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Association of Cells with Natural Killer (NK) and NKT Immunophenotype with Incident Cancers in HIV-Infected Women

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ABSTRACT

Evidence indicates that immunosupression is associated with the development of certain cancers. The pathogenesis of HIV disease includes an alteration in innate immunity, mediated through NK and NKT cells. The evaluation of innate immune status in HIV patients prior to cancer diagnosis may identify the specific immunological events preceding the development of malignant disease. We evaluated the association between immunophenotypically defined NK, NKT, and $CD8^+$ cell percentages and incident malignancies in 1817 HIV(+) women in the Women's Interagency HIV Study (WIHS) who were followed for a median of 7.5 years. A total of 52 incident cancers of 20 different sites were identified. Compared to cancer-free women, cancer cases were older ($p < 0.01$), more likely to be anti-HCV(+) ($p = 0.02$), and had higher baseline median HIV RNA levels than controls. The $CD8^+$, NK, and NKT percents at baseline were not related to cancer risk. However, when time-dependent values for NKT cells were used, higher levels of NKT cells were associated with a reduced risk of cancer (adjusted hazard ratio = 0.67, 95% CI = 0.50, 0.89 per NKT percentage point). In addition to the loss of $CD4^+$ lymphocytes and an increased risk of opportunistic infections, HCV coinfected individuals may also experience alterations in innate immunity, including reduced NKT and NK cell number and possibly their function. In time-dependent analyses, increased numbers of NKT cells were associated with a reduced risk of cancer. HIV-induced innate immune dysfunction may contribute to the eventual emergence of cancer in the setting of existing coinfections and altered immunosurveillance.

INTRODUCTION

A LARGE BODY OF EVIDENCE indicates that immunosuppression is associated with the development of certain cancers, but the mechanisms underlying the immune defects in cancer patients have not been fully elucidated.[1] In some cases, evidence of immunosupression is found at the time of cancer diagnosis, while in other cases, progressive tumor growth itself could lead to the development of cellular immune deficiency involving both T cell loss and dysfunction.[2] The evaluation of immune status in patients prior to cancer diagnosis may identify the specific immunological events leading to the development of malignant disease.[3]

The pathogenesis of HIV disease includes an alteration in natural killer (NK) cell function.[4] Decreased function and number of NK cells in the peripheral blood are associated with more rapid disease progression, indicating that defective major histocompatibility complex (MHC) unrestricted cytotoxicity may be associated with HIV-related clinical progression.[5] The overall loss of the $CD8^+$ cells and $CD56^+$ NK cell function, with

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an increase in immune deficiency, may be due to differential loss of the NK cell subsets or, alternatively, to the loss of NK activity.[6,7] Data suggest that expansion of the CD56(−) NK cell subset in HIV-viremic individuals primarily accounts for the impaired function of the total NK cell population.[8,9]

Natural killer T (NKT) lymphocytes share receptor structures and functions of classical T cells and NK cells.[10] Humans appear to have a more heterogeneous repertoire of NKT cells than mice, expressing both T cell receptor (TCR)/CD3 complex and NK cell markers. Consequently, these cells could be MHC-restricted as well as CD1d-dependent NKT cells,[11] also known as invariant NKT cells because they express invariant TCR $V_{\alpha 24}J_{\alpha 18}$ and limited $V_{\beta 11}$ chain usage. Invariant NKT cells mediate antitumor cytotoxicity directly or indirectly by secreting interleukin (IL)-2, thus activating NK cells.[12,13]

As NK and NKT cells are known to be responsible for anticancer surveillance and protection against tumors,[3] we evaluated the association between immunophenotypically defined NK, NKT cells, and $CD8^+$ cell percentages and incident malignancies in HIV-positive women in the Women's Interagency HIV Study (WIHS).

MATERIALS AND METHODS

Study population

A detailed description of the WIHS cohort has been published.[14] The current study used longitudinal data from the WIHS cohort that accrued 2059 HIV-1–positive and 569 HIV-negative women from October 1994 through November 1995. The median age of participants at baseline was 36.3 years. These participants were recruited from HIV clinics, street outreach, referral from other studies, and word of mouth in six WIHS study sites located throughout the United States, including New York City (two sites), Northern California, Southern California, Chicago, and Washington D.C.

