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**Permalink**
https://escholarship.org/uc/item/8z36v6bp

**Journal**
AIDS, 29(13)

**ISSN**
0269-9370

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**Publication Date**
2015-08-24

**DOI**
10.1097/qad.0000000000000687

Peer reviewed

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# HHS Public Access

Author manuscript

*AIDS*. Author manuscript; available in PMC 2016 August 24.

Published in final edited form as:

*AIDS*. 2015 August 24; 29(13): 1659–1664. doi:10.1097/QAD.0000000000000687.

# Dysregulated B-cell TLR2 expression and Elevated Regulatory B cell frequency precede the diagnosis of AIDS-related non-Hodgkin lymphoma

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## Abstract

**Objectives—**In Antiretroviral therapy (ART)-treated subjects, to determine if AIDS-related Non-Hodgkin Lymphoma (AIDS-NHL) is preceded by: i) elevated frequency of potentially malignant abnormal activated/ Germinal center (GC)-like B cells, ii) elevated serum prevalence of B-cell stimulatory TLR-ligands resulting from HIV-infection associated microbial translocation, iii) dysregulated B-cell TLR expression/signaling and iv) perturbations in the frequency of immunoregulatory cells.

**Design—**A case-control study nested with a cohort study of HIV-infected women.

**Methods—**Pre-diagnostic AIDS-NHL cases (n=14, collected 1-12 months pre diagnosis) and controls (n=42) from the Women's Interagency HIV Study (WIHS) cohort, were matched for HIV and ART status, age, race, and CD4 lymphocyte count. Serum levels of TLR ligands, the prevalence of malignancy-associated abnormal activated/GC-like ($CD19^+CD10^+CD71^+CD86^+AID^+$) B cells, TLR2 expression on B cells, expression of TLR2-modulating micro-RNA, and the frequency of regulatory T and B cells were assessed.

**Results—**Diagnosis of AIDS-NHL was preceded by a significantly elevated frequency of activated/GC-like $CD19^+CD10^+CD71^+CD86^+AID^+$ B cells (p=0.0072), elevated serum prevalence of TLR2-ligand and significantly elevated B-cell TLR2 expression (p=0.0015), positively correlating with the frequency of activated/GC-like B cells (rho=0.7273, p=0.0144). In cases, a purified subset of activated/GC-like B cells exhibited decreased expression of micro-RNAs that modulate TLR2-signaling, including: miR-21, 146a, 146b and 155. Finally, cases also

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exhibited significantly elevated frequencies of anti-tumor immunity inhibitory regulatory B cells (p=0.0024) but not regulatory T cells.

**Conclusions—**Our findings suggest that increased microbial translocation and dysregulated TLR expression/signaling, coupled with an elevated frequency of Bregs precede the diagnosis of AIDS-NHL in HIV-infected ART-treated subjects.

### Keywords

HIV; non-Hodgkin lymphoma (NHL); Toll-like receptor; regulatory B cells; microRNA; microbial translocation

## Introduction

Systemic chronic immune activation and inflammation are hallmarks of HIV infection and these conditions persist in patients on antiretroviral therapy (ART) with controlled viremia. Immune activation and inflammation can contribute to an increased risk of cardiovascular (CVD), liver, kidney and bone disease, as well as AIDS-related non-Hodgkin lymphoma (AIDS-NHL)[1]. AIDS-NHL is the most commonly occurring AIDS-related malignancy, accounting for 16%-19% of AIDS-related causes of death in developed countries [2-4]. There are several AIDS-NHL subtypes: Burkitt, small non-cleaved cell lymphoma (BL), diffuse large B cell lymphoma (DLBCL), primary central nervous system lymphoma (PCNSL) and primary effusion lymphoma (PEL). While virtually all AIDS-NHL subtypes are of B cell origin, there are significant differences in cellular phenotype and etiology [5].

Prior studies suggest that two key factors drive the genesis of AIDS-NHL: (i) attenuation of immunoregulatory cell functions and (ii) chronic B cell activation, which persists during ART [6, 7]. Though the mechanisms underlying HIV-associated B-cell activation are largely undefined, we demonstrated that Toll-like receptor ligands (TLR-L), resulting from HIV infection associated elevated microbial translocation [8, 9], likely contributes to B-cell hyperactivation [10]. Additionally, prior work demonstrated that HIV infection is associated with elevated levels of several B cell stimulatory molecules, with increasingly heightened levels observed preceding AIDS-NHL diagnosis [6, 7, 11, 12].

