopes as described in *Materials and Methods*. NK cells isolated from individuals who are homozygous for the CCR5 $\Delta 32$ mutation had significantly lower levels of cytotoxicity against K 562 after exposure to X4 HIV envelope. However, NK cells from individuals who are homozygous for the CCR5 $\Delta 32$ mutation had only slightly lower levels of cytotoxicity against K562 after exposure to R5 HIV envelope. *D*, Proliferation of freshly isolated NK cells isolated from individuals who are homozygous for the CCR5 $\Delta 32$ mutation in the presence of IL-2 was measured before and after exposure to HIV R5- and X4-subtype envelopes as described in *Materials and Methods*. NK cells from individuals who are homozygous for the CCR5 $\Delta 32$ mutation have impaired ability to proliferate in the presence of IL-2 after exposure to the X4 HIV envelope. However, these cells had only slightly impaired ability to proliferate in the presence of IL-2.

had profound impairments in both cytotoxicity and proliferative capacity after exposure to the X4 HIV envelope.

Effect of R5- and X4-subtype HIV envelopes on cytokine secretion by NK cells

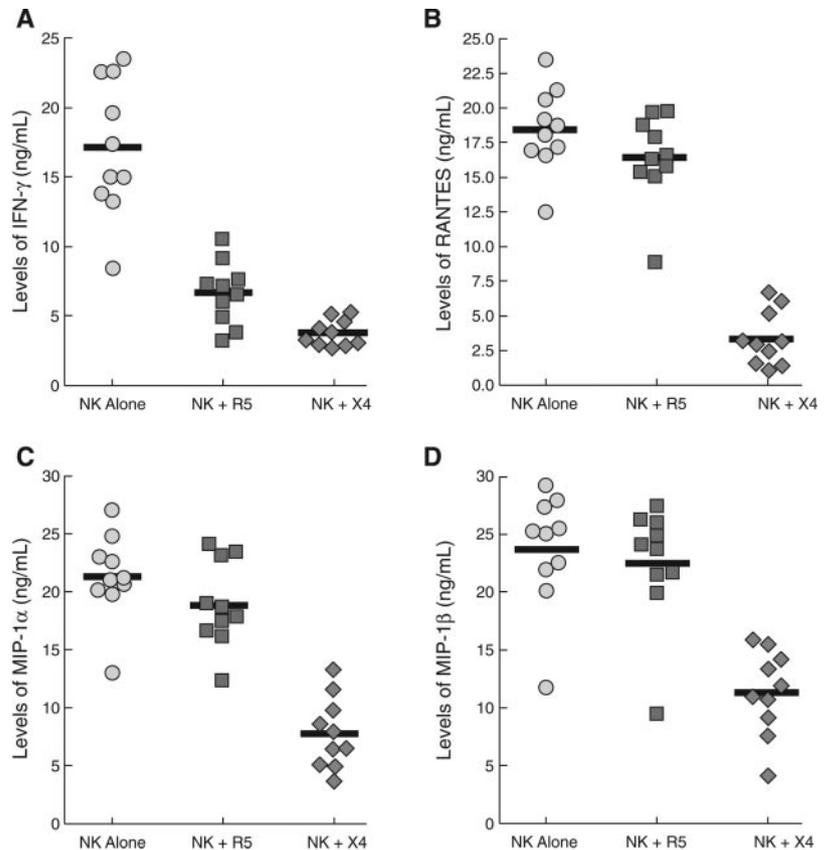
To further elucidate the effect of HIV envelopes on the secretory function of NK cells, we tested the ability of NK cells to secrete IFN- γ and CC chemokines (RANTES, MIP-1 α , and MIP-1 β) in the presence or absence of HIV envelopes (Fig. 3). Levels of IFN- γ measured in the culture supernatants from NK cells that were exposed overnight to R5- (6.6 ± 0.7 ng/ml) and X4-subtype (3.7 ± 0.3 ng/ml) HIV envelopes were significantly lower compared with that secreted by untreated NK cells (17.1 ± 1.6 ng/ml; $p < 0.001$; Fig. 3A). Culture supernatants from NK cells exposed to the X4-subtype HIV envelope produced significantly lower levels of CC chemokines RANTES,

