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TLR2 activated B cells are phenotypically similar to the abnormal circulating B cells seen preceding the diagnosis of AIDS related non-Hodgkin lymphoma (NHL) diagnosis

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Abstract

Background—AIDS-related non-Hodgkin lymphoma (AIDS-NHL) is a common AIDS-defining cancer. Prior studies suggest that chronic B cell activation precedes AIDS-NHL diagnosis.

Activation of B cells by multiple factors, including Toll-like receptor (TLR) signaling, leads to the expression of activation-induced cytidine deaminase (AID), a DNA mutating molecule that can contribute to oncogene translocations/mutations, leading to NHL. The goal of this study was to determine whether surface markers expressed on activated and/or germinal center (GC) B cells, and AID expression, were elevated on circulating B cells preceding AIDS-NHL, as well as to determine if TLR signaling contributes to this activated B cell phenotype.

Methods—Stored viable peripheral blood mononuclear cell (PBMC) specimens, obtained prior to AIDS-NHL diagnosis, were assessed by multi-color flow cytometry. Additionally, B cells isolated from PBMC were exposed to TLR ligands *in vitro*, after which B cell phenotype was assessed by flow cytometry.

Results—An elevated fraction of B cells expressing CD10, CD71, or CD86 was seen in those who went on to develop AIDS-NHL. AID expression was detected in some who developed AIDS-NHL, but not in HIV+ or HIV– controls. TLR2- stimulated purified B cells exhibited the activated B cell phenotype observed in HIV+ subjects prior to AIDS-NHL diagnosis.

Conclusions—These results indicate that an elevated fraction of B cells display an activated/GC phenotype in those HIV+ subjects who go on to develop AIDS-NHL, and suggest that TLR2-mediated activation may play a role in HIV infection-associated B cell activation, potentially contributing to the genesis of AIDS-NHL.

Conflicts: none to declare

Keywords

Immunophenotype; HIV; AIDS lymphoma; non-Hodgkin lymphoma; activation induced cytidine deaminase (AID); Toll-like receptor (TLR)

Introduction

AIDS-related non-Hodgkin lymphoma (AIDS-NHL) is a common AIDS-defining cancer. Compared with the general population, the incidence of AIDS-NHL is increased around 100–250 times in HIV infected persons^{1,2}. There are several AIDS-NHL subtypes: Burkitt, small non-cleaved cell lymphoma (BL), diffuse large B cell lymphoma (DLBCL), primary central nervous system (PCNS) lymphoma and primary effusion lymphoma (PEL). While virtually all AIDS-NHL subtypes are of B cell origin, there are significant differences in cellular phenotype, oncogenic molecular lesions, and in the frequency of Epstein-Barr virus (EBV) or Kaposi's sarcoma (KS) associated herpesvirus (KSHV) infection among these AIDS-NHL subtypes³.

Since the widespread use of highly active antiretroviral therapy (HAART), the incidence of AIDS-NHL has decreased, but not as profoundly as KS^{4–6}. Additionally, the HAART-associated decrease in AIDS-NHL incidence has been subtype specific, with a marked decrease in CNS lymphomas, but no decrease in BL in the post-HAART era. This may be due to essential differences in the etiology of these AIDS-NHL subtypes. It is believed that two major factors drive the genesis of AIDS-NHL: 1) loss of immunoregulatory control of EBV-infected B cells with progressive HIV-induced immune system damage, and 2) chronic B cell activation due to HIV infection^{7–9}. Though the mechanisms driving HIV-associated B-cell activation are largely undefined, there are several possible sources of B cell activation in HIV infection, including antigenic stimulation of B cells, elevated levels of B cell stimulatory cytokines, and direct activation of B cells by HIV virions containing cellular activation molecules, such as CD40 ligand (CD154)^{8,10–14}, we have demonstrated that Toll-like receptor (TLR) signaling, especially via TLR2, likely contributes to B-cell hyperactivation¹⁵. In prior work, we noted elevated levels of phenotypically aberrant B cells in the circulation of HIV+ persons, with an increased fraction of CD10 and CD71 (transferrin receptor, TfR) positive B cells¹⁶. Chronic B cell stimulation can result in the ongoing expression of activation-induced cytidine deaminase (AID). AID is an apolipoprotein B editing catalytic subunit (APOBEC) like enzyme, which is primarily expressed in germinal center (GC) B cells, and is responsible for immunoglobulin gene class switch recombination (Ig CSR) and somatic hypermutation (SHM) in GC B cells¹⁷. Chronic AID expression can contribute to lymphomagenic molecular lesions, such as oncogene translocations and oncogene mutation^{18,19}. In previous work, we found that AID gene expression in circulating B cells was elevated prior to the diagnosis of AIDS-NHL, with the highest levels seen in those who develop BL²⁰. HAART may be more effective in restoring effective anti-viral immunity to EBV-infected B cells than in preventing B cell activation. In fact, results from our recent study²¹ indicate that HAART decreases serum levels of several B cell-stimulatory factors, but does not result in the normalization to the levels seen in HIV uninfected persons.