Finally, during HIV infection there is elevated expression of the enzyme activation-induced cytidine deaminase (AID) in B cells [13, 14] and AID mediates DNA-modifying events. These events including somatic hypermutation (SHM) and cross switch recombination (CSR)[15], and its constitutive expression contributes to lymphomagenic molecular lesions [16, 17]. We have previously determined that prior to an AIDS-NHL diagnosis, circulating B cells exhibit elevated AID levels [15, 18]. Further, prior to an AIDS-NHL diagnosis, B cells exhibited abnormally elevated expression of activated and germinal center (GC) markers, including CD10, CD71, CD86, and AID, similarly to malignant cells identified in other B-cell malignancies [6, 19, 20]. Interestingly, we also determined that TLR2-stimulated B cells from healthy controls adopted this activated/germinal center-like phenotype [21].

In the current study, we extend our investigation to determine if an AIDS-NHL diagnosis in ART-treated subjects is preceded by: i) elevated frequency of abnormal activated/GC-like B

cells, ii) elevated serum prevalence of B-cell stimulatory TLR-ligands, iii) dysregulated B-cell TLR expression/signaling and iv) perturbations in the frequency of immunoregulatory cells.

## Methods

### Subjects and Specimens

Data and specimens were obtained from the Women's Interagency HIV Study (WIHS) cohort. The institutional review boards (IRB) of all participating institutions approved the study protocol and written informed consent was obtained from all women. Details of the study design, data collection methods, and baseline characteristics have been reported previously[22] [23]. In this pilot study, we selected specimens from HIV-infected ART-treated WIHS participants, 1-12 (median of 8) months pre-AIDS-NHL diagnosis. Following previously published criteria [24], the HIV-infected ART-treated controls (Table 1) were matched for race, age (median ages were 37.5 and 38 for controls and cases respectively), and CD4 lymphocyte counts (median CD4 counts were 332 and 234 for controls and cases respectively, p=0.2).

### Isolation of cells and flow cytometry

PBMC from the WIHS repository were thawed and incubated for 48 hours and during the final 5 hours of incubation the cultures were supplemented with Brefeldin A (1:100, BD), PMA (50ng/ml, Invivogen) and Ionomycin (1ug/ml, Invivogen). The cells were washed, stained for viable cells (LIVE/DEAD Aqua Fixable Dead Cell Stain Kit, Invitrogen), surface stained, fixed/permeabilized (Fix/Perm Kit BD Biosciences) and stained for intracellular AID-PerCP (IC39101C, RnD System). The following antibodies were used for immunophenotyping of PBMC: CD19-ECD (IM2708U, Beckman Coulter), CD86-AF-700, CD10-PE-Cy7, CD4-Pacific Blue, CD127-APC-H7, CD71-FITC, CD38-PE, CD25-PE, CD24-PercpCy5.5 (all antibodies were purchased from BD Biosciences except when stated otherwise). All samples were acquired on an LRSII (BD, Bioscience) flow cytometer and the data were analyzed using FlowJo software (Tree Star Inc).

### ELISA assay for detection of TLR2-ligand, lipoteichoic acid LTA in serum

Serum was diluted (1:10) in ELISA coating buffer (KPL, Gaithersburg) on microtiter plates and incubated overnight at 4°C. Bound protein was captured using rabbit polyclonal anti-LTA antibody (1:350, Abcam) and detected by goat polyclonal anti-rabbit-Horseradish-peroxidase (HRP) (1:10,000, Abcam) antibody, and optical density was measured at 450nm.

### MicroRNA Real-time quantitative PCR

After FACS purification of cells, using the TaqMAn MicroRNA Cells-to-CR, miRNAs specific primers were used in the cDNA synthesis (Applied Biosystems, Foster City, CA). miRNA levels were normalized to the expression of small nucleolar RNA, RNU48. CT values were obtained using ABI 7900 Real-Time machine (Applied Biosystems, Foster City, CA). Samples were run in duplicates.



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### Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) or as indicated. GraphPad Prism software, version 5.03 was used for all statistical analyses. The statistical significance p value between group parameters was determined using the Mann Whitney test. The statistical dependence between variables was calculated using the Spearman rank correlation analysis. P values of <0.05 were considered statistically significant.

## Results

We observed a significant heightened frequency of phenotypically aberrant, activated/GC-like B-cell subset ($CD19^+CD10^+CD71^+CD86^+AID^{hi}$, Figure 1a, p=0.0072). We also found a positive but not statistically significant correlation between CD4 counts and the frequency of activated/GC-like B-cells (Figure 1b, rho=0.5515, p=0.1019).