MIP-1 α , and MIP-1 β (3.4 ± 0.6 , 7.8 ± 1.0 , and 9.4 ± 1.0 ng/ml, respectively) compared with that secreted by untreated NK cells (18.5 ± 1.0 , 21.4 ± 1.2 , and 19.8 ± 1.3 ng/ml, respectively) ($p < 0.001$; Fig. 3, B–D). However, culture supernatants from NK cells exposed to R5-subtype HIV envelope produced similar amounts of RANTES, MIP-1 α and MIP-1 β (16.4 ± 1.0 ng/ml; 18.9 ± 1.3 and 18.8 ± 1.4 ng/ml) compared with that secreted by untreated NK cells (18.5 ± 1.0 ng/ml ($p = 0.14$); 21.4 ± 1.2 ng/ml ($p = 0.11$); and 19.8 ± 1.3 ng/ml ($p > 0.5$), respectively) ($p = 0.12$; Fig. 3, B–D).

Extended exposure to HIV envelopes induces apoptosis of NK cells

DNA microarray analyses showed that several genes in cluster IV that regulate apoptosis and programmed cell death were up-regulated upon exposure to HIV envelopes (R5 and X4 subtypes). To

FIGURE 3. Effects of R5- and X4-subtype HIV envelopes on cytokine secretion of NK cells. *A*, Levels of IFN- γ before and after exposure to HIV R5 and X4 envelopes were measured as described in *Materials and Methods*. Culture supernatants from NK cells exposed to X4- and R5-subtype HIV envelopes produced significantly lower amounts of IFN- γ when compared with untreated controls ($p < 0.001$). *B–D*, Levels of CC chemokines before and after exposure to HIV R5- and X4-subtype HIV envelopes were measured as described in *Materials and Methods*. Culture supernatants from NK cells treated with the R5-subtype HIV envelope produced comparable amounts of CC chemokines, RANTES (*B*), MIP-1 α (*C*), and MIP-1 β (*D*) to that of untreated NK cells ($p = 0.14$, $p = 0.11$, $p > 0.5$, respectively). Culture supernatants from NK cells treated with the X4-subtype HIV envelope produced significantly lower amounts of all three CC chemokines than that of untreated NK cells ($p < 0.001$).



validate the above findings, we performed Annexin V/PI staining of NK cells either untreated or exposed to R5- or X4-subtype HIV envelopes for 18 h. A representative flow cytometric analyses is shown in Fig. 4A and the mean levels of apoptosis are shown in Fig. 4B. As indicated in Fig. 4B, significantly higher levels of apoptosis were observed with NK cells exposed to either R5- ($21.6 \pm 1.2\%$) or X4-subtype ($23.9 \pm 0.9\%$) HIV envelopes than were observed with untreated NK cells ($2.4 \pm 0.4\%$; $p < 0.001$). To understand the underlying mechanisms involved in HIV envelope-induced apoptosis of NK cells, we performed CBA assay for the levels of activated caspase-3 on NK cell lysates with or without exposure to the R5- and X4-HIV envelopes. As shown in Fig. 4C, the activated caspase-3 levels were higher in the cell lysates from R5- (785 ± 55 pg/ml) and X4-HIV envelopes (946 ± 67 pg/ml) when compared with untreated NK cell lysates (539 ± 4 pg/ml).

Discussion

In the present study, we have demonstrated that HIV envelopes, both R5 and X4, alter the profiles of gene expression in NK cells by up-regulating genes involved in apoptosis and down-regulating genes involved in cell proliferation and survival. Our DNA microarray data were validated by a number of functional assays that confirmed reduced cytotoxicity, IFN- γ production, proliferative responses, and increased propensity to undergo apoptosis upon exposure to HIV envelopes.