Only HIV-positive women were included in these current analyses. Women were excluded if they had a current or prior diagnosis of cancer at entry into the cohort ($n = 32$), did not have at least two visits ($n = 211$), or did not have at least one measurement of NK percentage ($n = 9$). Ten women met multiple exclusion criteria. In total, 242 HIV-positive women were excluded who met one or more of these criteria. The 1817 eligible subjects were followed for a median of 7.5 years following the first visit (range = 7 days, 8 years). Of these women 52 were diagnosed with cancer during the follow-up period.

Data collection and laboratory analysis

WIHS participants are seen every 6 months and undergo a comprehensive interview, physical and gynecological examination, and extensive laboratory evaluations. Blood and cervicovaginal lavage (CVL) specimens are processed and stored according to a standardized WIHS protocol.[14] Demographic data collected at baseline include date of birth and race/ethnicity. Smoking status and injection drug use were determined at each study visit by a structured questionnaire.

We determined hepatitis C virus (HCV) status at baseline, and HIV status, HIV RNA, CD4 percent, and CD8 percent at each visit. NK cell [$CD3^-/CD(16/56)^+$] and NKT cell [$CD3^+/CD(16/56)^+$] percents were determined routinely for patients at visits 1 through 7 (WIHS I, 3.5 years of follow-up), but were subsequently discontinued (WIHS II and III).

The percentages of NK and NKT cell subsets were evaluated by two-color flow cytometric analysis using a Simultest reagent (BD Biosciences, San Jose, CA) according to the instructions of the manufacturer. We used the following monoclonal antibody (mAb) combinations: CD3 FITC/($CD16^+$CD56) PE mAb. Isotype controls using IgG1 FITC/IgG2a PE were included in each experiment. Samples were analyzed within 24 h using a FACSCalibur flow cytometer (Becton Dickinson) with Cell-Quest software for data acquisition and analysis. Data from a minimum of 5000 events were acquired in the lymphocyte gate and analyzed.

The percentages of CD4- and CD8-positive lymphocyte subsets were evaluated by two-color flow cytometric analysis using CD4PE/CD3FITC and CD8PE/CD3FITC antibodies (BD Biosciences, San Jose, CA) according to the instructions of the manufacturer. Briefly, 100 μl of blood was added to 20 μl of different fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAb combinations and incubated for 30 min at room temperature in the dark. After incubation, 1 ml of lysing solution diluted 1:10 was added to samples to lyse erythrocytes. After centrifugation and washing, the samples were fixed using 0.5 ml of 1% *p*-formaldehyde. The IgG1 FITC/IgG2a PE were included as isotype controls in each experiment. Samples were analyzed within 24 h using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software for data acquisition and analysis. Data from a minimum of 5000 events were acquired in the lymphocyte gate and analyzed. Flow cytometry and real-time testing for HIV RNA were performed at local sites certified through the National Institutes of Health, National Institute of Allergy and Infectious Diseases, Division of AIDS Quality Assurance Program using consensus protocols.[15–17]

Ascertainment of incident cancers

Cancers that occurred in women from the WIHS cohort were identified through the following methods and in this hierarchical order of priority: (1) linkage of WIHS participants with statewide cancer registries, (2) medical record confirmation of self-reported cancer diagnoses, and (3) WIHS-initiated gynecological biopsies. Cancers that were identified by self-reports or death certificates were included only if they were confirmed by medical record or cancer registry review. A central pathological review was conducted on all cases of reported cervical cancer. The majority of the breast cancer cases also underwent central pathological review. Participants were reclassified as not having cancer when review of pathology reports or central pathological review did not confirm a diagnosis of cancer. Cancers were classified as being incident if they were first diagnosed after enrollment into the WIHS. Seven cases were identified before the second follow-up visit.

