Markers of microbial translocation and risk of AIDS-related lymphoma

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Background: Depletion of gut-associated lymphocytes by HIV infection facilitates microbial translocation, which may contribute to non-Hodgkin lymphoma (NHL) risk via chronic immune activation and B-cell hyperstimulation.

Method: We therefore examined associations of four microbial translocation markers with subsequent NHL risk in a case–control study nested within four prospective cohort studies of HIV-infected individuals. Prediagnostic blood specimens for 56 NHL cases and 190 controls matched for age, sex, race, specimen type, cohort, and $CD4^+$ T-cell count were tested for the endotoxin lipopolysaccharide (LPS), anti-endotoxin core antibody (EndoCab), LPS-binding protein (LBP), and soluble CD14 (sCD14).

Results: Elevated levels of sCD14 were associated with significantly increased NHL risk [odds ratio (OR) 2.72 (95% confidence interval [95% CI] 1.29–5.76)]. In subgroup analyses, elevated LPS levels were also associated with significantly increased NHL risk [OR 3.24 (95% CI 1.10–9.53)]. EndoCab and LBP levels were not associated with NHL risk.

Conclusion: The association of sCD14 and LPS with NHL risk supports an etiologic role for gut microbial translocation in lymphomagenesis among HIV-infected individuals. Additional studies with larger sample sizes are needed to confirm these observations.

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Introduction

Individuals with uncontrolled HIV infection are at markedly elevated risk of multiple subtypes of non-Hodgkin lymphoma (NHL), including diffuse large B-cell lymphoma (DLBCL), primary central nervous system lymphoma (PCNSL), and Burkitt lymphoma [1]. This increased risk is believed to arise from HIV-related immunosuppression as well as loss of control of Epstein–Barr virus (EBV) replication. Indeed, NHL risk increases

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with declining CD4 T-cell counts and EBV is detected in all PCNSL and a substantial fraction of DLBCL [2,3]. Nevertheless, low CD4 cell count does not explain all of the excess NHL risk among HIV-infected individuals. Despite substantial increases in CD4 cell count and declines in NHL incidence since the introduction of HAART, NHL risk remains significantly elevated among HIV-infected individuals as compared with the general population [3,4].

Chronic immune activation and B-cell hyperstimulation also contribute to NHL risk, and elevated circulating levels of immune activation markers, such as IL-6, soluble CD27 (sCD27), sCD30, sCD44, and immunoglobulin light chains are associated with increased risk among HIV-infected individuals [5–8]. However, the causes of HIV-related immune activation are unclear. A potential source of immune activation among HIV-infected individuals is gut microbial translocation, the leakage of intestinal bacteria and bacterial products such as lipopolysaccharide (LPS), a component of Gram-negative bacterial cell membrane [9], into the bloodstream. This phenomenon induces the production of host response molecules, such as neutralizing antibodies against LPS-endotoxin core antigen [antiendotoxin core antibody (EndoCab)], lipopolysaccharide-binding protein (LBP), which is released from hepatocytes in response to LPS exposure, and sCD14, which is released by hepatocytes and peripheral macrophages in response to LPS exposure [10–12].

Markers of microbial translocation have been identified as significant predictors of HIV-related disease progression and death [13,14]. The relevance of microbial translocation in lymphomagenesis is as yet unknown. In this study, we undertook to evaluate the association of microbial translocation, as measured by circulating levels of LPS, EndoCab, LBP, and sCD14, with NHL risk in well characterized prospective cohort studies of HIV-infected individuals.

Methods

Study samples

We conducted a nested case–control study within four cohorts of HIV-infected individuals [8]: the District of Columbia/New York gay men's cohort (DCG) [15], the Multicenter Hemophilia Cohort Studies I and II (MHCS I and MHCS II) [16,17], and the AIDS Cancer Cohort Study (ACC) [18]. Institutional review boards at the National Cancer Institute (NCI) and collaborating institutions approved each study, and all participants gave written informed consent.