HIV infection is associated with several functional defects in NK cells (6, 7, 24, 28). These include increased expression of iNKR (7, 24), reduced expression of NCRs (7, 29), reduced ability to secrete CC chemokines and block HIV replication (6, 30), and reduced ability to lyse target cells in a receptor-mediated fashion (7, 29). However, the exact mechanisms by which HIV induces these changes remain unclear at present. It is conceivable that HIV exerts global suppressive effects on NK cells by means of gener-

alized immune activation associated with active viral replication, and also possibly by means of a direct effect of HIV or HIV products on NK cells in vivo. We have previously demonstrated that changes in the patterns of expression of certain receptors on NK cells (e.g., CCR5 expression) were induced by immune activation, while expression of other receptors (iNKR expression) were not influenced by immune activation (24). To better understand HIV-NK cell interactions, we performed a series of experiments in which freshly isolated NK cells from HIV-seronegative donors were incubated with R5- and X4-subtype HIV envelopes.

DNA microarrays have been used previously in the study of HIV infection to better understand the pathogenesis of HIV-induced immune defects (31–34). We performed DNA microarrays using RNA isolated from NK cells with or without exposure to R5- or X4-subtype HIV envelopes. Our results demonstrated profound suppressive effects of HIV envelope on cell proliferation, cell functions associated with immune defenses, cell division, and survival of NK cells, whereas they also showed up-regulation of genes that were involved in the suppression of cell proliferation, decreased survival, and the promotion of apoptosis. These results indicate that HIV envelopes are capable of affecting NK cell functions in a profound manner even after a short exposure. These early changes in gene expression could translate into functional defects in generic NK functions that include proliferation, cytotoxicity, and secretion of cytokines and chemokines.

To validate the DNA microarray findings at the functional level, we performed assays to test generic function of NK cells. When NK cell cytotoxicity assays were performed with or without the exposure to HIV envelopes, there was a substantial reduction in the level of cytotoxicity observed after exposure to HIV envelopes when compared with NK cells that were not exposed to HIV envelopes. Similar suppressive effects were also observed when the ability of NK cells to proliferate in the presence of IL-2 was tested