Statistical analysis

Cox proportional hazards regression was used to relate the time on study until first cancer diagnosis to the variables age, race/ethnicity, smoking status, injection drug use, HCV status, HIV RNA, CD4 percent, CD8 percent, and NK/NKT percent.

Participants were censored at death or their last follow-up contact on or prior to visit 17 (approximately 8 years). Associations of baseline visit levels of NK/NKT and CD8 with cancer risk were tested in models that were both unadjusted and adjusted for baseline values of age, race/ethnicity, smoking status, injection drug use, HCV status, HIV RNA, and CD4 percent, with and without further adjustment for baseline NK/NKT and CD8 values. NK/NKT and CD8 measured during follow-up visits were analyzed as time-dependent (longitudinal) variables using each measurement from the date obtained until the date at which the next NK/NKT or CD8 sample was obtained. Both unadjusted analyses and analyses adjusted for baseline age, race, and HCV status, and time-dependent variables for smoking status, injection drug use, CD4 percent, and HIV RNA were performed. Additional analyses further adjusted for time-dependent measures of NK/NKT and CD8 values. HIV RNA was also modeled as $\log_{10}$ of HIV RNA in place of HIV RNA copies/ml. All data were analyzed using SAS System for Windows, version 9.0, SAS Institute, Cary, NC.

RESULTS

Baseline demographic and laboratory measures for the women included in this study are shown in Table 1. A total of 52 incident cancers of 20 different types were identified, with non-Hodgkin's lymphoma being the most frequently reported ($n = 11$, 21.2%). Other cancer types included lung ($n = 8$), Kaposi's sarcoma ($n = 6$), and breast ($n = 5$). There were single cases of buccal cavity/pharynx, colon, connective tissue, glioblastoma, leukemia, multiple myeloma, ovarian, rectal, renal, and vaginal cancer. The median time from initial visit to cancer diagnosis was 33 months (interquartile range 16–55 months). Results were similar when the seven cases diagnosed before the second visit were excluded from the analyses. Cancer cases were more likely than noncases to be HCV seropositive (59% vs. 42%, $p = 0.02$). There was almost a 2-fold higher number of cancer cases when calculated as a percent of all HCV^+ versus HCV^- women in the study. There was no association between HCV seropositivity and any particular type of cancer (data not shown).

NK cell levels were reduced in HCV-infected patients compared to HCV-uninfected patients ($p < 0.0001$). NKT cell levels did not differ by HCV status. On multivariate Cox regression analysis (Table 2), women who were HCV positive at baseline were more likely to develop cancer (HR = 1.98, 95% CI = 1.03, 3.80). The same was true for those with higher baseline HIV viral load (HR = 1.04, 95% CI = 1.01, 1.08 per 10^5 copies/ml increase in viral load) and lower baseline $CD4^+$ (HR = 0.97, 95% CI = 0.94, 1.00 per percentage point increase in $CD4^+$ cells). The $CD8^+$, NK, and NKT percents measured at baseline were not related to cancer risk. However, when time-dependent values for NKT cells were used, higher levels of NKT cells were associated with a reduced risk of cancer (adjusted hazard ratio = 0.67, 95% CI = 0.50, 0.89 per percentage point increase). In the same analysis, time-dependent measures of NK cells and $CD8^+$ lymphocytes were not related to cancer risk. Results were similar when models were further adjusted for NK/NKT and CD8 values and when log (HIV RNA) was used in place of HIV RNA.

Table 1. Demographic and Baseline Laboratory Measures in the WIHS Analytic Cohort ($n = 1817$)[a]