Incident NHL cases were ascertained through active follow-up and pathologic confirmation. NHL cases with available serum or plasma specimens collected between 0 and 2 years before diagnosis were eligible for selection [median time 1 year (interquartile range [IQR] 0.7–1.2)]. HIV-positive cohort members who were NHL-free at the time of a case diagnosis were eligible for selection as controls. Up to 4 controls were selected for each case, matched on age (±5 years), sex, race, most recent CD4 T-cell count measured within 0–2 years before selection (categorized as 0–49, 50–99, 100–199, 200–499, and >500 cells/μl), parent cohort (DCG, MHCS I, MHCS II, and ACC), and specimen type (plasma vs. serum) [8]. Among the 3877 individuals included in the four cohort studies, 65 incident NHL cases occurred [8]. Of these, nine cases were excluded due to lack of appropriately matching controls. Thus, the current analysis included 56 NHL cases and 190 matched controls.

Laboratory measures

Serum or plasma specimens previously collected and stored at −70°C were measured for molecular markers. We utilized commercially available assays to measure LPS, EndoCab, LBP, and sCD14. The Limulus Amebocyte Lysate Assay (Lonza, Walkersville, Maryland, USA) was used to measure total LPS in specimens diluted 1 : 10 with endotoxin-free water and heated to 80°C for 15 min prior to testing. EndoCab was measured using an ELISA (Hycult Biotech, Uden, The Netherlands) at a 1 : 200 dilution. LBP was measured using an ELISA (Hycult Biotech) at a 1 : 1000 dilution. sCD14 was measured at a 1 : 200 dilution using the Quantikine ELISA (R&D Systems, Minneapolis, Minnesota, USA). Markers for cases and matched controls were measured on the same analytical batch.

For each marker, all specimens were tested in duplicate (technical replicates), with results reported as the mean of the duplicate values. These technical replicates were used to assess assay reproducibility by calculating coefficients of variation. For LPS, EndoCab, LBP, and sCD14, coefficients of variation were 6.4, 4.0, 2.6, and 6.7%, respectively. We also evaluated interbatch variability using blinded replicate samples (33 pairs for sCD14 and EndoCab; eight pairs for LPS and LBP). For EndoCab and sCD14, coefficients of variation for these specimens were 8.6 and 22.3%, respectively. For LPS and LBP, coefficients of variation were 18.9 and 21.4%, respectively.

We were concerned that fractionated blood products may contain compounds such as proteases, cholesterols, and other factors that neutralize LPS and would interfere with accurate measurement [19–21]. Therefore, we estimated LPS recovery by adding a known quantity of LPS to each specimen. Serum specimens spiked with 0.25 endotoxin unit (EU) of LPS were measured on the same plate with the paired unspiked specimen. Percentage recovery was calculated as: $[([C]_{post} - [C]_{pre})/0.25] \times 100$, where $[C]_{post}$ denotes the spiked LPS concentration, $[C]_{pre}$ denotes the unspiked LPS

concentration, and 0.25 is the amount of LPS added to each specimen. Recoveries within the range of 50–200% were considered within the manufacturer's recommended range [21].

Statistical analyses

Specimens with undetectable levels for any marker were assigned a value of half the lowest detectable limit [0.08 EU/ml, 10 MMU/ml (IgM median units/ml), and 4.4×10^6 pg/ml for LPS, EndoCab, and LBP, respectively]. Specimens with sCD14 measures above the maximal quantifiable value were assigned the highest detectable limit (3.2×10^6 pg/ml). Marker levels were analyzed as both categorical (dichotomized based on median levels among controls) and continuous (log-transformed). Conditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of each marker with NHL risk. Through matching, these conditional logistic models incorporated adjustment for age, sex, race, specimen type, cohort, and CD4 T-cell count. Correlations across markers and correlation of markers with CD4 T-cell count were assessed using Spearman's rank correlation coefficients.

Finally, a prior study from our group reported on the association of B-cell activation markers – circulating free kappa and lambda light chain concentrations – with NHL risk, using the same cases and controls included in the current study [8]. Given our hypothesis that the association of microbial translocation with NHL risk could, in part, be mediated through B-cell activation, we evaluated correlations of microbial translocation markers with B-cell activation markers using Spearman's rank correlation coefficients.

