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Research Article

B-Cell Stimulatory Cytokines and Markers of Immune Activation Are Elevated Several Years Prior to the Diagnosis of Systemic AIDS-Associated Non-Hodgkin B-Cell Lymphoma

Elizabeth Crabb Breen^{1,2}, Shehnaz K. Hussain^{3,5}, Larry Magpantay^{2,3,4}, Lisa P. Jacobson⁶, Roger Detels⁵, Charles S. Rabkin⁹, Richard A. Kaslow¹⁰, Daina Variakojis¹¹, Jay H. Bream⁷, Charles R. Rinaldo¹², Richard F. Ambinder⁸, and Otoniel Martinez-Maza^{2,3,4,5}

Abstract

Background: The risk of developing non-Hodgkin lymphoma (NHL) is greatly increased in HIV infection. The aim of this study was to determine whether elevated serum levels of molecules associated with B-cell activation precede the diagnosis of AIDS-associated NHL (AIDS-NHL).

Methods: Serum levels of B-cell activation-associated molecules, interleukin (IL)6, IL10, soluble CD23 (sCD23), sCD27, sCD30, C-reactive protein (CRP), and immunoglobulin E were determined in 179 NHL cases and HIV+ controls in the Multicenter AIDS Cohort Study, collected at up to 3 time points per subject, 0 to 5 years prior to AIDS-NHL diagnosis.

Results: Serum IL6, IL10, CRP, sCD23, sCD27, and sCD30 levels were all significantly elevated in the AIDS-NHL group, when compared with HIV+ controls or with AIDS controls, after adjusting for CD4 T-cell number. Elevated serum levels of B-cell activation-associated molecules were seen to be associated with the development of systemic [non-CNS (central nervous system)] NHL, but not with the development of primary CNS lymphoma.

Conclusions: Levels of certain B-cell stimulatory cytokines and molecules associated with immune activation are elevated for several years preceding the diagnosis of systemic AIDS-NHL. This observation is consistent with the hypothesis that chronic B-cell activation contributes to the development of these hematologic malignancies.

Impact: Marked differences in serum levels of several molecules are seen for several years prediagnosis in those who eventually develop AIDS-NHL. Some of these molecules may serve as candidate biomarkers and provide valuable information to better define the etiology of NHL. *Cancer Epidemiol Biomarkers Prev*; 20(7): 1303–14. ©2011 AACR.

Introduction

The risk of developing B-cell non-Hodgkin lymphoma (NHL) is greatly increased in the setting of HIV infection (1–5). NHL is now the most common AIDS-related malignancy (6) and is a major cause of HIV infection-associated death in developed countries, where NHL accounts for 23% to 30% of AIDS-related causes of death (7–9). Although a decreased incidence of AIDS-associated NHL (AIDS-NHL) is associated with the use of highly active antiretroviral therapy (HAART), not all subtypes of AIDS-NHL have decreased in incidence in the HAART era (9–11). In fact, HAART may allow some HIV+ subjects who would have died of opportunistic infections to survive longer with continued immunosystem dysfunction, resulting in an increased opportunity for developing lymphoma over time (12). Also, some forms of AIDS-NHL occur relatively early in the course of HIV disease, so these lymphomas often arise prior to the initiation of HAART, or even prior to the recognition of HIV infection.

Authors' Affiliations: ¹Department of Psychiatry and Biobehavioral Sciences, ²UCLA AIDS Institute, ³Jonsson Comprehensive Cancer Center, and ⁴Departments of Obstetrics and Gynecology, and Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, and ⁵Department of Epidemiology, UCLA School of Public Health, Los Angeles, California; ⁶Departments of ⁶Epidemiology and ⁷Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, and ⁸Department of Oncology, Johns Hopkins School of Medicine, Baltimore, Maryland; ⁹Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, Maryland; ¹⁰Departments of Epidemiology, Medicine, Microbiology and Genetics, University of Alabama at Birmingham, Birmingham, Alabama; ¹¹Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, Illinois; ¹²University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania

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Corresponding Author: Elizabeth Crabb Breen, David Geffen School of Medicine at UCLA, 300 Medical Plaza, Room 3160A, Box 957076, Los Angeles, CA 90095-7076. Phone: 310-206-5738; Fax: 1-310-794-9247. E-mail: ebreen@mednet.ucla.edu

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There are several subtypes of AIDS-NHL, including Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), primary central nervous system lymphomas (PCNSL), and primary effusion lymphoma (13–16). These different AIDS-NHL subtypes differ in anatomical distribution, Epstein-Barr virus (EBV) or human herpesvirus type 8 infection of neoplastic cells, and in the frequency of various molecular lesions, including chromosomal translocations and mutations involving immunoglobulin (Ig) genes and/or oncogenes. Many AIDS-NHLs, including virtually all PCNSLs, are EBV-positive (EBV+) lymphomas (17). Loss of immunoregulatory control of EBV-infected B cells as a result of HIV infection-induced immunodeficiency is believed to contribute directly to the genesis of such EBV+ AIDS-NHL (5, 14). In fact, a study from the Amsterdam Cohort Study on AIDS showed that EBV-specific CTL decreased in 5 of 5 patients who went on to develop EBV+ AIDS-NHL, but not in the 2 subjects who developed EBV-negative lymphomas (18, 19). In the HAART era, the incidence of those forms of AIDS-NHLs that are typically EBV+ has decreased much more than that of those subtypes of AIDS-NHLs that are not necessarily EBV+ (10, 12, 14, 20), consistent with the view that these EBV+ tumors result from loss of EBV-specific T-cell function, which is restored by HAART.

In contrast, most AIDS-NHLs of the BL subtype are not EBV+, and are characterized by a chromosomal translocation that involves the rearrangement of the *c-MYC* oncogene (*MYC*) and the Ig heavy chain gene (*IgH*) switch region (14). The *MYC:IgH* rearrangement is believed to result from an error in *IgH* class switch recombination (CSR), a somatic DNA recombination event that occurs normally in activated B cells, which shifts the variable region from μ to another Ig heavy chain gene (γ , α , ϵ), and results in the production of antibodies of isotypes different from IgM. *IgH* CSR is driven by exposure to various B-cell stimulatory cytokines and is mediated by the activity of activation-induced cytidine deaminase (AICDA), a DNA modifying enzyme. Similarly, many AIDS-NHLs of the DLBCL subtype also are EBV-negative. These lymphomas are associated with the mutation, translocation, and/or overexpression of *BCL-6* and other oncogenes, and they tend to occur earlier in the course of HIV disease progression than PCNSL (15, 16, 21–25). The mutation of *BCL-6* and other NHL-associated oncogenes is believed to occur during the process of somatic hypermutation (SHM), another DNA-modifying event that occurs normally following B-cell activation, which results in the generation of antibodies that have enhanced binding affinity for antigen. SHM of Ig gene variable regions also is mediated by AICDA.