Activated B-cell and/or GC-like phenotypes have been associated with B-cell malignancies^{10,22,23}. The goal of this study, therefore, was to investigate if markers of B-cell activation and/or a GC-like phenotype, as well as AID, were prevalent in circulating B cells isolated preceding the diagnosis of AIDS-NHL. We also sought to elucidate novel HIV-infection associated mechanisms likely contributing to this activated B-cell phenotype, including stimulation of B cells by TLR ligands.

Materials and Methods

Cases and controls

All cases and controls were from the UCLA center of the Multicenter AIDS Cohort Study (MACS). The MACS is a prospective cohort study of the natural and treated history of HIV/AIDS, and consists of 6978 adults homosexual and bisexual men from four metropolitan areas: Baltimore, Chicago, Pittsburg and Los Angeles. Participants were enrolled in 1984–1985, 1987–1991, and 2001–2003, and are followed with a semiannual visit, at which blood and clinical information are collected²⁴. Viably frozen peripheral blood mononuclear cells (PBMC) are available from these semiannual study visits. We utilized viably frozen PBMC from a total of 12 AIDS-NHL cases: six BL AIDS-NHL cases and six non-BL AIDS-NHL cases (4 DLBCL and 2 PCNS). The EBV status of the tumors was known for three of the BL cases: based on EBV EBER and LMP1 DNA qPCR, one of the three BL cases was EBV positive, the other two EBV negative. In addition to this, we obtained viably frozen PBMC from six HIV seropositive (HIV+) and six HIV seronegative (HIV-) persons. For the each AIDS-NHL case, we obtained PBMC from two pre-lymphoma diagnosis visits, based on the date of lymphoma diagnosis: >3 years and 1–3 years before NHL diagnosis. All of the samples were viably frozen PBMC. In HIV+ group, the samples were selected to have a range of CD4 T cell counts that was similar to those seen in the pre-lymphoma samples from the AIDS-NHL cases.

Isolation of cells and flow cytometry

Viably frozen PBMC from the UCLA MACS repository were thawed, viability confirmed and then counted. Based on the number of PBMC, RNA was extracted from at least 2×10^6 ($2-4 \times 10^6$) cells using Trizol (GIBCO/BRL) for assessment of *AID* expression using real-time quantitative PCR. The remaining cells (around $3-8 \times 10^6$) were used for assessment of cell surface expression of various B cell-associated molecules by flow cytometry. First, cells were fixed in 3% of formaldehyde solution, 1 hour at 4°C, then 0.2% Tween 20 buffer was used to permeabilize the cells, by exposure for 15 minutes at 37°C. After these two steps, cells were incubated with primary anti-AID antibody or isotype control (EK2 5G9 rat monoclonal antibody, Cell Signaling Technology; isotype control was pure rat IgG, Jackson Immuno Research) for 45 minutes at room temperature, followed by the addition of goat anti-rat IgG second antibody combined with Alexa Fluor 488 (Alexa Fluor 488 goat anti-rat IgG, Invitrogen), for 30 minutes at room temperature protected from light. After intracellular staining, cells were stained for the expression of cell surface molecules. Cells were exposed to the relevant antibodies for 20 minutes at 4°C, then washed in 1% BSA-PBS. Antibodies specific for CD10, CD19, CD28, CD38, CD71 and CD86, and isotype controls, were conjugated with APC, PEcy7, PEcy5, FITC, PE and APC separately (Becton Dickinson - BD). PE-conjugated antibody specific for CD257 (BAFF) also was used (eBioscience). All specimens were analyzed on a BD LSR flow cytometer. Data files were acquired and analyzed for each specimen by using BD FACSDiva software. We used the Ramos BL B cell line as a positive control: almost all Ramos cells were seen to be AID positive (99.3%). We used the Jurkatt T cell leukemia cell line as a negative control: all Jurkatt cells were negative for AID expression.