A P value of less than 0.05 was considered statistically significant. All analyses were performed using STATA 11 (StataCorp., College Station, Texas, USA).

Results

Sample characteristics

The study included 56 incident NHL cases and 190 controls (Table 1). The NHL cases included 23 DLBCLs, nine PCNSL, three Burkitt lymphomas, and 21 lymphomas of other/unknown type. Cases and controls were similar with respect to the matching variables (i.e. age, sex, race, $CD4^+$ T-cell count, cohort, and specimen type), and did not differ significantly by antiretroviral treatment or HIV-1 viral load. Most patients were not on HIV treatment.

Table 1. Comparison of demographic and HIV-related factors by case–control status.

Variable	Cases ($N=56$)	Controls ($N=190$)	P
Sex[a]			
Male	53 (94.6)	178 (93.7)	
Female	3 (5.4)	12 (6.3)	0.79
Age categories[a]			
<30	15 (26.8)	37 (19.5)	
30–36	11 (19.6)	51 (26.8)	
37–42	17 (30.4)	52 (27.4)	
>43	13 (23.2)	50 (26.3)	0.52
Race[a]			
White	48 (85.7)	158 (83.2)	
Black/Hispanic	8 (14.3)	32 (16.8)	0.65
Cohort[a]			
MHCS-I	30 (53.6)	100 (52.6)	
MHCS-II	2 (3.6)	6 (3.2)	
ACC	16 (28.6)	62 (32.6)	
DCG	8 (14.2)	22 (11.6)	0.92
Specimen type[a]			
Plasma	46 (82.1)	164 (86.3)	
Serum	10 (17.9)	26 (13.7)	0.44
CD4 T-cell count[a,b]			
<50	18 (32.1)	73 (38.4)	
50–99	15 (26.8)	42 (22.1)	
100–199	7 (12.5)	25 (13.2)	
200–499	12 (21.4)	38 (20.0)	
500+	4 (7.2)	12 (6.3)	0.91
HIV viral load			
<4.96 log copies/ml	16 (28.6)	37 (19.5)	
≥4.96 log copies/ml	14 (25.0)	37 (19.4)	0.76
Missing	26 (46.4)	116 (61.1)	
HIV treatment			
None	44 (78.6)	151 (79.5)	
HAART	7 (12.5)	26 (13.7)	
Other	5 (8.9)	13 (6.8)	0.86

[a]Matching factor.
[b]cells/μl.

Association of markers of microbial translocation with non-Hodgkin lymphoma risk

The associations of the four markers, treated both continuously and categorically, with NHL risk are presented in Table 2. On a continuous scale, levels of LPS (OR 1.21, $P=0.11$), EndoCab (OR 1.04, $P=0.83$), LBP (OR 0.71, $P=0.11$), and sCD14 (OR 2.36, $P=0.12$) were not significantly associated with NHL risk. When marker levels were categorized based on median levels among controls, levels of LPS, EndoCab, and LBP were not significantly associated with NHL risk. In contrast, elevated levels of sCD14 were significantly associated with increased NHL risk [OR 2.72 (95% CI = 1.29–5.76); $P<0.01$]. Associations did not materially change when models incorporated simultaneous adjustment for all four markers (data not shown). Likewise, results did not change when analyses were adjusted for antiretroviral treatment or for HIV-1 viral load. For example, the association of sCD14 with NHL risk remained significant after adjustment for antiretroviral treatment [OR 2.67 (95% CI 1.25–5.69); $P=0.01$] or HIV viral load [OR 4.00 (95% CI 0.72–22.1); $P=0.11$]. Finally, the association of elevated sCD14 concentrations and NHL risk did not differ significantly when analyses were stratified by the median time between serum sampling and NHL diagnosis/control selection: less than 1 year [OR 3.93 (95% CI 0.76–20.3)] vs. at least 1 year [OR 1.35 (95% CI 0.44–4.19); $P_{\text{interaction}}=0.85$].

Table 2. Association of markers of microbial translocation with non-Hodgkin lymphoma risk.