Therefore, at least 2 general types of pathogenetic mechanisms seem to contribute to the genesis of AIDS-NHL: (i) loss of immunoregulation of EBV-infected B cells, due to the loss of T-cell function that is associated with HIV infection, and (ii) chronic B-cell hyperactivation, which is associated with DNA-modifying events

(SHM and *IgH* CSR) that can contribute to lymphoma-genic molecular lesions (oncogene mutations/translocations; ref. 5).

HIV infection is characterized by chronic B-cell hyperactivation, driven by the overproduction of B-cell stimulatory cytokines such as interleukin (IL)6, as well as by chronic antigenic stimulation (4, 26, 27). It has been known for some time that IL6 levels are elevated prior to the development of AIDS-NHL (28). Also, high expressor genotypes for 2 B-cell stimulatory cytokines, SDF-1 and IL10, and for CCR5, a receptor for B-cell stimulatory chemokines, are associated with the development of AIDS-NHL (29–31). In our prior study examining a single time point close to but preceding the diagnosis of AIDS-NHL, HIV+ subjects who developed AIDS-NHL had especially elevated serum levels of several B-cell stimulatory molecules [IL6, IL10, CXCL13, and soluble CD23 (sCD23)] and molecules associated with immune activation (sCD27, sCD30, sCD44, and IgE), higher than the already elevated levels seen in HIV+ subjects who did not develop lymphoma (4, 29, 32–39). In addition, elevated expression of the *AICDA* gene (*AICDA*) in circulating lymphocytes was seen for several years preceding lymphoma diagnosis (40). However, little is known about the length of time preceding AIDS-NHL diagnosis that serum levels of these B-cell stimulatory molecules are elevated.

In this study, we sought to better define the longitudinal expression, preceding AIDS-NHL diagnosis, of B-cell stimulatory cytokines (IL6 and IL10), of a cytokine-like B-cell activation-associated molecule that is associated with the induction of *IgH* CSR (sCD23; refs. 41, 42), of an Ig isotype that indicates the activity of *IgH* CSR (IgE), of 2 molecules from the tumor necrosis factor (TNF) receptor superfamily that reflects immunosystem activation (sCD27 and sCD30; ref. 43), and of a molecule that is a marker for the activity of proinflammatory cytokines [C-reactive protein (CRP)]. This was done using up to 3 archival, prelymphoma diagnosis serum specimens from subjects in the Multi-center AIDS Cohort Study (MACS) who developed AIDS-NHL, as well as from appropriate MACS HIV-seropositive controls who had not developed AIDS-NHL. Elevated levels of several of these B-cell stimulation-associated molecules were seen for an extended period of time (more than 3 years) preceding the diagnosis of AIDS-NHL, especially in those who developed systemic (non-PCNSL) AIDS-NHL which would be expected to be associated with chronic B-cell hyperactivation.

Materials and Methods

Study design

This study is a nested case-control design, utilizing serum samples from participants in the MACS. MACS participants are homosexual men who have had study visits at 6 month intervals to examine the natural history of HIV infection and AIDS (44). Subjects were utilized from men who were enrolled in 1984 to 1985 or in 1987 to 1991. At each visit, detailed histories and blood samples

were obtained, and serum archived in a central repository. Clinical information from each visit was obtained from the participants and by review of external registries and confirmed by medical record review. The protocols and questionnaires utilized in the MACS have been approved by the institutional review board at each center.

Cases consisted of HIV-infected MACS subjects diagnosed with AIDS-NHL prior to April 2003, for whom at least 1 serum sample from a time point preceding AIDS-NHL diagnosis was available in the MACS repository, and a matched HIV-infected (HIV+) control could be identified as described next ($n = 179$). Longitudinal serum samples were obtained at up to 3 time points prior to NHL diagnosis: more than 3 years pre-NHL (closest to 4 years), 1 to 3 years pre-NHL (closest to 2 years), and 0 to 1 year pre-NHL (closest to 0.5 year). The median time between serum samples at each time point and NHL diagnosis was 4.0, 2.0, and 0.6 years, respectively.

For each case, all possible HIV+ controls were identified from among HIV-infected MACS subjects who had not developed lymphoma as of April 2003, matched on: (i) actual length of infection with HIV on the basis of known date of HIV seroconversion (21 cases), or date of entry ± 1 year into MACS as HIV seroprevalent (158 cases), and (ii) expected sample availability at equivalent time points ± 1 year. One unique HIV+ control was randomly selected for each case; the median time that cases and HIV+ controls had been HIV-infected or seroprevalent at the time of AIDS-NHL diagnosis in the case was 6.8 and 6.9 years, respectively. In the subset of 21 cases and matched controls with a known date of seroconversion, the median HIV+ time to AIDS-NHL was 6.7 years (range 2.7–10.4) and 6.4 years (range 2.3–10.3), respectively. The characteristics of all AIDS-NHL cases and HIV+ controls, as determined by the information obtained from the MACS database, are shown in Table 1. AIDS-NHL cases were stratified by primary tumor location as CNS (brain or other CNS site, $n = 58$) or systemic (all other sites, $n = 121$); systemic cases with known tumor subtype were further stratified into BL/BL-like ($n = 21$), DLBCL ($n = 61$), and other subtypes ($n = 3$).

In a subset of the AIDS-NHL cases ($n = 100$), NHL was diagnosed subsequent to another AIDS-defining condition, according to the 1993 definition of the Centers for Disease Control and Prevention, excluding cases defined by CD4 T-cell count alone (45). For this subset of cases, one unique AIDS control was randomly selected for each case from all MACS subjects with an AIDS diagnosis other than NHL, matched on: (i) year of AIDS diagnosis (± 1 year), (ii) post-AIDS survival time (i.e., survived NHL-free longer than the case following AIDS diagnosis), and (iii) expected sample availability at equivalent time points (± 1 year). The median time between the initial AIDS diagnosis and NHL diagnosis was 1.2 years. The characteristics of this subset of AIDS-NHL cases and AIDS controls are also shown in Table 1.