TLR-stimulation assay

Using the MACS (Miltenyi Biotec, Cambridge) B cell Isolation kit®, B cells were purified from PBMCs of healthy controls and cultured with were incubated with medium alone or with 10ug/ml CpG-B ODN2006 (TLR9L, Invivogen), 2µg/ml LPS (TLR4L, Invivogen), 2µg/ml PAM3CSK4 (TLR2L, Invivogen), and CD40L (2ug/ml, Invivogen) for 48 hours, after which markers of activation assessed by flow cytometry.

RNA extraction for quantitative real-time PCR (qPCR)

Total RNA was extracted from around 3×10^6 PBMC with TRIzol. The real time PCR assay for *AID* and the construction of standard curves has been described previously^{11,20,25}.

Statistics

The results are presented as mean. Statistical significance was determined using Student's T-test.

Results

Clinical and biological characteristics AIDS-NHL cases and controls

The age of the AIDS-NHL cases ranged from 29 to 56 years, at the time when they were diagnosed with lymphoma. The pre-lymphoma diagnosis viable PBMC samples chosen for this study were collected at MACS study visits from one to seven years prior to lymphoma diagnosis. The CD4 T cell counts of pre-lymphoma samples ranged from 68 to 820 cells/mm³. In the HIV+ (non-lymphoma) control group, the age at blood draw ranged from 42 to 68, CD4 T cell counts ranged from 41 to 689 cells/mm³. In the HIV- control group the age ranged from 34 to 47, the CD4 T cell counts were from 452 to 1269 cells/mm³. The EBV infection status of the tumors was available for few individuals (see Material and Methods). EBV DNA load in plasma and serum was not available. Although EBV is known to play a role in the development of some forms of AIDS-lymphoma, pre-diagnosis EBV DNA load was not seen to correlate with NHL development²⁶

Elevated levels of CD10, CD71, and CD86-positive circulating B cells were seen preceding AIDS-NHL diagnosis

HIV-infection associated chronic B-cell hyperactivation, with resulting aberrant AID expression, is believed to contribute to the genesis of AIDS-NHL⁹. Further, HIV infection is associated with elevated prevalence of phenotypically abnormal B cells²⁷ and published studies report that B cells with an activated/GC-like phenotype are associated with B-cell malignancies^{24,28-30}. This prompted us to investigate if such an activated/GC-like aberrant phenotype is prevalent in AIDS-NHL patients prior to NHL diagnosis. Using PBMC isolated from the subjects described above, around 30,000 lymphocytes (gated based on forward scatter (FSC) and side scatter (SSC)) events per tube were acquired and analyzed by flow-cytometry. In the lymphocyte population, CD19 positive cells were subgated as the B cell population, and CD10, CD28, CD38, CD71, CD86, BAFF (B cell activating factor, CD257) and AID expression was analyzed on CD19+ cells. Representative flow cytometry plots are shown in Figure 1. It is apparent that elevated levels of CD71 B cells co-expressing AID protein were seen preceding the diagnosis of AIDS-associated BL.(Figure 1). The proportion of B cells expressing these different molecules was assessed, this is expressed as the percentage of B cells that were positive for each molecule (Table 2).

The mean percentage of CD10(CALLA) positive B cells was 14% in the pre-lymphoma group, more than four times the percentage seen in the HIV- group, and more than three times that seen in the HIV+ group (Table 2). This represents a statistically significant elevation in the fraction of B cells expressing CD10 in those individuals who went on to develop AIDS-NHL, when compared both to HIV+ (P=0.0049) and HIV-negative controls (P=0.0081).

CD71 also was expressed at higher levels on B cells from the pre-lymphoma group, when compared to the HIV+ and HIV-negative control groups (Table 2). The fraction of CD71+ B cells seen in those who developed AIDS-NHL was 1.7 times higher than that seen in the

HIV+ control group ($p=0.05$) and almost twice as high as that seen in the HIV- group ($p=0.01$).