Marker	Cases ($N=56$)	Controls ($N=190$)	$OR_{continuous}$ (95% CI)[a,b]	$OR_{categorical}$ (95% CI)[a]	P
LPS (EU/ml)					
Median (IQR)	0.24 (0.04–0.49)	0.16 (0.04–0.35)	1.21 (0.96–1.55)		0.11
<0.16	23 (41.1)	96 (50.5)		1.0	
≥0.16	33 (58.9)	94 (49.5)		1.51 (0.77–2.96)	0.23
EndoCab (MMU/ml)					
Median (IQR)	44.2 (21.2–73.6)	41.2 (24.5–65.4)	1.04 (0.69–1.58)		0.83
<41.2	27 (48.2)	95 (50.0)		1.0	
≥41.2	29 (51.8)	95 (50.0)		1.10 (0.56–2.16)	0.78
LBP ($\times 10^6$ pg/ml)[c]					
Median (IQR)	14.5 (7.3–21.7)	14.2 (8.8–23.4)	0.71 (0.47–1.08)		0.11
<14.2	27 (48.2)	95 (50.3)		1.0	
≥14.2	29 (51.8)	94 (49.7)		1.16 (0.59–2.29)	0.67
sCD14 ($\times 10^6$ pg/ml)					
Median (IQR)	2.0 (1.63–2.34)	1.76 (1.45–2.39)	2.36 (0.80–6.95)		0.12
<1.76	19 (33.9)	96 (50.5)		1.0	
≥1.76	37 (66.1)	94 (49.5)		2.72 (1.29–5.76)	<0.01

CI, confidence interval; EndoCab, antiendotoxin core antibody; IQR, interquartile range; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MMU/l, IgM median units/ml; NHL, non-Hodgkin lymphoma; sCD14, soluble CD14.
[a]Conditional logistic regression with controls individually matched to cases on sex, age, race, cohort, specimen type, CD4 cell count.
[b]Odds ratios (ORs) reflect the change in NHL risk per log unit increase in marker concentration.
[c]Control missing due to inadequate specimen volume ($n=1$).

The median LPS percentage recovery among cases and controls combined was 60% (IQR 45.5–92.7). Twenty-eight cases (50.0%) and 80 (42.1%) controls had LPS recovery within the recommended range of 50–200%. Of note, median LPS concentrations were significantly higher for individuals with recoveries within the recommended range vs. those outside the range (0.24 vs. 0.10 EU/ml; Wilcoxon $P<0.001$). Given this variability, we conducted sensitivity analyses stratifying our study sample by LPS recovery (within vs. outside 50–200%). Among individuals within the recommended range of LPS percentage recoveries, LPS levels above the median were associated with significantly increased NHL risk [OR 3.24 (95% CI 1.10–9.53); $P=0.03$]. In contrast, among individuals with percentage recoveries outside the recommended range, LPS levels were not associated with NHL risk [OR 0.92 (95% CI 0.34–2.52; $P=0.88$].

Associations with CD4 T-cell count and correlations across markers among controls

Among controls, we evaluated associations of marker levels with CD4 T-cell counts and assessed correlations across markers. Levels of LPS and EndoCab were not associated with CD4 T-cell count. In contrast, LBP and sCD14 were significantly inversely correlated with CD4 T-cell count (LBP: rho $=-0.25$; $P<0.01$ and sCD14: rho $=-0.33$; $P<0.001$).

We observed a strong positive correlation between LBP and sCD14 among controls (rho $=0.64$; $P<0.01$), whereas EndoCab levels were weakly, yet significantly, correlated with sCD14 (rho $=0.18$; $P=0.01$) and LBP (rho $=0.27$; $P<0.01$).

Correlation of microbial translocation markers with kappa and lambda free light chain concentrations

We evaluated correlations of microbial translocation markers with levels of B-cell activation markers (kappa and lambda free light chains), which were previously measured and reported by our group [8]. Among controls, levels of sCD14, LBP, and EndoCab were positively correlated with levels of both kappa [rho $=$ 0.24 ($P<0.01$), 0.24 ($P<0.01$), and 0.30 ($P<0.01$), respectively] as well as lambda [rho $=0.49$ ($P<0.01$), 0.47 ($P<0.01$), and 0.39 ($P<0.01$), respectively] free light chains. Among cases, levels of sCD14 were positively correlated ($P=0.02$) with levels of lambda free light chains (rho $=0.29$).