Determination of immune markers in serum samples

Serum levels of immune markers were assessed by ELISA. Assays for sCD23 (detection limit = 13 units/mL) and sCD30 (detection limit = 6 units/mL) were carried out according to the manufacturer's protocols (Bender MedSystems USA). sCD27 was determined using the PeliKine-compact ELISA Kit and Toolset according to the manufacturer's protocol (CLB/Sanquin), with 1:20 dilutions on all samples (detection limit = 32 units/mL, taking dilution into account). CRP was measured using the high-sensitivity protocol (detection limit = 0.25 $\mu\text{g/mL}$), as described by the manufacturer (Virgo CRP 150, Hemagen). IL6 was measured using an ultrasensitive assay (Biosource/Invitrogen) with color development time extended to 40 minutes to ensure consistent low-level detection (detection limit = 0.2 pg/mL). IL10 was measured using a human IL10-specific assay (Biosource/Invitrogen; detection limit = 2 pg/mL) that does not cross-react with viral IL10 of EBV, as previously described (29). Total serum IgE was determined utilizing the CIA-7.12 and CIA-4.15 monoclonal antibodies (46), as previously described (47), with the following modifications: plates were blocked with 10% fetal bovine serum, serum samples were diluted 1:10, and 50 μL per well of diluted sera and all subsequent reagents were incubated on a rotator (500 rpm). The IgE standard was pooled normal serum (generously provided by A. Saxon and Ke Zhang); when referenced to the World Health Organization IgE standard NIBSC 75/502 (which is also pooled human sera), the mean conversion factor was 0.67 ng per international unit (IU). By taking the dilution into account, the limit of detection was 8 ng/mL.

Data analysis

IL6, CRP, sCD23, sCD27, sCD30, and IgE data were natural log-transformed and treated as continuous variables, with results below the detection limit (value of the lowest standard on standard curve) set equal to half the value of the detection limit; univariate analyses were done using t tests. IL10 data were analyzed as binary variables (detectable IL10 ≥ 2 pg/mL vs. undetectable), using chi square statistic. Correlations were calculated separately for cases and controls at each time point for continuous variables using Spearman's correlation coefficients.

Absolute CD4 T-cell count (CD4 count) data were available from the MACS database and were used to control for possible differences in HIV disease progression. At each of the pre-NHL time points, age and CD4 count-adjusted conditional logistic regression models were generated to compare AIDS-NHL cases with matched HIV+ controls. ORs and 95% CIs were generated to estimate the risk of AIDS-NHL associated with one log-unit increase in concentration for continuous variables or detectable versus undetectable for IL10. Models were also generated adjusting for rate of change of CD4 count (CD4 slope, cells/ mm^3/year) or baseline HIV RNA viral load (VL log10 copies/mL, 18 months after first

Table 1. Characteristics of AIDS-NHL cases and controls

	All AIDS-NHL cases	HIV+ controls	AIDS-NHL subset with prior AIDS diagnosis	AIDS controls
<i>n</i>	179	179	100	100
Age at time of case NHL diagnosis, median years (range)	41 (24–60)	39 (24–60)	40 (24–57)	38 (23–68)
Ethnicity, <i>n</i> (%)				
White, non-Hispanic	149 (83)	156 (87)	81 (81)	87 (87)
White, Hispanic	19 (11)	8 (4)	11 (11)	6 (6)
Black, non-Hispanic	11 (6)	10 (6)	8 (8)	4 (4)
Other	0	5 (3)	0	3 (3)
CD4 T cells, median cells/mm ³ (range) ^a				
>3 years pre-NHL	387 (5–1349)	561 (3–2030)	369 (5–1227)	397 (19–1123)
1–3 years pre-NHL	209 (6–1178)	522 (37–1544)	144 (6–1178)	238 (9–1132)
0–1 year pre-NHL	74 (0–707)	468 (4–1255)	32 (0–691)	93 (0–1159)
HAART at the time of sample, <i>n</i> (%) ^a				
>3 years pre-NHL	1 (1)	0	1 (1)	0
1–3 years pre-NHL	3 (2)	4 (3)	1 (1)	1 (1)
0–1 year pre-NHL	5 (5)	5 (5)	1 (1)	2 (2)
Tumor subtype, <i>n</i> (%)				
Systemic NHL tumor	121 (68)		56 (56)	
Diffuse large B cell	61 (50)		27 (48)	
BL/BL-like	21 (17)		8 (14)	
Other subtypes	6 (5)		3 (5)	
Not specified	33 (27)		18 (32)	
CNS NHL tumor	58 (32)		44 (44)	
Tumor EBV status, <i>n</i> (%)				
Not tested	92 (51)		44 (44)	
Tested	87 (49)		56 (56)	
Negative	28 (32)		16 (29)	
Positive	59 (68)		40 (71)	

^aCases and controls who had serum available for that study visit.

HIV-seropositive MACS study visit; refs. 48, 49), which included fewer case-control pairs due to missing data, especially for VL (data not shown). Age- and CD4 count-adjusted models were further tested with stratification by systemic versus CNS AIDS-NHL, and by NHL subtype (DLBCL and BL) among systemic AIDS-NHLs.

Similar models were generated to compare the subset of AIDS-NHL cases with prior AIDS to AIDS controls, adjusted only for age because they were matched on the basis of progression to AIDS. An additional model adjusted for age and CD4 count did not meaningfully affect the OR estimates (data not shown).

Results

Increased serum levels of multiple immune biomarkers are seen up to 3 or more years prior to AIDS-NHL diagnosis

Univariate analyses of AIDS-NHL cases compared with HIV+ controls, without regards to matching, showed significantly higher mean serum levels of IL6,

CRP, sCD27, and sCD30 in cases at all 3 time points (>3 years, 1–3 years, and 0–1 year) prior to lymphoma diagnosis (Fig. 1A–D, $P \leq 0.002$). sCD23, a soluble form of the low-affinity cell surface Fc receptor for IgE, which has B-cell stimulatory properties, showed elevated serum levels in cases, but was only statistically significantly elevated >3 years prior to lymphoma diagnosis (Fig. 2A, $P < 0.001$). Serum levels of IgE showed no significant differences between AIDS-NHL cases and HIV+ controls at any time point (Fig. 2B). As expected from previous study on subjects from this same cohort study (29), the majority of all HIV+ subjects had undetectable levels (<2 pg/mL) of serum IL10. However, consistent with those prior observations, the frequency of detectable serum levels of IL10 (≥ 2 pg/mL) was clearly and significantly elevated in AIDS-NHL (Fig. 2C), but only at the time point immediately preceding lymphoma diagnosis (0–1 years prior, $P < 0.001$).