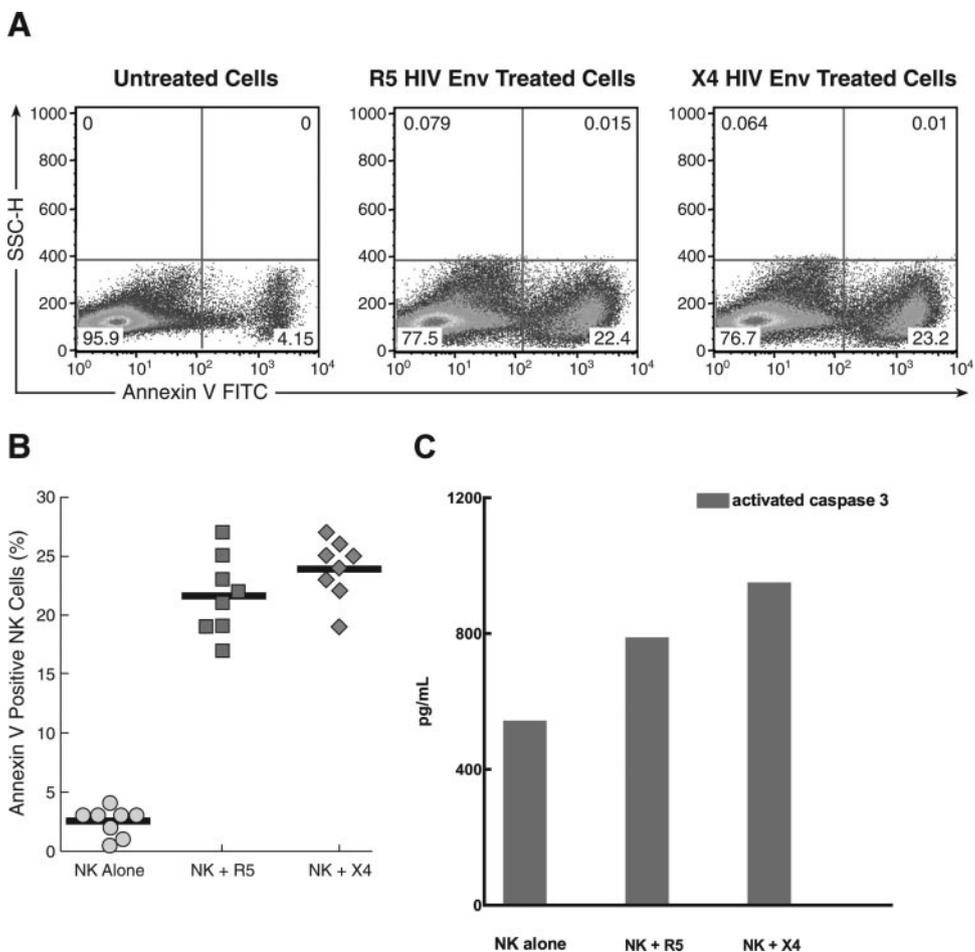


FIGURE 4. Extended exposure to HIV envelopes induce apoptosis of NK cells. Annexin V staining of NK cells was performed as described in *Materials and Methods*. A representative FACS dot plot is shown in A indicating annexin V-positive NK cells after exposure to R5- or X4-subtype HIV envelopes. B, NK cells exposed to either R5- or X4-subtype HIV envelopes exhibited significantly higher levels of annexin V staining (early apoptosis) than those of untreated NK cells ($p < 0.001$). C, Activated caspase-3 levels were higher in the cell lysates from R5- and X4-HIV envelopes when compared with untreated NK cell lysates.

with or without exposure to HIV envelopes. Furthermore, there was a substantial suppression of IFN- γ secretion by those NK cells that were exposed to the HIV envelope, when compared with untreated NK cells. Previously reported studies have shown that NK cells are a major source of CC chemokines *in vivo* (6, 30, 35) and also that levels of CC chemokines were significantly reduced in patients with detectable HIV viremia (6). The ability to secrete CC chemokines and suppress HIV replication is considered to be a potentially important innate defense mechanism exhibited by NK cells against HIV *in vivo* (6, 30). To understand the effect of HIV envelopes on the ability of NK cells to secrete CC chemokines *in vitro*, we measured the levels of CC chemokines in the supernatant of NK cells with or without exposure to HIV envelopes. Exposure to X4-subtype HIV envelope profoundly suppressed the ability of NK cells to secrete CC chemokines when compared with untreated cells; however, NK cells exposed to the R5-subtype HIV envelope secreted levels of CC chemokines that were comparable to those secreted by untreated NK cells. The exact reason for this dichotomous response of different HIV envelope subtypes is not completely understood. One possible explanation is that the R5-subtype HIV envelope bound to the CCR5 receptor could block the binding of CC chemokines to their natural receptor thereby resulting in the accumulation of the chemokines in the supernatant. Another possible explanation is that even though both HIV envelope subtypes are quite similar in their effects on NK cells, it is possible that they may bind to separate receptors on the surface of NK cells and induce certain distinct effects. In support of this argument, DNA microarray data indicate that of all 128 genes up-regulated by HIV envelopes, there were 44 genes that were common to both R5- and X4-subtype HIV envelopes, while 56 and 28 were

uniquely up-regulated by R5- and X4-subtype HIV envelopes, respectively. Of all 173 genes down-regulated by HIV envelopes, there were 94 genes that were common to both R5- and X4-subtype HIV envelopes, while 49 and 30 were uniquely up-regulated by R5- and X4-subtype HIV envelopes, respectively. These data imply that even though both R5- and X4-subtype HIV envelopes share several pathways in their interactions with NK cells, they also exert certain unique effects on gene expression in NK cells. When we compared the gene expression levels of these cytokines identified by DNA microarray with those obtained with functional assays, the four cytokines tested by ELISA (RANTES, MIP-1 α , MIP-1 β , IFN- γ) were not significantly modulated in the microarray study. These results suggest that differential modulation of these cytokines in the culture medium is regulated posttranscriptionally (data not shown).