Age (years)	
0–30	439 (24.2)
31–40	886 (48.8)
41–50	418 (23.0)
51+	74 (4.1)
Median	42.3 (36.5,41.6)
Race	
White (non-Hispanic)	331 (18.2)
African-American	1009 (55.5)
Latina	430 (23.7)
Other	447 (2.6)
Current smoking status	
No	801 (44.2)
Yes	1010 (55.8)
Current injection drug use	
No	1619 (89.5)
Yes	190 (10.5)
HCV positive	
No	1014 (57.6)
Yes	748 (42.4)
Antiretroviral therapy	
None	675 (37.2)
Mono	598 (33.0)
Combination	528 (29.1)
HAART	13 (0.7)
HIV RNA (copies/ml $\times 10^3$)	20 (4–93)
NK cells: $CD3^-/CD(16/56)^+$ (%)	5 (3,8)
NKT cells: $CD3^+CD(16/56)^+$ (%)	1 (0,2)
$CD4^+$ lymphocytes (%)	22.2 (14.0,30.2)
$CD8^+$ lymphocytes (%)	53.6 (44.9,61.6)

[a]Numbers in table are *n* (%) for categorical variables and median (interquartile range) for continuous variables.

DISCUSSION

In this report we present data on innate immunological factors associated with the development of cancer among women infected with HIV. Recent reports indicate that the host immune system plays a critical role not only in promoting host protection against cancer but also in selecting tumors that can better escape host immunosurveillance. The two main cell types thought to play a key role in the generation of antitumor immunity are the NK and NKT cells. The relationship between NK cell activity, as defined by lysis of K562 cells, and prevalent cancers was first reported by Kastelan *et al.*[18,19] and Norris *et al.*[20] Accumulating data suggest that NKT cells play a pivotal role in modulating antitumor responses.[21] Our data, demonstrate that time-dependent measures of NKT cell values adjusted for age and other covariates, were significantly associated with a reduced cancer risk. We found that immunophenotypically defined NKT cell levels measured at baseline were not predictive of cancer diagnosis. Only the time-dependent measurements showed a significant association, suggesting that the development of cancer is associated with more recent NKT cell levels rather than reflecting a long-term effect. Given the important immunoregulatory functions of these cells, they may play a critical role in HIV-1 pathogenesis as these cells also facilitate protective immune responses during bacterial[22–25] and viral infections.[26–31]

TABLE 2. MULTIVARIATE COX REGRESSION ANALYSIS: WIHS INCIDENT CANCERS

	Number of cancer cases in analyses (unadjusted/adjusted)	*Unadjusted hazard ratio (95% CI)*	*Unadjusted* p-*value*	*Adjusted hazard ratio (95% CI)*[a]	*Adjusted* p-*value*[a]
Baseline values					
Age	52/45	1.09 (1.06,1.12)	<0.01	1.07 (1.04,1.11)	<0.01
Race					
White	15/12	1.00		1.00	
African-American	24/20	0.51 (0.27,0.97)	0.04	0.48 (0.23, 0.99)	0.05
Latina	11/11	0.54 (0.25,1.17)	0.12	0.67 (0.29,1.54)	0.35
Other	2/2	0.92 (0.21,4.00)	0.91	1.37 (0.30,6.15)	0.68
Current smoking	52/45	0.86 (0.50,1.48)	0.58	0.90 (0.47,1.73)	0.75
Injection drug use	52/45	0.73 (0.26,2.03)	0.55	0.40 (0.12,1.39)	0.15
HCV	49/45	2.03 (1.15,3.60)	0.01	1.98 (1.03,3.80)	0.04
CD4 percent	51/45	0.97 (0.94, 0.99)	0.01	0.97 (0.94,1.00)	0.04
HIV RNA $\times 10^5$	49/45	1.05 (1.02,1.07)	<0.01	1.04 (1.01,1.08)	0.01
NK percent	52/45	1.04 (1.01,1.08)	<0.01	1.03 (0.99,1.07)	0.14
NKT percent	52/45	0.99 (0.89,1.11)	0.91	0.99 (0.87,1.12)	0.82
CD8 percent	51/45	1.02 (0.99,1.04)	0.14	0.99 (0.97,1.02)	0.50
NK/NKT/CD8 percents as time-dependent variables[b]					
NK percent	52/47	1.05 (1.02,1.08)	<0.01	1.02 (0.98,1.05)	0.43
NKT percent	52/47	0.68 (0.53,0.89)	<0.01	0.67 (0.50,0.89)	0.01
CD8 percent	51/47	1.02 (1.00, 1.04)	0.13	1.00 (0.98,1.03)	0.83

[a]Adjusted for baseline age, race, smoking, injection drug use, HCV, CD4 percent, and HIV RNA.
[b]Adjusted for smoking, injection drug use, CD4 percent, and HIV RNA as time-dependent variables; age, race, and HCV were fixed variables.