CRP, which is produced by hepatocytes in response to IL6 and can be considered a marker for the bioactivity of IL6 (50), was significantly correlated with IL6 at all time

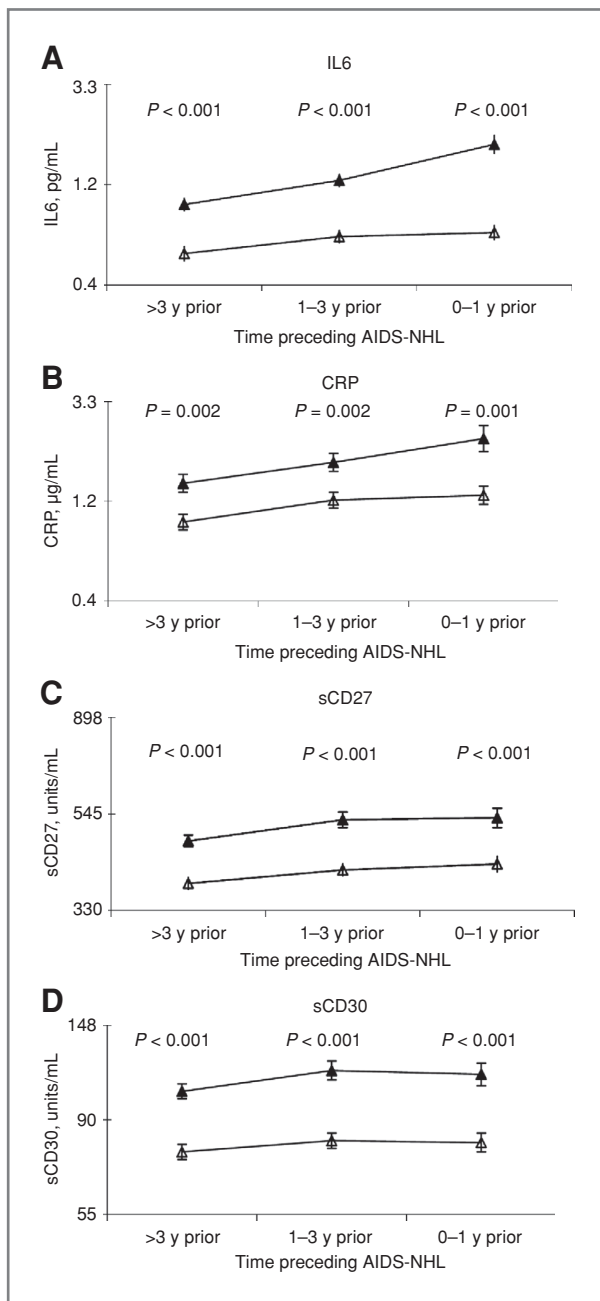


Figure 1. Mean serum levels of several B-cell activation-associated molecules are consistently elevated more than 3 years preceding AIDS-associated lymphoma diagnosis. Natural log-transformed mean values ($\pm$ SEM) for HIV-infected subjects who went on to develop AIDS-NHL (filled triangles) and HIV-infected controls without lymphoma (open triangles) for (A) IL6, (B) C'-reactive protein (CRP), (C) sCD27, and (D) sCD30 in the years prior to NHL diagnosis in the case; *P* values (univariate *t* test) are shown only if less than 0.05.

points ($r = 0.37$ – 0.48 , Supplementary Table S1). sCD27 and sCD30, which are both members of the TNF receptor superfamily, showed strong correlations to each other at all time points ($r = 0.55$ – 0.75), as well as to sCD23

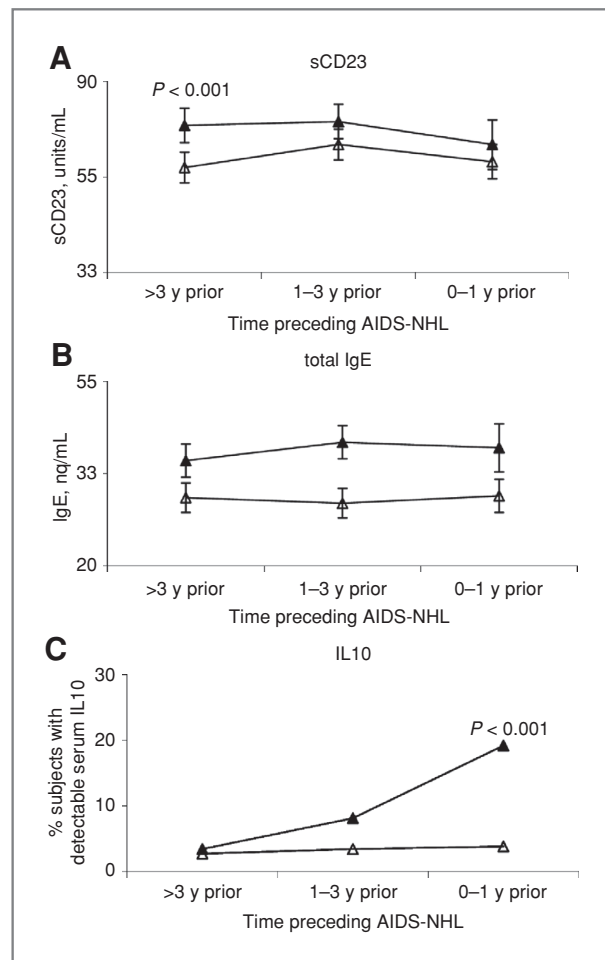


Figure 2. Mean serum levels of additional B-cell activation-associated molecules may be elevated prior to lymphoma. Natural log-transformed mean values ($\pm$ SEM) for (A) sCD23, (B) total IgE, and (C) percentage of samples with detectable (≥ 2 pg/mL) serum IL10 are shown for all AIDS-NHL subjects (filled triangles) and HIV+ controls (open triangles); *P* values for continuous variables (univariate *t* test) and frequency of detectable IL10 (chi square) are shown only if less than 0.05.

($r = 0.28$ – 0.46); sCD23 showed no correlation with IgE (Supplementary Table S1).

Certain immune biomarkers are increased prior to NHL, even when diagnosis of lymphoma occurs after some other AIDS-defining condition

Among the 179 subjects who developed AIDS-NHL, 100 subjects were diagnosed with lymphoma after previously being diagnosed with another AIDS-defining condition. These cases were compared with a second group of HIV-infected controls who also had AIDS, matched by time of first AIDS diagnosis (AIDS controls).

There were no significant differences between NHL cases with prior AIDS and AIDS controls in serum levels of CRP, sCD23, or IgE at any time point pre-lymphoma (data not shown). At the time point furthest

from lymphoma diagnosis (>3 years prior), there were no significant differences between AIDS-NHL cases and AIDS controls in mean serum levels of IL6, sCD27, sCD30, or IL10 (Fig. 3). However, at 1 to 3 years before lymphoma diagnosis, mean IL6 levels rose in the AIDS-NHL group, but stayed relatively stable in the AIDS control group, resulting in a significant difference (Fig. 3A). Both cases and controls showed higher mean IL6 levels 0 to 1 year before lymphoma and so did not differ significantly at that time point. In contrast, mean serum levels of sCD27 (Fig. 3B) and sCD30 (Fig. 3C) were relatively stable in the AIDS-NHL cases over the time approaching lymphoma diagnosis, whereas AIDS controls showed declining mean levels over time, leading to significant differences at the time closest to lymphoma. For IL10, NHL cases with prior AIDS (Fig. 3D) were similar to AIDS-NHL cases overall (Fig. 2C), with increased frequency of detectable IL10 seen only at 0 to 1 year prior to lymphoma diagnosis.