We determined that in the WIHS pre-AIDS-NHL diagnosis cases, B-cells express significantly elevated levels of TLR2 cell surface expression compared to controls (Figure, 1c, p=0.0015). Further, we determined a significant positive correlation between B-cell TLR2 expression and the frequency of activated/GC-like B cells (Figure 1d, rho=0.7273, p=0.0379). Finally, we determined that compared to controls, cases exhibited higher, but not statistically significant (p=0.1), serum levels of the TLR2 ligand, lipoteichoic (LTA); LTA is a potent mediator of B-cell activation and elevated LTA serum levels in HIV-infected subjects is indicative of microbial translocation[10, 21].

After determining that heightened B-cell TLR2 expression in pre-AIDS-NHL cases was associated with the activated/GC-like B cells, we further characterized TLR2-signaling in the cases. In a subset of the activated/GC-like B cells ($CD19^+CD71^{hi}CD86^{hi}$) and $CD19^+CD71^{lo}CD86^{lo}$ B cells cases exhibited significantly higher frequency of $CD19^+CD71^{hi}CD86^{hi}$ B cells when compared to controls, Figure 1e, p=0.0367). We determined that the activated/GC-like subset ($CD19^+CD71^{hi}CD86^{hi}$ B cells) exhibited reduced expression of miR-21 (Figure 1f, 3,5 fold), miR-146a (Figure 1f 3-fold), miR-146b (Figure 1f, 3-fold), and miR-155a (Figure 1f, 5-fold, p=0.0379), compared to $CD19^+CD71^{lo}CD86^{lo}$ B cells. There were no changes in expression of miR-19a, 19b, 105 and 147 (not shown).

We observed that compared to controls, cases exhibited a significantly higher frequency of Bregs (Figure 1g, p=0.0024) but comparable frequencies of Tregs (not shown).

## Discussion

An important sequelae of persistent immune activation is the significantly elevated risk of developing malignancies, including AIDS-NHL, and this risk is still evident among HIV-infected individuals taking ART [4]. Elucidating the mechanisms underlying the genesis of AIDS-NHL could lead to novel prophylactic or therapeutic interventions. Our study found an association between B-cell TL2-expression/signaling and the etiology of AIDS-NHL. An association between the serum levels TLR4-ligand lipopolysaccharide (LPS) and the etiology of AIDS-NHL has been reported [25], suggesting that LPS stimulation contributes

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to B-cell hyperactivation, a critical driver of AIDS-NHL genesis. However, our previous data suggest TLR2 stimulation is a potent mediator of B-cell activation and HIV-infected subjects on ART exhibit higher serum levels of the TLR2-ligand lipoteichoic acid (LTA)[10] .

Our prior data indicate that TLR2-activated B cells from healthy subjects mimic a peripheral activated/germinal center (GC)-like phenotype ($CD19^{+}CD10^{+}CD71^{+}CD86^{+}AID^{hi}$) that was observed in the blood of men in the MACS cohort prior to AIDS-NHL diagnosis[21]. AIDS-NHL largely develops from the malignant transformation of GC or post-GB B cells [26], suggesting that these activated/GC-like B-cells observed pre-AIDS-NHL diagnosis are potentially precursors of a malignant population. The expression of AID is upregulated in GC cells and persistent expression of AID contributes to lymphomagenesis characteristic molecular lesions, such as oncogenic translocations and oncogenic mutations [16, 17]. Interestingly, some GC-B cells also express the activation marker CD71 [27] and CD71 was identified as a non-immunoglobulin partner of *BCL6* translocation, a common abnormality in NHL [28]. Finally, data by Guech-Ongey et al [29], suggested that in some AIDS-NHL cases malignant cells require functional CD4 lymphocytes to thrive. Supporting this finding, we determined a positive correlation between the frequency of the potentially malignant abnormal activated/GC-like B cells and CD4+ T cell count.

Data from multiple studies have identified a role for dysregulated B-cell TLR expression/ signaling in the etiology of lymphomas [30, 31]. Our finding that B cells from cases exhibit significantly high levels of TLR2 expression support these reports. In lymphomagenesis, TLR expression has multiple implications, most importantly, TLR signaling can promote the growth of malignant cells, leading to accumulation and eventually autonomous growth [31]. Prior to an AIDS-NHL diagnosis, there was a significant positive correlation between B-cell TLR2 expression and the frequency of activated/GC-like cells. Furthermore, the elevated serum levels of TLR2-ligand lipoteicoich acid (LTA), which is indicative of microbial translocation [10, 21], suggest persistent TLR2 stimulation was associated with the development of AIDS-NHL. Interestingly, we did not find a difference in the serum levels of TLR9-ligand or bacterial DNA (not shown).