DNA microarray analyses also demonstrated up-regulation of several genes that promote apoptosis in NK cells upon exposure to both R5- and X4-subtype HIV envelopes. To determine whether exposure to HIV envelopes increases the propensity of NK cells to undergo apoptosis, we measured the level of early apoptosis of NK cells with or without exposure to HIV envelopes. There was a significant increase in the degree of apoptosis exhibited by NK cells exposed to both R5- and X4-subtype HIV envelopes when compared with that observed with untreated NK cells. Previous studies have shown that HIV envelope-induced apoptosis was mediated by activated caspase-3 in CD4⁺ T cells (9). When we investigated the role of activated caspase-3 levels in HIV envelope-induced apoptosis of NK cells, we detected elevated levels of activated caspase-3 in NK cell lysates treated with R5- and X4-HIV envelopes. These results suggest that similar pathways of induction of

apoptosis are involved in the induction of HIV envelope-induced apoptosis in both NK cells and CD4⁺ T cells. These data indicate that HIV envelopes can suppress generic NK cell functions and induce apoptosis and contribute to in vivo loss of NK cells in HIV-infected individuals.

In this study, we have demonstrated profound effects of HIV envelopes on NK cell gene expression, function, and survival. However, the precise molecular mechanism(s) whereby HIV envelopes interact with NK cells is unclear. It is known that HIV binds to the CD4 molecule expressed on lymphocytes and monocytes; in addition, the virus uses CCR5 and CXCR4 as coreceptors for entry into these cells (36–39). Because NK cells are not known to express the CD4 molecule on their surface, HIV envelopes could not have used the CD4 receptor to exert their effects (40). However, NK cells have been shown to express CCR5 and CXCR4 receptors on their surface and we have shown recently that HIV infection increases the level of CCR5 receptor expression on the surface of NK cells (24). Therefore, it is possible that both R5- and X4-subtype HIV envelopes could bind to these receptors and thereby exert their effects on NK cell function. In this regard, it has been demonstrated that even though HIV envelopes can bind and trigger chemokine receptors on T cells, simultaneous engagement of the CD4 molecule is essential for the maximal effect on T cells (8, 37). Our data obtained from individuals who are homozygous for the CCR5 Δ 32 mutation suggest that, although the presence of CCR5 receptor is essential for the maximal suppressive effect of HIV envelope on NK cells, there was still some effect of HIV envelopes on NK cells even among those who lacked expression of CCR5 receptor. This would imply that the profound changes seen with exposure of NK cells to the HIV envelope might not be explained entirely by engagement of chemokine receptors alone. It is conceivable that there could be other receptors on NK cells for which the HIV envelope may serve as a ligand. The HIV envelope is one of the most heavily glycosylated molecules and NK cells have several surface receptors that belong to the C-type lectin family, which are natural ligands to polysaccharides. One or several of these receptors could serve as a potential HIV receptor on NK cells similar to the DC-SIGN molecule that has been described to aid in the entry of HIV into target cells (41). The existence of such molecule(s) on NK cells is presently unknown.