AIDS-NHL risk is significantly associated with increased prediagnosis levels of serum IL6, sCD27, sCD30, and IL10, even when taking differences in CD4 T-cell numbers into account

To discriminate between effects associated with the development of NHL and those associated with the progression of HIV disease over time, all AIDS-NHL cases and HIV+ controls were matched based on duration of HIV infection. After matching, cases and controls differed in their absolute CD4 T helper cell counts, especially as the cases approached the time of lymphoma diagnosis (Table 1). In age-adjusted analyses, a decrease of 50 cells/mm³ in CD4 count was associated with an increased risk of NHL at >3 years (OR 1.15, 95% CI = 1.08–1.22), 1 to 3 years (OR 1.42, 95% CI = 1.26–1.60), or 0 to 1 year (OR 1.45, 95% CI = 1.26–1.67) prior to NHL diagnosis. Independent of NHL risk, CD4 counts are an important indicator of immune system status in the context of HIV disease and may impact circulating immune biomarker levels. Therefore, to adjust for this potential confounder, logistic regression analyses for lymphoma risk in association with increased serum levels of immune biomarkers were done controlling for CD4 counts at the time of each sample.

For all AIDS-NHL cases compared with HIV+ controls, increased serum levels of IL6, sCD27, and sCD30 remained statistically significant at all 3 time points after CD4 count adjustment (Table 2, upper half). Interestingly, in spite of the significant correlation seen between IL6 and CRP levels, adjusted ORs for CRP were no longer statistically significant at the time point closest to lymphoma diagnosis (where the CD4 differences were greatest and the sample size was smallest). Consistent with the univariate analyses, sCD23 levels were significantly increased only at the time furthest from lymphoma diagnosis, frequency of detectable IL10 was significantly increased only at 0 to 1 year prior to lymphoma, and

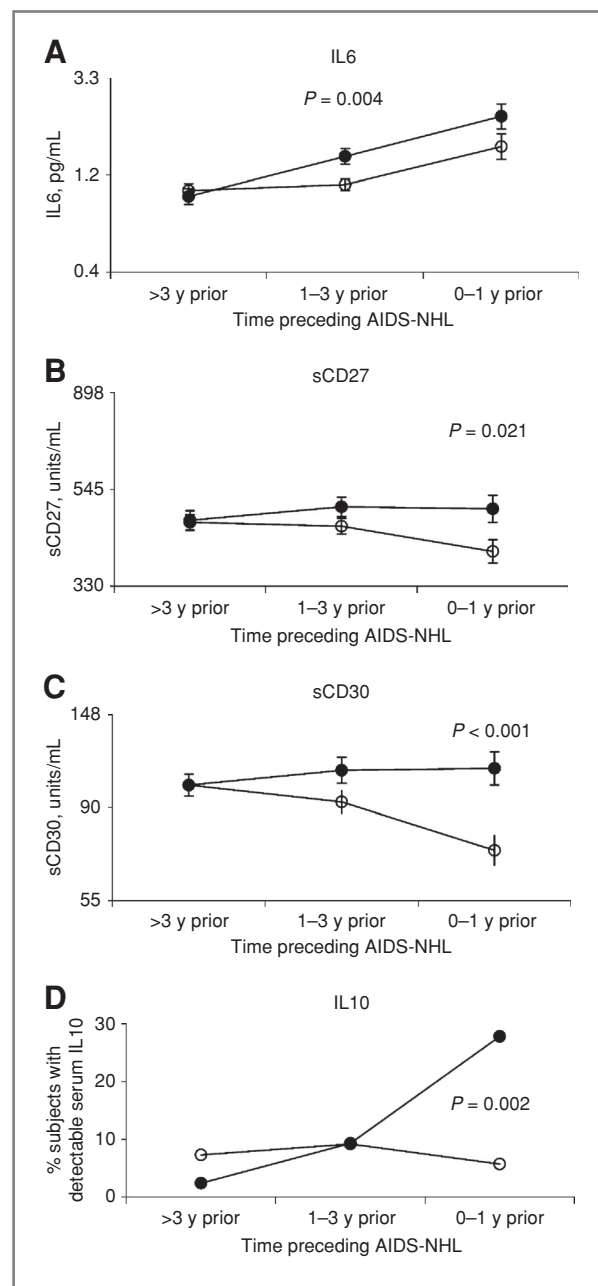


Figure 3. Some B-cell activation markers are elevated prior to lymphoma in those AIDS-NHL cases that had another AIDS-defining condition prior to NHL. Natural log-transformed mean values ($\pm$ SEM) for (A) IL6, (B) sCD27, (C) sCD30, and (D) percentage of subjects with detectable serum IL10 are shown for the subset of AIDS-NHL subjects ($n = 100$) who had a primary AIDS diagnosis other than lymphoma, then went on to develop NHL (filled circles), and controls who had an AIDS diagnosis but did not develop lymphoma (open circles); P values for continuous variables (univariate t test) and frequency of detectable IL10 (chi square) are shown only if less than 0.05.

IgE levels were not significantly increased at any time point preceding lymphoma diagnosis.

To confirm that the observed associations between AIDS-NHL and increased serum levels of IL6, sCD27,

Table 2. ORs and 95% CIs for increased cytokine concentrations in NHL cases

	>3 years prior			1–3 years prior			0–1 year prior		
	<i>n</i> , sets ^a	OR ^b	95% CI	<i>n</i> , sets ^a	OR ^b	95% CI	<i>n</i> , sets ^a	OR ^b	95% CI
All NHL cases vs. HIV+ controls ^c									
IL6	132	2.37	1.51–3.72	129	4.14	1.81–9.49	88	6.01	2.07–17.45
CRP	141	1.65	1.25–2.18	139	1.55	1.08–2.22	100	1.31	0.84–2.07
sCD27	141	3.72	1.68–8.25	139	6.98	2.23–21.83	100	3.81	1.21–11.96
sCD30	141	3.22	1.75–5.91	139	8.64	3.09–24.14	101	4.77	1.63–13.90
sCD23	136	2.02	1.17–3.47	136	1.74	0.93–3.26	99	1.37	0.78–2.42
IgE	141	1.08	0.88–1.31	139	0.99	0.78–1.25	100	0.98	0.76–1.27
IL10	141	0.78	0.17–3.64	139	1.26	0.28–5.57	100	7.74	1.06–56.59
NHL after other AIDS diagnosis vs. AIDS controls ^d									
IL6	79	1.00	0.60–1.69	81	2.08	1.13–3.86	49	1.47	0.90–2.40
CRP	82	1.34	0.94–1.92	86	1.34	0.98–1.85	54	1.03	0.77–1.38
sCD27	82	1.04	0.45–2.40	86	1.79	0.82–3.93	54	2.47	1.02–5.96
sCD30	82	0.93	0.51–1.68	86	2.00	1.02–3.91	54	4.85	1.92–12.28
sCD23	82	1.30	0.75–2.28	86	1.34	0.81–2.21	53	1.31	0.77–2.24
IgE	82	1.23	0.99–1.55	86	1.13	0.95–1.36	54	1.12	0.91–1.39
IL10	82	0.33	0.07–1.65	86	1.03	0.35–2.97	54	9.08	1.87–44.03

