Circulating Mediators of Inflammation and Immune Activation in AIDS-Related Non-Hodgkin Lymphoma

Brian M. Nolen1, Elizabeth Crabb Breen2,3, Jay H. Bream4, Frank J. Jenkins1,5, Lawrence A. Kingsley6, Charles R. Rinaldo5, Anna E. Lokshin1,5,7,8

1 University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 2 Department of Psychiatry & Biobehavioral Sciences, David Geffen School of Medicine at UCLA, University of California Los Angeles, Los Angeles, California, United States of America, 3 UCLA AIDS Institute, University of California Los Angeles, Los Angeles, California, United States of America, 4 Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, United States of America, 5 Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 6 Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 7 Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 8 Department of Ob/Gyn, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America

Abstract

Background: Non-Hodgkin lymphoma (NHL) is the most common AIDS-related malignancy in developed countries. An elevated risk of developing NHL persists among HIV-infected individuals in comparison to the general population despite the advent of effective antiretroviral therapy. The mechanisms underlying the development of AIDS-related NHL (A-NHL) are not fully understood, but likely involve persistent B-cell activation and inflammation.

Methods: This was a nested case-control study within the ongoing prospective Multicenter AIDS Cohort Study (MACS). Cases included 47 HIV-positive male subjects diagnosed with high-grade B-cell NHL. Controls were matched to each case from among participating HIV-positive males who did not develop any malignancy. Matching criteria included time HIV+ or since AIDS diagnosis, age, race and CD4+ cell count. Sera were tested for 161 serum biomarkers using multiplexed bead-based immunoassays.

Results: A subset of 17 biomarkers, including cytokines, chemokines, acute phase proteins, tissue remodeling agents and bone metabolic mediators was identified to be significantly altered in A-NHL cases in comparison to controls. Many of the biomarkers included in this subset were positively correlated with HIV viral load. A pathway analysis of our results revealed an extensive network of interactions between current and previously identified biomarkers.

Conclusions: These findings support the current hypothesis that A-NHL develops in the context of persistent immune stimulation and inflammation. Further analysis of the biomarkers identified in this report should enhance our ability to diagnose, monitor and treat this disease.

Introduction

Non-Hodgkin lymphoma (NHL) is the most common AIDS-related malignancy in developed countries [1], where it accounts for 23–30% of AIDS-related death [2–4]. Although NHL also affects HIV-uninfected individuals, the risk of developing AIDS-associated NHL (A-NHL) is estimated to be 60 times greater in HIV-infected (HIV+) persons [5,6]. A recent study regarding the incidence of AIDS-related cancer in the years prior to and during widespread administration of highly active antiretroviral therapy (HAART) indicated that while HAART implementation is associated with a reduction in NHL rates among HIV+ persons, rates of A-NHL development remain significantly higher than those observed in uninfected populations [7]. Immunosuppression of any type appears to be a major risk factor for NHL.
development, with duration of HIV infection and nadir CD4-positive (CD4+ T cell counts positively and inversely associated with the risk of A-NHL, respectively [8,9]. The incidence of primary central nervous system lymphoma (PCNSL), a less prevalent subtype of NHL associated with immune deficiency, has been reported to be over 1000 times higher in persons with AIDS compared to the general population [10,11], although recent evidence suggests that this incidence is declining dramatically with the advent of HAART [12].

The precise pathogenic mechanism underlying A-NHL, development is a current focus of intense scrutiny, and several basic themes have emerged. Immune deficiency appears to contribute to the development of A-NHL through the loss of T-cell mediated control over B-cell proliferation. This mechanism appears to be particularly important in the development of Epstein-Barr virus (EBV)-positive A-NHL (reviewed in [13]). The persistently elevated incidence of A-NHL despite the advent of HAART argues for additional pathogenic mechanisms including hyperactivation of B-cells. Chronic B-cell hyperactivation associated with genetic abnormalities due to EBV or human herpesvirus-8 (HHV-8) infection, c-MYC and BCL-6 rearrangements, RAS or p53 mutations, and 6 q deletions is believed to play a role in A-NHL [6,14,15]. Direct integration of HIV into the genome of malignant B-cells has not been observed experimentally, however persistent antigenic stimulation of B-cells during HIV infection may promote hyperactivation and transformation [16–19]. Another proposed mechanism suggests that HIV-infected macrophages contribute stimulatory signals in order to create a microenvironment permissive to malignant B-cell growth [20].