There are several in vivo implications relevant to these findings. The model system used in this study, i.e., the HIV envelope interaction with NK cells, recapitulates several of the functional defects that are seen with NK cells isolated from chronically HIV-infected individuals in vivo. Previous studies have demonstrated defects in NK cell cytotoxicity and ability to secrete cytokines and chemokines associated with HIV viremia (6). Recently, it has been shown that NK cells from HIV-infected viremic individuals are defective phenotypically and functionally and manifest a dichotomous expression of iNKR and NCRs ex vivo (7, 24). However, we were not able to demonstrate changes in the expression of iNKR and NCRs on NK cells after exposure to HIV envelopes (data not shown) as was reported in patients with chronic HIV viremia (7, 24). Conceivably, these latter changes could be induced by prolonged exposure to HIV envelopes in vivo rather than exposures in vitro for a brief period of time. Hence, some of the defects, especially those seen with expression of iNKR and NCRs (7, 24), probably reflect consequences of prolonged exposure of NK cells to HIV Ags as seen with the chronic HIV viremic state. It should also be noted that HIV uses several mechanisms to circumvent immune effector mechanisms and these may include HIV Ags other than envelopes (42, 43). Therefore, it is possible that several of the defects that are described with regard to NK cell phenotype and functions (6, 7, 24, 28) could be a direct conse-

quence of interactions with HIV Ags other than envelopes. It should also be noted that the state of HIV viremia up-regulates CCR5 expression and this phenomenon could contribute to an enhanced effect of HIV R5-subtype envelope-mediated suppression of NK cells (24). Control of HIV viremia by means of antiretroviral therapy would minimize the HIV envelope-induced direct suppressive effects on NK cells and help to partially restore NK cell functions (6, 7, 24, 28). Furthermore, identification of target receptors on NK cells for HIV would enable better understanding of HIV-NK cell interactions and ultimately further delineate the mechanism(s) used by HIV to circumvent innate immune defenses and establish chronic infection.

Acknowledgments

This research was performed in whole by the Intramural Research Program of the National Institutes of Health (National Institute of Allergy and Infectious Diseases).

Disclosures

The authors have no financial conflict of interest.

References

- Lanier, L. L. 2001. On guard—activating NK cell receptors. *Nat. Immunol.* 2: 23–27.
- Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47: 187–376.
- Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M. C. Mingari, and L. Moretta. 1996. Receptors for HLA class-I molecules in human natural killer cells. *Annu. Rev. Immunol.* 14: 619–648.
- Lanier, L. L., and J. H. Phillips. 1996. Inhibitory MHC class I receptors on NK cells and T cells. *Immunol. Today* 17: 86–91.
- Barao, I., and J. L. Ascensao. 1998. Human natural killer cells. *Arch. Immunol. Ther. Exp.* 46: 213–229.
- Kottlilil, S., T. W. Chun, S. Moir, S. Liu, M. McLaughlin, C. W. Hallahan, F. Maldarelli, L. Corey, and A. S. Fauci. 2003. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J. Infect. Dis.* 187: 1038–1045.
- Mavilio, D., J. Benjamin, M. Daucher, G. Lombardo, S. Kottlilil, M. A. Planta, E. Marceano, C. Bottino, L. Moretta, A. Moretta, and A. S. Fauci. 2003. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. *Proc. Natl. Acad. Sci. USA* 100: 15011–15016.
- Cicala, C., J. Arthos, M. Ruiz, M. Vaccarezza, A. Rubbert, A. Riva, K. Wildt, O. Cohen, and A. S. Fauci. 1999. Induction of phosphorylation and intracellular association of CC chemokine receptor 5 and focal adhesion kinase in primary human CD4⁺ T cells by macrophage-tropic HIV envelope. *J. Immunol.* 163: 420–426.
- Cicala, C., J. Arthos, A. Rubbert, S. Selig, K. Wildt, O. J. Cohen, and A. S. Fauci. 2000. HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4⁺ T cells. *Proc. Natl. Acad. Sci. USA* 97: 1178–1183.
- Cicala, C., J. Arthos, S. M. Selig, G. Dennis, Jr., D. A. Hosack, D. Van Ryk, M. L. Spangler, T. D. Steenbeke, P. Khazanie, N. Gupta, et al. 2002. HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication. *Proc. Natl. Acad. Sci. USA* 99: 9380–9385.
- Arthos, J., A. Rubbert, R. L. Rabin, C. Cicala, E. Machado, K. Wildt, M. Hanbach, T. D. Steenbeke, R. Swofford, J. M. Farber, and A. S. Fauci. 2000. CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J. Virol.* 74: 6418–6424.
- Kornfeld, H., W. W. Cruikshank, S. W. Pyle, J. S. Berman, and D. M. Center. 1988. Lymphocyte activation by HIV-1 envelope glycoprotein. *Nature* 335: 445–448.
- Weissman, D., R. L. Rabin, J. Arthos, A. Rubbert, M. Dybul, R. Swofford, S. Venkatesan, J. M. Farber, and A. S. Fauci. 1997. Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* 389: 981–985.
- Hivroz, C., F. Mazerolles, M. Soula, R. Fagard, S. Graton, S. Meloche, R. P. Sekaly, and A. Fischer. 1993. Human immunodeficiency virus gp120 and derived peptides activate protein tyrosine kinase p56^{lck} in human CD4 T lymphocytes. *Eur. J. Immunol.* 23: 600–607.
- Briant, L., N. Coudronniere, V. Robert-Hebmann, M. Benkirane, and C. Devaux. 1996. Binding of HIV-1 virions or gp120-anti-gp120 immune complexes to HIV-1-infected quiescent peripheral blood mononuclear cells reveals latent infection. *J. Immunol.* 156: 3994–4004.
- Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R. P. Sekaly, and T. H. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J. Exp. Med.* 176: 1099–1106.
- Chirmule, N., and S. Pahwa. 1996. Envelope glycoproteins of human immunodeficiency virus type 1: profound influences on immune functions. *Microbiol. Rev.* 60: 386–406.