^aMatched case-control sets.^bFor all markers except IL10, ORs are in terms of 1 unit increase in natural log-transformed values; for IL10, ORs are in terms of detectable versus undetectable. Statistically significant ORs are shown in bold.^cSets matched on duration of HIV infection, and analyses adjusted for age and CD4 T cell count.^dSets matched on time of initial AIDS diagnosis, and analyses adjusted for age.

sCD30, and IL10 were not merely a reflection of HIV disease progression, immune biomarker analyses were repeated controlling for age plus other prognostic indicators of HIV disease: rate of change of CD4 count (CD4 slope) or baseline HIV VL (48, 49). Increases in IL6, sCD27, and sCD30 levels remained significantly associated with increased risk of AIDS-NHL at all time points in both analyses (data not shown). Detectable IL10 remained significant at 0 to 1 year before lymphoma diagnosis with CD4 slope adjustment ($n = 93$ case-control sets, OR 7.40, 95% CI = 1.91–28.60), but lost significance when adjusted for VL, which may reflect instability in the estimates due to a reduced number of case-control sets ($n = 75$, OR 4.76, 95% CI = 0.86–26.27). In age-adjusted analyses, a decrease in CD4 slope of 50 cells/mm³/year (OR 1.55, 95% CI = 1.23–1.94) or an increase in baseline VL of 1 log₁₀ copies/mL (OR 6.31, 95% CI = 3.13–12.72) was independently associated with an increased risk of AIDS-NHL.

Logistic regression analyses were also done in the subset of NHL cases with prior AIDS and AIDS controls (Table 2, lower half). ORs were similar to the results obtained in univariate analyses, with significantly elevated ORs seen at 1 to 3 years before lymphoma for IL6, and at 0 to 1 year for sCD27, sCD30, and IL10. In addition, sCD30 had an OR of borderline significance at 1 to 3 years prelymphoma.

Elevated serum levels of immune biomarkers were seen to be associated with the development of systemic AIDS-NHL, but not with the development of PCNS AIDS-NHL

As compared with similar forms of NHL in the non-HIV-infected population, primary tumors in AIDS-NHL are more likely to occur at sites other than lymph nodes (1, 12). In HIV-infected subjects with advanced HIV disease and low CD4 counts, a notable proportion of AIDS-NHL cases presents with PCNSL (28, 51). Among the 179 cases of AIDS-NHL in this study, 58 of 179 (32%) of the primary tumors were located within the CNS, whereas the remaining 68% of cases presented with primary tumors outside the CNS (systemic). The AIDS-NHL cases were stratified on the basis of primary tumor location, and CD4 count-adjusted logistic regression analyses were performed by comparing their matched HIV+ controls.

The stratification of AIDS-NHL cases according to CNS or systemic primary tumor location revealed stronger associations of elevated levels of biomarkers with the risk of systemic lymphoma (Fig. 4). Detectable IL10 could not be evaluated at 0 to 1 year and 3 to 5 years prior to PCNSL due to sparse data, and was not significantly associated with PCNSL at 1 to 3 years prior to diagnosis. Although elevated ORs were seen for several of the markers other than IL10, especially at >3 years

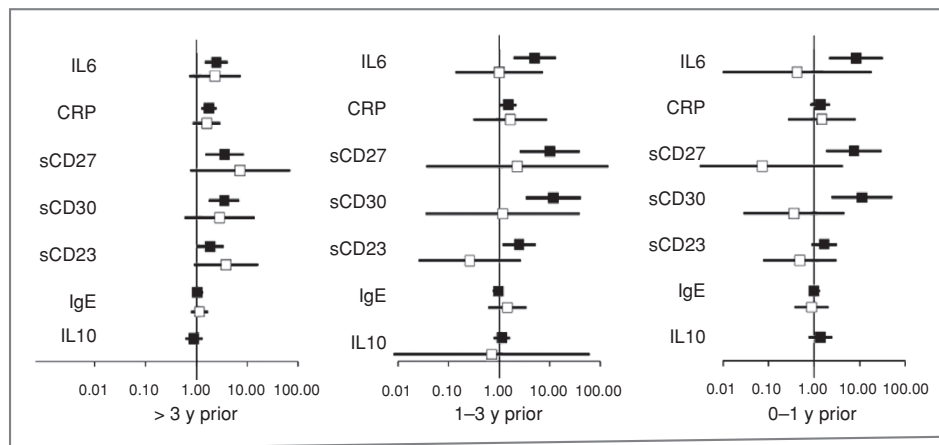


Figure 4. Elevated levels of B-cell activation markers prior to AIDS-NHL are seen only in those subjects who develop systemic lymphoma. CD4-adjusted ORs $\pm$ 95% CI for increased serum cytokine levels are shown for AIDS-NHL cases compared with matched HIV+ controls, stratified according to primary tumor location outside of the CNS (systemic, filled squares) or within the CNS (open squares). For all markers except IL10, ORs are in terms of 1 unit increase in natural log-transformed values; for IL10, ORs are in terms of detectable versus undetectable. ORs are missing for IL10 for CNS tumors at 2 time points (>3 years and 0-1 year) due to failure of the logistic regression model to converge on account of sparse data.

pre-PCNSL, no statistical significant association was seen at any time point prior to PCNSL. The systemic NHL cases recapitulated the results seen in the AIDS-NHL cases overall, with elevated serum levels of IL6, sCD27, and sCD30 significantly associated with AIDS-NHL at all time points preceding lymphoma diagnosis, and CRP at all but 0 to 1 year prelymphoma. Interestingly, elevated sCD23 levels, which were significantly associated only at >3 years prior to AIDS-NHL cases overall, emerged in the analyses of systemic AIDS-NHL cases as also significantly associated at 1 to 3 years prior to lymphoma diagnosis. In contrast, detectable IL10 was no longer significantly associated at the time point closest to systemic NHL diagnosis. No significant association was seen at any time point between serum IgE levels and systemic AIDS-NHL.