Finally, additionally evidence supporting our hypothesis that aberrant TLR signaling contributes to NHL-genesis is provided by our finding reduced miRNA that modulate TLR signaling in activated/GC-like cells[32].

Finally, our finding that cases exhibited a significantly elevated frequency of Bregs, which can inhibit anti-tumor immunity, corroborates reports in other malignancies [33]. Although we did not find an elevated Tregs frequency, it is feasible that Bregs driven Treg differentiation can profoundly compound the anti-tumor immunity inhibitory milieu.

Our study has a few limitations including: the small sample size, the heterogeneity of the NHL types and the proximity of sample collection to AIDS-NHL diagnosis: though we did not find a correlation between time of AIDS-NHL diagnosis: and B-cell TLR2 expression nor frequency of activated/GC-like B cells (Table 1). Nonetheless, this is the first report identifying a potentially malignant activated/GC-like B cell subset preceding the diagnosis of AIDS-NHL, associated with a novel role for TLR2 and anti-tumor immunity inhibitory

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Bregs in AIDS-NHL. A larger study and one examining earlier pre-AIDS-NHL time points, would offer further insight into these highly pertinent mechanisms underlying the etiology of AIDS-NHL.

## Acknowledgments

BS, AL and OM-M designed study, BS and JTP performed experiments, and BS wrote manuscript. JTP, OM-M, NH, MC, AL edited manuscript.

Data in this manuscript were collected by the Women's Interagency HIV Study (WIHS) Collaborative Study Group with centers (Principal Investigators) at New York City/Bronx Consortium (Kathryn Anastos); Brooklyn, NY (Howard Minkoff); Washington DC Metropolitan Consortium (Mary Young); The Connie Wofsy Study Consortium of Northern California (Ruth Greenblatt); Los Angeles County/Southern California Consortium (Alexandra Levine); Chicago Consortium (Mardge Cohen); Data Coordinating Center (Stephen Gange). The WIHS is funded by the National Institute of Allergy and Infectious Diseases (UO1-AI-35004, UO1-AI-31834, UO1-AI-34994, UO1-AI-34989, UO1-AI-34993, and UO1-AI-42590) and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (UO1-HD-32632). The study is co-funded by the National Cancer Institute, the National Institute on Drug Abuse, and the National Institute on Deafness and Other Communication Disorders. Funding is also provided by the National Center for Research Resources (UCSF-CTSI Grant Number UL1 RR024131).

**Funding:** The Women's Interagency HIV Study (WIHS) is funded by the National Institute of Allergy and Infectious Diseases and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development. The study is co-funded by the National Cancer Institute, the National Institute on Drug Abuse, and the National Institute on Deafness and Other Communication Disorders. Funding is also provided by the National Center for Research Resources.