18. Cauda, R., M. Tumbarello, L. Ortona, P. Kanda, R. C. Kennedy, and T. C. Chanh. 1988. Inhibition of normal human natural killer cell activity by human immunodeficiency virus synthetic transmembrane peptides. *Cell. Immunol.* 115: 57–65.
19. Peruzzi, M., C. Azzari, M. E. Rossi, M. De Martino, and A. Vierucci. 2000. Inhibition of natural killer cell cytotoxicity and interferon γ production by the envelope protein of HIV and prevention by vasoactive intestinal peptide. *AIDS Res. Hum. Retroviruses* 16: 1067–1073.
20. Nair, M. P., and S. A. Schwartz. 1997. Inhibition of natural killer cell activities from normal donors and AIDS patients by envelope peptides from human immunodeficiency virus type I. *Cell. Mol. Biol.* 43: 969–979.
21. Nair, M. P., and S. A. Schwartz. 2000. Reversal of human immunodeficiency virus type 1 protein-induced inhibition of natural killer cell activity by α interferon and interleukin-2. *Clin. Diagn. Lab. Immunol.* 7: 101–105.
22. Corbeil, J., D. Sheeter, D. Genini, S. Rought, L. Leoni, P. Du, M. Ferguson, D. R. Masys, J. B. Welsh, J. L. Fink, et al. 2001. Temporal gene regulation during HIV-1 infection of human CD4⁺ T cells. *Genome Res.* 11: 1198–1204.
23. Geiss, G. K., R. E. Bumgarner, M. C. An, M. B. Agy, A. B. van 't Wout, E. Hammersmark, V. S. Carter, D. Upchurch, J. I. Mullins, and M. G. Katze. 2000. Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays. *Virology* 266: 8–16.
24. Kottlil, S., K. Shin, M. Planta, M. McLaughlin, C. W. Hallahan, M. Ghany, T. W. Chun, M. C. Sneller, and A. S. Fauci. 2004. Expression of chemokine and inhibitory receptors on natural killer cells: effect of immune activation and HIV viremia. *J. Infect. Dis.* 189: 1193–1198.
25. Oh, S. K., W. W. Cruikshank, J. Raina, G. C. Blanchard, W. H. Adler, J. Walker, and H. Kornfeld. 1992. Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. *J. Acquir. Immune Defic. Syndr.* 5: 251–256.
26. Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* 98: 5116–5121.
27. Castriconi, R., C. Cantoni, M. Della Chiesa, M. Vitale, E. Marcenaro, R. Conte, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2003. Transforming growth factor β 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. USA* 100: 4120–4125.
28. Mavilio, D., G. Lombardo, J. Benjamin, D. Kim, D. Follman, E. Marcenaro, A. O'Shea, M. A. Kinter, C. Kovacs, A. Moretta, and A. S. Fauci. 2005. Characterization of CD56⁻/CD16⁺ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc. Natl. Acad. Sci. USA* 102: 2286–2291.
29. De Maria, A., M. Fogli, P. Costa, G. Murdaca, F. Puppo, D. Mavilio, A. Moretta, and L. Moretta. 2003. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur. J. Immunol.* 33: 2410–2418.