The systemic AIDS-NHL cases were further stratified to determine if there was a pattern of elevated serum biomarkers associated with either of the major subtypes, DLBCL ($n = 61$) and BL ($n = 21$). In a univariate comparison of mean levels between DLBCL and BL cases, the only significant difference was higher levels of sCD23 at 1 to 3 years preceding the diagnosis of BL ($P = 0.02$, data not shown). In CD4 count-adjusted logistic regression models comparing DLBCL or BL cases with matched HIV+ controls (Supplementary Table S2), some markers and time points could not be evaluated due to small sample size. Although there were elevated ORs for many markers in both the DLBCL and BL cases, statistically significant associations were seen only with elevated levels of IL6, sCD27, and sCD30 more than 3 years and/or 1 to 3 years prior to DLBCL. It is likely that the lack of any significant association for any markers or time points with BL is due at least in part to the small number of cases of this subtype.

Discussion

In this study, we have examined the longitudinal levels of several serum molecules associated with B-cell and/or immune system activation, over a period of several years preceding AIDS-NHL diagnosis. These included 2 B-cell stimulatory cytokines, IL6 and IL10, and sCD23, a soluble Fc receptor for IgE that has cytokine-like B-cell stimulatory properties (41, 42). In addition, we assessed serum levels of IgE, which is induced by Th2 cytokines, as well as serum levels of sCD27 and sCD30, 2 TNF receptor superfamily molecules that reflect immune system activation (43), and CRP, a marker for inflammation, the production of which is driven by proinflammatory cytokines.

Elevated serum levels of IL6, CRP, sCD27, and sCD30 were consistently seen at all time points tested preceding lymphoma diagnosis, when comparing all AIDS-NHL cases ($n = 179$) with HIV+ controls (Fig. 1). Even after adjustment for CD4 T-cell count (Table 2), CD4 slope, or HIV VL, to control for differences possibly related to HIV disease progression rather than AIDS-NHL, an increased association clearly persisted between the development of AIDS-NHL and elevated serum levels at all time points for IL6, sCD27, and sCD30. Increased frequency of detectable IL10 was also seen preceding AIDS-NHL cases both with and without CD4 adjustment, but only at the time closest to lymphoma diagnosis. With CD4 adjustment, CRP was no longer significant at 0 to 1 year prior to NHL, which may reflect an increasing contribution of more general HIV disease-related inflammation obscuring the earlier relationship with development of AIDS-NHL and/or may be due to the smaller number of samples available closest to lymphoma diagnosis. Similarly, elevated serum levels of sCD23 only at >3 years

prelymphoma may suggest increasing levels in HIV+ controls with time obscure earlier differences. IgE showed no significant association with AIDS-NHL in any analysis, in contrast to an early report (36), but consistent with later studies (38), which is likely due to differences in study design and differences in the immunoassays utilized. Hence, even when taking into account lower CD4 counts (or more rapid CD4 decline or higher baseline VL) over the same length of time of HIV infection, our data indicated the presence of a sustained environment of B-cell and immunosystem hyperactivation for more than 3 years in those subjects who went on to develop AIDS-NHL.

A little more than half of the AIDS-NHL cases in the overall analyses (100 of 179) were diagnosed with lymphoma after some other AIDS-defining condition. We compared this subset of cases with an independent set of controls matched on time of AIDS diagnosis, and saw elevated serum levels of IL6, sCD27, and sCD30, but only at 0 to 1 and/or 1 to 3 years prior to lymphoma diagnosis. This suggests that although a prior AIDS-defining condition may disrupt an earlier prelymphoma B-cell stimulatory environment and/or confound our ability to detect it, evidence of increased immune activation does emerge, especially in the year immediately preceding AIDS-NHL diagnosis. In light of differences seen between systemic and CNS AIDS-NHL cases (as discussed shortly), less immune activation might also be attributable at least in part to the fact that PCNSL occurred in a higher proportion of post-AIDS diagnosis cases (44%) than in the overall AIDS-NHL cases (32%).

IL6 is a pluripotent cytokine that can stimulate B-cell proliferation and differentiation and promote cell survival and tumor growth (52–55). IL6 production is driven by multiple stimuli, including exposure to lipopolysaccharide or HIV virions (56–58). IL6 is also associated with proinflammatory responses, as well as with Th17 responses, which are associated with autoimmunity (59, 60). Interestingly, those forms of autoimmunity that involve B-cell activation and are mediated by autoantibody production have been seen to be associated with an enhanced risk for the development of B-cell NHL (61). Previous cross-sectional studies have documented elevated IL6 preceding AIDS-NHL (28, 33). The observation that elevated serum levels of IL6 are repeatedly associated with AIDS-NHL for more than 3 years prior to diagnosis is consistent for a role for IL6-driven B-cell stimulation in the development and/or growth of these lymphomas.

CD30 and CD27 are the receptors for CD30 ligand (CD153) and CD70, respectively, which are TNF-like immunostimulatory molecules (62–65). CD30 is characteristically expressed on HL Reed–Sternberg cells, but is rarely expressed by NHL cells. sCD30 is produced by activated T cells and may be a marker for Th2 immune responses that support B-cell activation and differentiation into antibody-secreting plasma cells (66). CD27 is expressed on the surface of B cells following activation and is a marker for memory B cells (67–70). In prior cross-

sectional studies, we noted strong correlations between these 2 related molecules and have reported elevated serum levels of both sCD30 and sCD27 preceding the diagnosis of AIDS-NHL (32, 35). Like IL6, the consistent elevation of these molecules for more than 3 years prelymphoma provides strong evidence for the role of sustained B-cell activation in the development of AIDS-NHL. Elevated serum sCD30 has also been reported preceding the diagnosis of non-AIDS-NHL (71), suggesting that an environment rich in B-cell activation may be a feature that precedes many B-cell lymphomas, regardless of HIV status.

IL10 is a B-cell stimulatory cytokine that can also suppress Th1 responses, including antiviral cell-mediated responses (72, 73). Our observations of IL10 in this study are consistent with our previous study, in which we found elevated serum levels of IL10 immediately preceding AIDS-NHL, as well as an association between IL10 genotype and the development of AIDS-NHL (29, 74). An elevated frequency of detectable IL10 only at the time point closest to lymphoma diagnosis (<1 year pre-NHL) suggests that this cytokine is either tumor-produced, consistent with a report that IL10 is an autologous growth factor for AIDS-NHL (75), and/or is produced by, or in response to, the inflammatory microenvironment of the tumor.