The proposed pathogenic pathways underlying the development of A-NHL suggest a complex network of interactions between various components of the immune system. Cytokines and other inflammatory mediators represent the chief means by which these components mediate those interactions. A number of groups have evaluated serum biomarkers in an effort to more fully characterize the dysregulated cytokine signalling underlying the immunological mechanisms at work in the development of A-NHL. A number of proteins involved in B-cell activation, stimulation and other inflammatory functions have been implicated by the findings of those groups including: IL-6 [21,22], IL-10 [21–23], IP-10 [22], neopterin [22], immunoglobulin free light chains [22], sCD30 [21,24], sCD23 [21,25], sCD44 [26], CRP [21], CXCL13 [27,28], and TNGfs [22]. Many of these proteins were observed at elevated serum levels in subjects prior to the diagnosis of A-NHL with a lead time ranging from several months to several years [21,23,24,26]. These findings suggest that the analysis of serum biomarker levels in HIV+ individuals may help in risk assessment strategies for NHL or in the development of prophylactic or preventive measures through an improved understanding of the early events of malignancy.

In the current study, we sought a broad evaluation of serum biomarkers that could possibly be associated with the development of NHL in HIV+ individuals. To that end, multiplexed bead-based immunoassays were utilized in the evaluation of serum levels of 161 proteins in subjects diagnosed with A-NHL and controls.

Materials and Methods

Ethics Statement

All subjects involved in this study were over the age of 18 and provided written informed consent. Specimen collection procedures were approved by the respective Institutional Review Board (IRB) at each collection site: Johns Hopkins Medicine IRB, Northwestern University IRB, University of California, Los Angeles IRB, University of Pittsburgh IRB. The current study was approved by the University of Pittsburgh IRB.

Study Population

The Multicenter AIDS Cohort Study (MACS) is an ongoing prospective study of the natural and treated histories of HIV-1 infection in homosexual and bisexual men conducted at sites located in Baltimore, Chicago, Pittsburgh and Los Angeles [29]. A total of 6,972 men have been enrolled. For the current analysis we obtained archived sera from HIV+ subjects diagnosed with high grade B-cell NHL (according to the MACS data repository) for which serum was available either immediately prior to (≤12 months, n = 37) or shortly after (≤3 months, n = 10) NHL diagnosis. For each case, a single matched control was identified from among HIV+ participants who did not develop any malignancy. Cases for which NHL was AIDS-defining (n = 25) were matched to controls who had not yet received an AIDS diagnoses at the time of blood collection, based on duration of HIV+ status (+/−1 year). Cases for which NHL developed subsequent to an AIDS diagnosis (n = 22) were matched to controls who had developed an AIDS-defining opportunistic infection (OI), based on time since AIDS diagnosis (+/−1 year). Matching criteria also included age (+/−5 yrs) and absolute CD4+ T cell count (+/−100 cells/mm3) at the time of blood collection in the case or matched visit in the control, and race (white or black). Following matching, a total of 49 cases and 49 controls were selected. Sera obtained for three subjects (2 cases, 1 control) were not evaluable due to insufficient volume or hemolysis. In the resulting groups of 47 cases and 48 controls (Table 1), age, race distribution, CD4+ T cell counts, and duration of HIV positivity did not differ significantly (p>0.05), reflecting the original matching criteria. The final experimental cohort included 46 matched pairs. 46 of the cases included in the current study overlap with those used in recent studies utilizing the MACS cohort [21,27,30]. In the majority of overlapping cases, the date of serum collection for the current study was closer to or after the A-NHL diagnosis date, compared to previous studies, as determined by the specific selection criteria. Information on NHL subtypes was obtained from the MACS data repository. HIV viral load values obtained at time of blood draw for biomarker analysis were available from the MACS data repository for 36 case and 22 control subjects. Viral load values were not utilized for matching but were utilized in subsequent biomarker analyses. Information on antiretroviral therapy was obtained from the MACS data repository for each case and control. HAART regimens were defined according to the DHHS/Kaiser guidelines [31]. Subjects were classified as current recipients if they received HAART at the same visit as their blood draw or during the interim time in between this visit and the previous MACS visit. Subjects were classified as former recipients if they received HAART prior to but not during this time period. Subjects classified as never receiving HAART may have received other antiretroviral therapy not classified as HAART. The characteristics of the study population are presented in Table 1.

Sources of bead-based immunoassays

A combination of 161 bead-based immunoassays for a diverse set of serum biomarkers available on the Lumixen platform was utilized in this study (Table 2). This list of biomarkers was assembled based on a literature review of proteins and families of proteins of interest in all areas of HIV and NHL research. Biomarkers were selected from this list on the basis of suitable immunoassay availability. A total of 29 multiplexed and individual assays were utilized (Table S1). Bead-based immunoassays
targeting ErbB2, EGFR, Cytokeratin 19 (Cyfra 21-1), mesothelin (MSLN), AFP, CA 72-4, EPCAM, PSA, transthyretin (TTR), angiostatin (AS), thrombospondin (TSP), endostatin (ES), TgII, HE4, CA 15-3, CA 125, CEA, and Kallikrein 10 (Klk10) were developed by the UPCI Luminex Core Facility according to strict quality control standards[32]. Assays for MMP 1, 2, 3, and 7 and TIMP 1–4 were obtained from R&D Systems (Minneapolis, MN). Assays for PDGF-BB, RANTES, SCGF-B, and NGF were obtained from Bio-Rad (Hercules, CA).