Discussion

Chronic immune activation leading to increased B-cell activity has been proposed as a potential mechanism in the cause of NHL [2]. Given the recent recognition of gut microbial translocation as a potential cause of chronic immune activation among HIV-infected individuals [9,22], we investigated the association of four markers of microbial translocation with NHL risk. Elevated circulating levels of sCD14 were associated with a 2.5-fold increased NHL risk. Additionally, in a subgroup analysis, elevated LPS levels were associated with a three-fold increased risk of NHL. In contrast, levels of EndoCab and LBP were unrelated to NHL risk.

The significant association of LPS and sCD14 with NHL risk in our study supports an etiologic role for gut

microbial translocation in lymphomagenesis among HIV-infected individuals. These associations persisted even after controlling for CD4 T-cell count, HIV viral load, and antiretroviral treatments, arguing against confounding and supporting an independent role of these markers with NHL risk [23,24]. Furthermore, elevated levels of LPS and sCD14 preceded NHL by 1–2 years prior to diagnosis. LPS is a potent immunogenic component of Gram-negative bacterial cell membranes, and the presence of LPS in the blood stream is a marker of the breakdown of the gut-mucosal immune barrier [9,22]. sCD14, a marker of immune activation, is a receptor molecule produced primarily by macrophages and hepatocytes as part of the innate immune response to LPS [10,12,25]. sCD14 functions as a co-factor along with LBP to mediate LPS recognition and response by Toll-like receptor 4 (TLR-4), which is found on a wide range of immune cells, including B cells [26,27]. Our results therefore suggest that microbial translocation (as evidenced by elevated circulating LPS levels) and the resultant immune activation (as evidenced by elevated sCD14 levels) contribute to lymphomagenesis, potentially through B-cell hyperstimulation. In support of this hypothesis was our finding that markers of microbial translocation were significantly correlated with kappa and lambda free light chain levels, two markers of B-cell activation.

We acknowledge the limitations of our study. We found that levels of other LPS-associated immune activation markers, LBP and EndoCab, were not associated with NHL risk. Likewise, we did not find significant correlations between LPS levels and sCD14 levels. These observations are not entirely consistent with our proposed model. Nevertheless, similar to our observations, recent studies of microbial translocation and HIV-related mortality have also reported consistent associations for sCD14, but inconsistent associations across LPS, LBP, and EndoCab [14]. These inconsistent associations across markers of microbial translocation have led to the suggestion that markers of LPS bioreactivity as opposed to LPS exposure are perhaps more reliable measures for microbial translocation-related outcomes [14].

Our study was based on a limited number of incident NHL cases, which precluded more detailed analysis across specific NHL subtypes. Although we utilized pre-diagnostic serum/plasma specimens, given the short interval between specimen collection and NHL diagnosis (1–2 years), we cannot entirely rule out reverse causation as a potential explanation. Additional studies with a wider range of time between specimen collection and NHL diagnosis are needed to further understand the association of microbial translocation with NHL risk. Additionally, the significant association between LPS levels and NHL risk needs to be interpreted within the context of a subgroup analysis based on spike-in recovery values. LPS is difficult to measure in blood fractions due to the presence of interfering compounds [21]. For example, Balagopal *et al.* [28] recently reported a high level of interference in LPS measurement using the LAL assay, which was used in our study. Indeed, we found wide variability in LPS recovery, and elevated LPS levels were associated with NHL risk only among individuals with recoveries within the recommended range. Therefore, our results need replication. Our study also has several strengths, including control for key confounders, such as CD4 T-cell counts, and the use of prediagnostic specimens.

Our study is the first, to our knowledge, to investigate the role of microbial translocation with NHL risk. The association of elevated sCD14 and LPS with increased NHL risk supports an etiologic role for gut microbial translocation in lymphomagenesis among HIV-infected individuals. Additional studies are needed to replicate our findings and further evaluate the potential role of microbial translocation in lymphomagenesis among HIV-infected populations.

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Conflicts of interest

There are no conflicts of interest.

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