When the results from those who developed PCNSL were compared with those who had systemic non-CNS NHL, it was seen that significantly elevated serum levels of B-cell activation-associated molecules were seen only with the development of systemic lymphomas. This suggests that elevated serum levels of molecules associated with B-cell activation are not associated with an increased risk for the development of PCNSL, which is characterized by EBV infection of tumor cells. Rather, elevated levels of B-cell activation-associated molecules were seen preceding the development of those forms of AIDS-NHLs (systemic AIDS-NHL, including BL and DLBCL) that contain molecular lesions believed to result from errors in B-cell activation-associated DNA-modifying events. These findings are consistent with the view that EBV+ PCNSL result primarily from loss of effective immunoregulation of EBV infection, whereas systemic lymphomas, which are often EBV-negative tumors, are associated with chronic B-cell activation over a prolonged period of time, resulting in the accumulation of genetic lesions due to *IgH* CSR or SHM (5, 76). The recent observation that elevated levels of *AICDA* expression occur pre-AIDS-NHL, but only in those who developed non-CNS lymphomas, is consistent with this conclusion (39). *AICDA* is a B-cell activation-associated DNA-mutating molecule that plays a central role in both *IgH* CSR and SHM in germinal center B cells (77). Interestingly, *AICDA* expression can be induced in B cells by exposure to HIV (78) and other lymphomagenic viruses, including EBV and HCV (79–81), suggesting a direct role for HIV in the induction of B-cell activation. Studies to quantify *AICDA* expression and its association with HIV

levels in the same cases and controls included in this study are planned.

The assessment of additional cytokines and immunostimulatory molecules other than those that were quantified in this study would have been informative. However, we focused on those molecules for which we had compelling preliminary evidence in prior preliminary cross-sectional studies, and for which robust immunoassays were available, given the limited volume of pre-NHL diagnosis serum that is available. As multiplexed immunoassays are becoming more sensitive and reliable, we hope to assess additional biomarkers in the future.

In summary, levels of B-cell stimulatory cytokines and molecules associated with immune activation are elevated several years preceding the diagnosis of AIDS-NHL. These results are consistent with the hypothesis that chronic B-cell hyperactivation contributes to the development of these lymphomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Table 1: Correlations^a between serum levels of immune markers in all NHL cases and in HIV+ controls^b

>3 years pre-NHL (controls n=141; cases n=142)						
	IL6	CRP	sCD27	sCD30	sCD23	IgE
IL6	1	0.37*	0.40*	0.26*	0.22*	-0.01
CRP	0.48*	1	0.18*	0.15	0.09	0.03
sCD27	0.30*	0.05	1	0.75*	0.41*	-0.04
sCD30	0.25*	0.10	0.64*	1	0.34*	0.01
sCD23	0.13	-0.004	0.41*	0.45*	1	-0.13
IgE	0.05	0.25*	-0.09	-0.14	-0.11	1
1-3 years pre-NHL (controls n=144; cases n=139)						
	IL6	CRP	sCD27	sCD30	sCD23	IgE
IL6	1	0.41*	0.22*	0.07	-0.11	0.06
CRP	0.43*	1	0.06	-0.05	-0.07	-0.03
sCD27	0.41*	0.01	1	0.70*	0.34*	-0.03
sCD30	0.42*	0.16	0.61*	1	0.44*	-0.11
sCD23	0.25*	0.07	0.37*	0.43*	1	0.04
IgE	-0.04	0.13	-0.03	-0.08	0.07	1
0-1 year pre-NHL (controls n=104; cases n=91)						
	IL6	CRP	sCD27	sCD30	sCD23	IgE
IL6	1	0.40*	0.27*	0.27*	-0.07	0.10
CRP	0.39*	1	0.03	0.25*	-0.02	0.02
sCD27	0.31*	0.16	1	0.69*	0.28*	0.07
sCD30	0.18	0.21*	0.55*	1	0.42*	0.04
sCD23	0.08	0.29*	0.28*	0.46*	1	-0.01
IgE	0.05	0.22*	0.09	-0.06	0.05	1

a: Spearman's correlation coefficients; p value <0.05 indicated by *

b: data above the shaded cells are from NHL cases, below the shaded cells are from HIV+ controls

Supplemental Table 2: Odds Ratios (OR) and 95% confidence intervals (CI) for increased cytokine concentrations in systemic diffuse large B cell lymphoma (DLBCL) or Burkitt lymphoma/Burkitt-like lymphoma (BL) cases

	>3 years pre NHL			1-3 years pre NHL			0-1 year pre NHL		
	N, sets ^a	OR ^b	95% CI	N, sets	OR	95% CI	N, sets	OR	95% CI
	all diffuse large B cell lymphoma (DLBCL) cases vs. HIV+ controls								
IL6	45	3.08	1.31 - 7.21	47	1.29	0.32 - 5.11	unable to estimate ^c		
CRP	48	1.40	0.91 - 2.15	51	1.10	0.63 - 1.92	41	2.23	0.72 - 6.92
sCD27	48	4.04	0.90 - 18.17	51	32.5	1.29 - 819.0	41	15.56	0.98 - 247.74
sCD30	48	4.32	1.36 - 13.8	51	21.6	1.53 - 304.9	unable to estimate		
sCD23	47	2.41	0.85 - 6.82	49	3.16	0.65 - 15.21	39	1.86	0.77 - 4.45
IgE	48	1.06	0.76 - 1.48	51	0.90	0.58 - 1.38	41	1.17	0.77 - 1.78
IL10	48	0.81	0.50 - 1.30	51	1.12	0.66 - 1.89	41	1.46	0.55 - 3.88
	all Burkitt lymphoma (BL)/BL-like cases vs. HIV+ controls								
IL6	12	1.58	0.48 - 5.25	unable to estimate			10	13.99	0.37 - 529.86
CRP	14	1.27	0.43 - 3.69	14	2.61	0.93 - 7.33	13	1.19	0.56 - 2.53
sCD27	14	8.40	0.67 - 106.13	14	5.95	0.56 - 63.02	13	2.76	0.45 - 16.91
sCD30	14	19.47	0.79 - 477.83	unable to estimate			13	2.72	0.56 - 13.17
sCD23	13	2.14	0.51 - 9.08	14	2.88	0.69 - 11.91	13	2.49	0.61 - 10.19
IgE	14	0.87	0.43 - 1.75	14	1.12	0.61 - 2.03	13	0.41	0.15 - 1.16
IL10	unable to estimate			unable to estimate			13	1.21	0.38 - 3.85

a: matched case-control sets

b: for all markers except IL10 ORs are in terms of one unit increase in natural log-transformed values; for IL10, ORs are in terms of detectable vs. undetectable.

Statistically significant ORs are shown in bold.

c: unable to estimate ORs and CI due to sparse data