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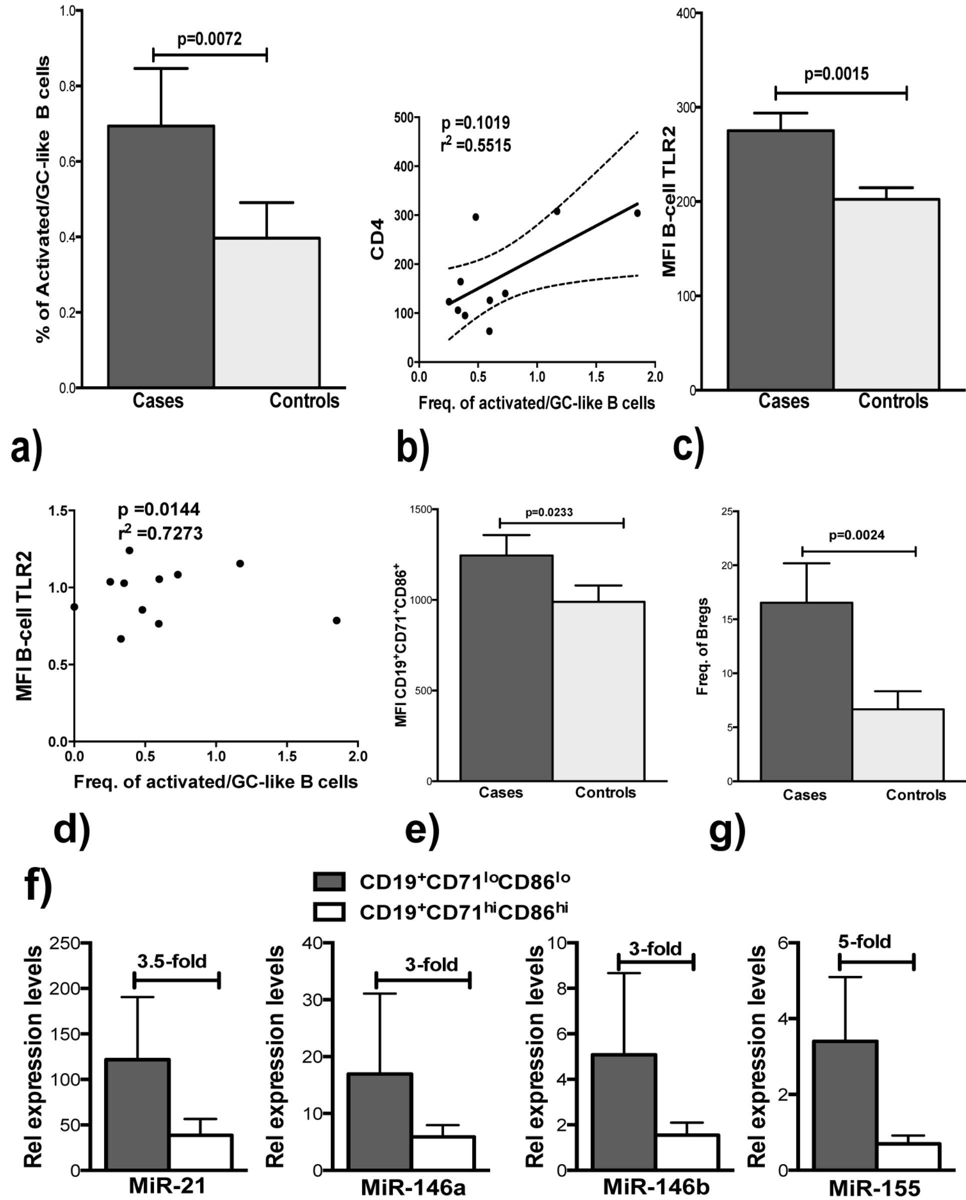


**Figure 1. Elevated frequencies of regulatory B cells, abnormal activated/germinal-center-like B cells and dysregulated B-cell TLR2-expression/signaling precede the diagnosis of AIDS-NHL**

In pre-AIDS NHL diagnosis samples from cases (n=12) and matched controls (n=42), by flow-cytometry, **(a)** the frequency of abnormal activated/germinal center (GC)-like B cells ($CD19^+CD10^+CD71^+CD86^+AID^+$), **(e)** the frequency of a subset of the activated/GC-like B cells ($CD19^+CD71^+CD86^+$), **(c)** B-cell expression of TLR2, and **(g)** the frequency of regulatory B cells ($CD19^+CD24^{hi}CD38^{hi}$) were determined. In the cases, the relationship between the frequency of activated/germinal center (GC)-like B cells ($CD19^+CD10^+CD71^+CD86^+AID^+$) and **(b)**, CD4 count **(d)**, B-cell TLR2 expression were determined. Finally, **(f)**, in $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+CD24^{lo}CD38^{lo}$ purified cells

from cases, the expression of miR21,146a, 146b and 155 was determined by qPCR. P values are indicated, MFI= mean fluorescence intensity, Freq.= frequency, rel= relative.

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## Table 1

| ID | time prediagnosis (months) | MFI B-cell TLR2 | % of NHL phenotype |
|---|---|---|---|
| 10205294 | 5 | 279 | 0.39 |
| 10205713 | 7 | 324 | 1.17 |
| 20100181 | 11 | 255 | 0.596 |
| 20100802 | 9 | 422 | 1.85 |
| 20101830 | 8 | 266 | 0.329 |
| 30203725 | 12 | 284 | 0.73 |
| 40203296 | 5 | 189 | 0.254 |
| 40512102 | 1 | 315 | 0.352 |
| 40615100 | 11 | 184 | 0 |
| 40718108 | 7 | 273 | 0.599 |
| 41027560 | 12 | 325 | 0 |
| 10101840 | 8 | 286 | 0.481 |
| | **Median =8** | | |
| | **p=** | **0.855** | **0.4429** |
| | **r=** | **0.05887** | **0.2569** |