30. Oliva, A., A. L. Kinter, M. Vaccarezza, A. Rubbert, A. Catanzaro, S. Moir, J. Monaco, L. Ehler, S. Mizell, R. Jackson, et al. 1998. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J. Clin. Invest.* 102: 223–231.
31. Chun, T. W., J. S. Justement, R. A. Lempicki, J. Yang, G. Dennis, Jr., C. W. Hallahan, C. Sanford, P. Pandya, S. Liu, M. McLaughlin, et al. 2003. Gene expression and viral production in latently infected, resting CD4⁺ T cells in viremic versus aviremic HIV-infected individuals. *Proc. Natl. Acad. Sci. USA* 100: 1908–1913.
32. Kim, S. Y., J. Li, G. Bentsman, A. I. Brooks, and D. J. Volsky. 2004. Microarray analysis of changes in cellular gene expression induced by productive infection of primary human astrocytes: implications for HAD. *J. Neuroimmunol.* 157: 17–26.
33. Moir, S., A. Malaspina, O. K. Pickeral, E. T. Donoghue, J. Vasquez, N. J. Miller, S. R. Krishnan, M. A. Planta, J. F. Turney, J. S. Justement, et al. 2004. Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *J. Exp. Med.* 200: 587–599.
34. Wang, Z., G. Trillo-Pazos, S. Y. Kim, M. Canki, S. Morgello, L. R. Sharer, H. A. Gelbard, Z. Z. Su, D. C. Kang, A. I. Brooks, et al. 2004. Effects of human immunodeficiency virus type 1 on astrocyte gene expression and function: potential role in neuropathogenesis. *J. Neurovirol.* 10(Suppl. 1): 25–32.
35. Fehniger, T. A., G. Herbein, H. Yu, M. I. Para, Z. P. Bernstein, W. A. O'Brien, and M. A. Caligiuri. 1998. Natural killer cells from HIV-1⁺ patients produce C-C chemokines and inhibit HIV-1 infection. *J. Immunol.* 161: 6433–6438.
36. Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272: 1955–1958.
37. Arthos, J., C. Cicala, S. M. Selig, A. A. White, H. M. Ravindranath, D. Van Ryk, T. D. Steenbeke, E. Machado, P. Khazanie, M. S. Hanback, et al. 2002. The role of the CD4 receptor versus HIV coreceptors in envelope-mediated apoptosis in peripheral blood mononuclear cells. *Virology* 292: 98–106.
38. Moore, J. P., A. Trkola, and T. Dragic. 1997. Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* 9: 551–562.
39. Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272: 872–877.
40. Brenner, B. G., A. Dascal, R. G. Margolese, and M. A. Wainberg. 1989. Natural killer cell function in patients with acquired immunodeficiency syndrome and related diseases. *J. Leukocyte Biol.* 46: 75–83.
41. Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, et al. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances *trans*-infection of T cells. *Cell* 100: 587–597.
42. Janvier, K., C. Petit, E. Le Rouzic, O. Schwartz, and S. Benichou. 2000. HIV auxiliary proteins: an interface between the virus and the host. *AIDS* 14(Suppl. 3): S21–S30.
43. Stevenson, M. 2003. HIV-1 pathogenesis. *Nat. Med.* 9: 853–860.