About 5% of B lymphocytes were positive for CD86 in pre-AIDS-NHL group, in contrast, to 0.75% and 2% CD86-positive B cells in the HIV+ ($p<0.01$) and HIV-negative control groups ($p<0.01$), respectively (Table 2).

The mean percentage of AID-expressing cells was higher in the pre-AIDS-NHL group than in the HIV+ and HIV- control groups (1.2% vs 1.0% and 0.5%, respectively) (Table 2). This was statistically significant only when comparing the pre-AIDS-NHL group ($P=0.025$), or the pre-BL subset ($p=0.039$), to the HIV-negative group (Table 2). To confirm this result, we performed real-time qPCR to assess AID gene (*AID*) expression by quantifying *AID* mRNA. We found that none of the B cell preparations from either of the HIV+ and HIV-negative control groups had detectable *AID* expression, while two of the subjects in the pre-BL group and two in non-BL AIDS-NHL group had detectable *AID* expression by qPCR (not shown).

No significant difference in the expression of CD28, CD38 or CD257 on B cells was noted, when comparing pre-lymphoma specimens to those isolated from the HIV+ and HIV- groups. Additionally, to determine if there were any differences between NHL and controls in the expression of CD71 on CD10+ B cells, we subgated CD10 positive B lymphocytes and further analyzed the expression of CD71. No significant difference was seen in CD71+ CD10+ double-positive B cells among pre-lymphoma, HIV+ and HIV- groups (not shown).

Changes in the expression of B cell activation markers with time preceding lymphoma diagnosis

After determining that circulating B cells pre-AIDS-NHL patients exhibit an abnormal activated/GC-like (CD10+, CD71+, CD86+, AID+) phenotype, we further investigated if prevalence of this characteristic phenotype was different with time pre-NHL diagnosis, or with NHL subtype. However, no significant differences were seen in the the percentage of B cells expressing these markers when comparing BL vs non-BL AIDS-NHL subtypes, or samples that were more or less than three years pre-NHL diagnosis (not shown).

TLR2-stimulated B cells exhibit the CD10+CD71+CD86+AID+ phenotype observed *in vivo* preceding the diagnosis of AIDS-NHL

HIV-infection associated chronic B-cell hyperactivation, which does not normalize completely after successful HAART²¹, is believed to play a key role in the genesis of AIDS-NHL^{31,32}. Although the mechanisms underpinning B-cell hyperactivation remain largely undefined, TLR-signaling has been implicated^{33,34} and we have also demonstrated that TLR2 is a potent mediator of B-cell activation (Siewe et al, manuscript submitted). Further, TLR expression and signaling has been demonstrated in lymphomas³⁵⁻³⁷, prompting us to investigate if TLR-mediated activation contributes to the genesis of AIDS-NHL.

We exposed purified B cells from healthy controls to TLR2, TLR4 or TLR9 agonists, and assessed the expression of CD10, CD71, CD86 and AID on B cells isolated from healthy HIV-negative persons by flow cytometry. We found that TLR2 signaling was a more potent stimulator than TLR9 and TLR4 (Figure 2a). Interestingly, only TLR2 stimulation led to co-expression of CD10+CD71+CD86+AID+ on B cells ($p=0.0346$, compared to medium control, Figure 2b), demonstrating the role for TLR2 in B-cell activation, and suggesting a role for TLR2 stimulation in the genesis of AIDS-NHL.

Discussion

HIV infection and AIDS are accompanied by severe disruptions in the B cell compartment, including the emergence of a significant subpopulation of phenotypically-aberrant circulating B cells, characterized by the expression of cell-surface molecules that are not typically seen on B cells in the circulation of healthy HIV– subjects. Some of these molecules are associated with B cell activation and/or a GC-like phenotype, and also may be expressed in B-cell malignancies^{22,23}. Further, HIV infection is associated with elevated expression of several B-cell activation markers, which are often elevated preferentially in those HIV+ persons who go on to develop AIDS-NHL^{8,10}. In this study, we assessed the expression of lymphoma associated B-cell activation and GC-like markers, including CD10, CD28, CD38, CD71, CD86, BAFF and AID, in circulating B cells isolated from HIV-infected subjects prior to AIDS-NHL diagnosis. Since B-cell activation is a major contributor to the genesis of AIDS-NHL, we further determined the role of TLR-signaling in inducing B-cell activation.