The finding that immunophenotypically defined NKT cell level is related to a subsequent diagnosis of cancer has not previously been reported in an HIV-infected population. Nonetheless, these findings are in agreement with the proposed role of NKT cells as an immunosurveillance mechanism involved in the control of cell and tissue growth.[22–25]

The mechanism by which NKT cells become depleted prior to cancer development is unknown. However, observations by others have suggested the possibility of redistribution of NKT cells from the periphery to the site of viral replication or tissue injury, and thus unavailable for other functions. NKT cells are found at higher levels in livers of HCV patients than in their peripheral blood.[32,33] Due to shared routes of transmission, HCV is common among HIV-infected women with a history of injection drug use.[14] Since many women in our cohort are coinfected with HCV, it is possible that our results reflect the sequestration of peripheral NKT cells in the liver, as has been suggested.[34–36] In this regard, Deignan and colleagues found that NKT cell numbers were reduced in the peripheral blood of HCV-infected compared to HCV-uninfected individuals.[37] Further, levels of NKT cells have been shown to be 20-fold higher in the livers of HCV-infected patients as compared to their levels in peripheral blood, whereas the ratio in HCV-uninfected individuals is 6.5:1.[26] It is thus possible that the consequence of NKT cell depletion during concomitant HIV-1 and HCV infections may be the loss of the NKT cell immunosurveillance function due to their depletion and/or sequestration at the site(s) of infection. In addition to these findings, an impairment of function and frequency of NKT[38] cells in HCV-infected individuals has also been described. Interestingly, and in contrast to NKT cells, it seems that a decreased number of peripheral NK cells in HCV and HIV coinfected patients is not due to accumulation in the liver but rather due to a common mechanism underlying the NK cell abnormalities in these two unrelated chronic virus infections.[39]

We observed that HCV status was associated with a diagnosis of cancer ($p = 0.01$ in unadjusted, $p = 0.04$ in adjusted analyses), even in the absence of any hepatic cancers in our cohort. The association of HCV with lymphoma has previously been described,[40,41] and lymphoma was the most common cancer diagnosed in the WIHS cohort. The possible association of HCV with other cancers has not been reported and will require additional study.

This study was limited by the fact that we were unable to study NK or NKT cell activity in our patients, relying upon cell numbers instead. Our data are limited to measurements of peripheral NK and NKT cell numbers. As we have no data about the dynamics of tissue-residing NKT cells, it is plausible that there is redistribution of NKT cells in those with solid tumors leading to lower levels of NKT cells in the peripheral blood. Also, our flow cytometry data obtained with a two-color panel may underestimate NK cells with a downregulated CD56 marker as well as NKT cells without a CD16 marker. However, several investigators use the same antibody panel to measure NK or NKT cells.[42–46]

Furthermore, although the WIHS cohort is large, we have had relatively few incident cancer cases in the cohort, limiting the power of our analyses and forcing us to consider all cancers together as our dependent variable. Nonetheless, we have shown a relationship between increased NKT cell numbers over time and a decreased risk of cancer, similar to the results obtained by others who have studied the function of these cells. Furthermore, this is the first such observation in a group of HIV-infected individuals.

In addition to the loss of $CD4^+$ lymphocytes and an increased risk of opportunistic infections, HIV-1–infected individuals may also experience further consequences of their immune dysfunction, such as eventual emergence of cancer in the setting of genetic alterations, and/or immune dysfunction, including that related to NK/NKT cell depletion. It will be important to elucidate the mechanism by which HIV-1 is associated with this important subset of T cells, which links innate and adaptive immune functions, and to determine its possible role in the development of AIDS-related infections or malignancies.

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