The results presented here identify a distinctive phenotypic abnormality in circulating B cells isolated from those HIV+ subjects who went on to later develop AIDS-NHL: an elevated fraction of B cells were seen to express CD10, CD71 or CD86 in the pre-AIDS-NHL group when compared to controls. AID also was seen to be frequently expressed in B cells from those who went on to develop AIDS-NHL, as previously noted^{20,38}, although a significant difference in AID expression was not seen between the HIV+ and AIDS-NHL groups.

CD10 is a 100kDa type-2 cell surface metallo-endopeptidase, expressed on GC B cells, and also on immature B cells in bone marrow; CD10 is expressed during the first stage of immunoglobulin heavy chain rearrangement in pre-B cells³⁹. It also can be expressed in the cytoplasm of some lymphomas, such as follicular lymphoma and BL^{29,40}. In a prior study, we noted that an increased percentage of circulating CD10+ B cells was seen in AIDS patients, suggesting that HIV infection can result in elevated levels of circulating B cells that express this GC B cell marker¹⁶. No lymphoma cases were included in that earlier study. The results of the current study indicate that a marked elevation of CD10 positive circulating B cells is seen preceding AIDS-NHL diagnosis, correlating with similar reports in follicular lymphoma patients^{29,40}.

CD71 is the transferrin receptor, a marker of B-cell activation, with heightened expression seen on malignant B cells²⁸ and during HIV infection¹⁶. Similarly upregulated on B cells during HIV infection is CD86 (B7-2), the ligand for CD28 and CTLA-4, which is expressed on antigen-presenting cells that provide costimulatory signals necessary for T cell activation, with emerging evidence that CD86 may promote proliferation of malignant B-cells³⁰. Consistent with prior reports delineating heightened expression of CD71 and CD86 in B-cell malignancies, in this study we found B cell CD71 or CD86 expression to be elevated in subjects who went on to develop AIDS-NHL, when compared with those in the HIV– and HIV+ control groups, although this difference did not reach statistical significance when comparing the NHL group to the HIV+ control group. Although both CD10 and CD71 were seen to be expressed on a larger fraction of B cells in those who went on to develop AIDS-NHL, it is interesting to note that the same B cell population does not appear to co-express CD10 and CD71. Only about 9% of CD10 positive B cells co-expressed CD71, suggesting that these molecules represent distinct B cell populations. We cannot, from the information presented here, determine if there is any relationship between these two populations. It is possible that the CD71+ B cell subset represents recently activated B cells, while the CD10+ B cell population is indicative of a GC-like B cell subset, both of which are seen in the circulation of those HIV+ subjects who go on to develop AIDS-NHL.

AID expression on B cells, assessed either by flow cytometry or by qPCR, was often elevated in those HIV+ subjects who went on to develop AIDS-NHL. This result was in accordance with a previous study, in which frequent *AID* expression was noted in PBMC from those who went on to develop AIDS-NHL, especially non-CNS lymphomas²⁰. *AID* expression was seen in both CD71+ and in CD10+ B cell populations.

Finally, our results indicate that purified B cells from healthy controls exposed to TLR2 ligands exhibit an activated phenotype similar to that observed *in vivo* in HIV+ subjects who went on to develop AIDS-NHL. Multiple published studies implicate TLR ligands in the pathogenesis of B-cell lymphoid malignancies^{36,41} and HIV-infection is associated with systemic prevalence of TLR ligands following microbial translocation⁴². Combined with the observed phenotypic abnormalities seen preceding AIDS-NHL diagnosis, this suggests that TLR2-mediated B-cell activation may contribute to the genesis of AIDS-NHL. Certainly, additional studies are needed to define the association of *in vivo* levels of TLR2 ligands and the development of AIDS-NHL.

To our knowledge, this is the first report documenting distinctive phenotypic abnormalities in circulating B cells isolated from those HIV+ subjects who went on to later develop AIDS-NHL, specifically, an elevated fraction of B cells that express CD10, CD71 or CD86 in the pre-AIDS-NHL group. Further, *AID* expression was frequently expressed in B cells from those who went on to develop AIDS-NHL. Finally, TLR2-mediated stimulation of B cells induced a phenotype reminiscent of that observed to occur pre-AIDS-NHL. Together, these results define these as potentially predictive markers for the development of B cell lymphoma in HIV infected subjects, and provide insights into the pathogenesis of these cancers.

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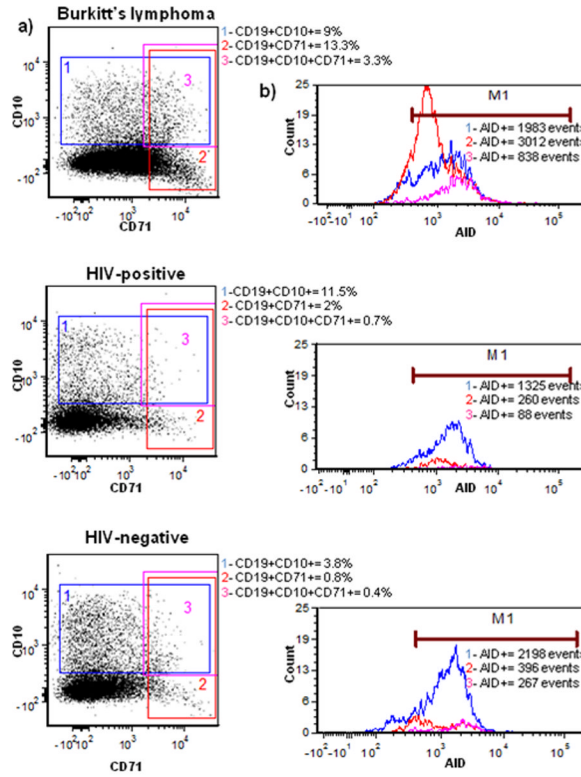


Figure 1. Higher levels of activated B cells (CD71+ cells) and AID-positive B cells are present in HIV+ subjects who went on to develop lymphoma
 PBMC collected prior to BL diagnosis, as well as PBMC from HIV-positive and HIV-negative control subjects, were tested. PBMC were gated on CD19 expression, to identify B cells. Panel “a” shows CD10 (1 - marked in blue), CD71 (2 – marked in red), and CD10/CD71 (3 – marked in fushia) expression on CD19 cells. Panel (b) shows expression of AID, assessed by intracellular staining, in CD10+ (blue), CD71+ (red) or CD71+CD10+ (fushia) B cells.

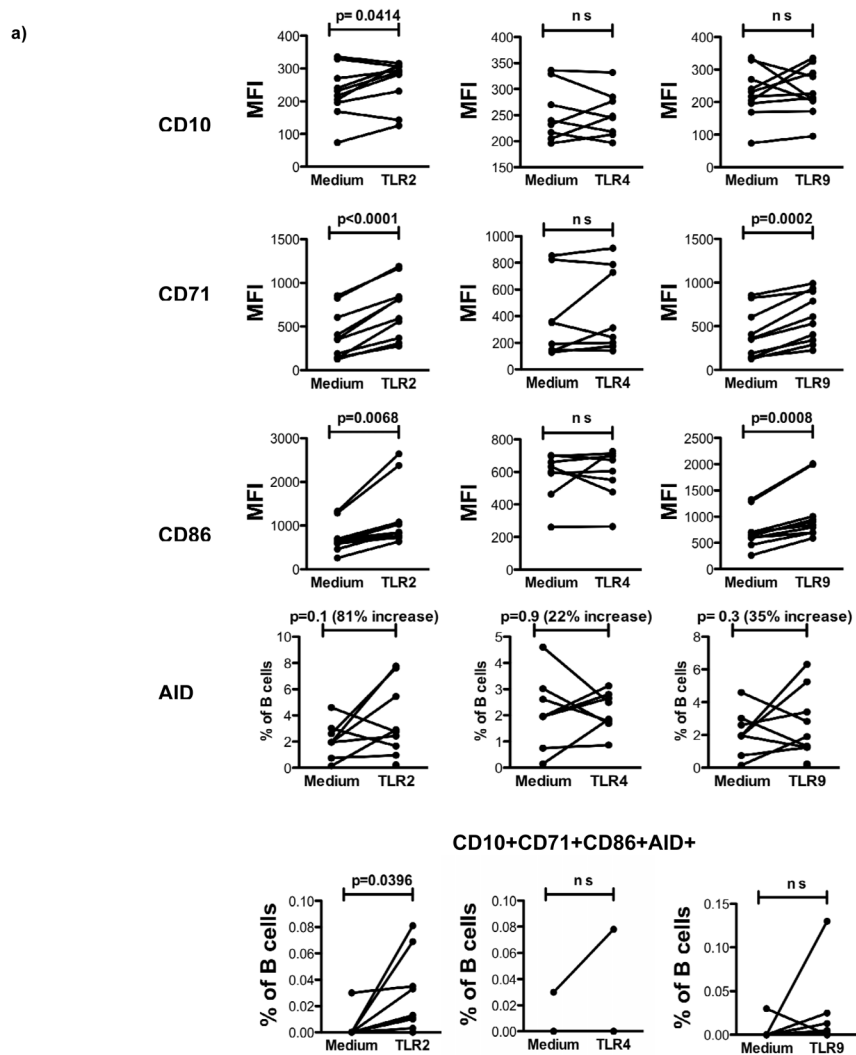


Figure 2. TLR2-activated B cells express CD10, CD71, CD86, and AID
 Purified blood B cells from HIV- controls (n=10), were exposed to TLR2, 4 and 9 ligands, and expression of **(a)** CD10, CD71, CD86, AID and **(b)** CD10+CD71+CD86+AID+ were then assessed by flow-cytometry (MFI = mean fluorescence intensity, ns = not significant). MFI was used to assess activation markers, as this is a better measure of total expression than the percent of positive cells.

Table 1

Clinical and biological characteristics of pre-AIDS-NHL cases

Subtype of lymphoma	Age at diagnosis	Age at death	Time from blood collection to lymphoma diagnosis	CD4 cell count	B cell %
BL	42	42	1: 4 years	643	8.4
			2: 1 year and 8 months	632	17.9
BL	45	46	1: 5 years and 7 months	252	22.3
			2: 2 years and 1 month	87	23.7
BL	37	39	1: 4 years and 3 months	421	4.8
			2: 1 year and 5 months	541	10
BL	32	32	1: 4 years and 1 month	408	11.6
			2: 1 year and 5 months	751	---
BL	31	---	1: 2 years and 10 months	751	13.1
			2: 1 year and 10 months	820	15.3
BL	29	29	1: 4 years	330	35
			2: 7 months	47	61
CNS	49	49	1: 5 years and 3 months	386	6.4
			2: 2 years and 3 months	71	14.4
CNS	39	39	1: 4 years	407	15.1
			2: 2 years and 2 months	130	26
DLBCL	46	47	1: 5 years and 6 months	371	2
DLBCL	39	39	1: 6 years	219	26.7
			2: 3 years and 2 months	68	---
DLBCL	45	45	1: 6 years and 9 months	487	19
			2: 2 years and 4 months	228	---
DLBCL	56	---	1: 7 years and 4 months	523	13.2
			2: 1 year and 4 months	229	9.7

Abbreviation: BL: Burkitt Lymphoma; CNS: Central Nervous System Lymphoma; DLBCL: Diffusion Large B cell Lymphoma; B cell %: the percentage of B cell in peripheral blood mononuclear cell. ---: PBMC were not viable on thawing.

Table 2

The phenotypes of circulating B cells in pre-lymphoma and control cases

Group	CD10	CD71	CD28	CD38	CD86	CD257	AID
Pre-lymphoma	14 (1.0–46)	14 (2.5–45)	5.5 (1.7–19)	55 (27–90)	5.0 (0.8–11)	1.6 (0–14)	1.2 (0.1–8.1)
HIV+	4.0 (0.3–14)	8.2 (3.2–38)	5.8 (2.0–19)	43 (5.9–81)	2.0 (0.6–3.1)	0.9 (0.2–4.4)	1.0 (0.2–4.6)
HIV–	3.4 (0.9–5.8)	6.9 (2.4–14)	3.2 (1.5–5.9)	45 (5.2–69)	0.8 (0–0.9)	0.4 (0–4)	0.5 (0.1–12)
P value ^a	<0.01	<0.05	>0.05	>0.05	<0.01	>0.05	>0.05
P value ^b	<0.01	<0.01	>0.05	>0.05	<0.01	>0.05	<0.05

The numbers in each group represent the mean percentage of B cells positive for these markers with the range of values seen shown in parentheses.

^a comparing pre-lymphoma and HIV+ group.

^b comparing pre-lymphoma and HIV-negative group.