Assays for HSP27, IL-1α, IL-1β, IL-3, IL-5, IL-7, IL-8, IL-12p40, IL-13, IL-17, TNF-α, IFNγ, GM-CSF, MCP-1, MCP-3, MIP-1α, MIP-1β, IP-10, Eotaxin (CCL11), RANTES, DR5, EGF, FGFβ, G-CSF, HGF, VEGF, and GROα were obtained from Life Technologies/Biosource (Grand Island, NY). All other assays were obtained from Merck/Millipore (Durnstadt, Germany). All commercial immunoassays were performed according to manufacturer’s protocols while UPCI Luminex Core Facility assays were performed as previously described [32,33].

Collection and statistical analysis of biomarker data

Each serum sample was analyzed for each multiplex immunoassay using the Bio-Plex suspension array system and the Bio-Plex Manager software (version 4.1) (Bio-Rad Laboratories, Hercules, CA). For each analyte, 100 beads were analyzed and the median fluorescence intensity was determined. Analysis of serum sample biomarker data was performed using five-parameter logistic curve fitting to standard analyte values. A number of samples fell below lower limit of extrapolation of the Bio-Plex Manager software for certain analytes and were therefore reported as out of range low (OOR). The percentages of cases and controls classified in this manner for each analyte are listed in Table S2. Observed concentration values were assigned to each of the OOR samples by manual extrapolation using the curve fitting parameters determined by the Bio-Plex software. Hence, no samples were excluded from the analysis on the basis of undetectability. The entire biomarker dataset has been deposited with the Center for Analysis and Management of Multicenter AIDS Cohort Study (CAMACS).

Biomarker distributions among the A-NHL and control groups were analyzed using both parametric and non-parametric statistical tests. In each analysis the minimum level of significance was p < 0.05. For the parametric analysis, exact conditional logistic regression was performed on the biomarker data obtained for the A-NHL and control groups (n = 46 matched case-control pairs). The results were further analyzed using the Bonferroni Step-Down (Holm) correction for multiple comparisons. For the non-parametric analysis, the Mann-Whitney U test was utilized on data from 47 cases and 48 controls without regard to matching. Here, the False Discovery Rate (FDR) was controlled at 5% according to the method described by Benjamini and Hochberg[34]. Biomarker variations among several subtypes of NHL were evaluated using a one-way ANOVA with Tukey’s multiple...
Results

Viral load were evaluated using the Spearman test for correlation. Comparison test. Correlations between biomarker levels and HIV viral load were evaluated using the Spearman test for correlation.

Comparison test. Correlations between biomarker levels and HIV viral load were evaluated using the Spearman test for correlation.

Pathway Analysis

The Ingenuity Pathway Analysis (IPA) software package (Ingenuity Systems, Inc., Redwood City, CA) was used to examine pathways and interactions associated with the most promising biomarkers identified in our analysis described above. Additional serum biomarkers demonstrating associations with A-NHL in previous reports, which were not measured in the current investigation, were also included in this analysis. The list of additional biomarkers included IL-6 [21], IL-10 [21,23,30], sCD30 [21,24], sCD23 [21], sCD44 [26], sCD27 [21], CRP [21], and CXCL13 [27,28]. Some of these reports utilized a number of overlapping MACS NHL cases as described above. The IPA analysis identifies interactions based solely on the identity of the biomarkers into the software and did not utilize biomarker concentrations.

Table 2. Complete List of Evaluated Biomarkers.

| Tumor Markers | AFP, CA 15-3, CA 19-9, CA125, CA22-4, CEA, EPCAM, HE4, Kallikrein-10 (Klk10), Mesothelin (MLN), PSA, squamous cell carcinoma antigen (SCC), tissue transglutaminase (Tgl) |
| Growth/Angiogenesis Factors | Angiostatin (AS), EGF, EGFRI, Endostatin (ES), ErbB2, FGFb, HGF, IGFBP-1-7, NGF, PDGF-BB, SCGF-B, sVEGF-1, sVEGFRI-2, TGFα, Thrombospondin (TSP) |
| Hormones | ACTH, Cortisol, FSH, GH, Insulin, LH, Prolactin, PTH, TSH |
| Apoptosis Factors | Cyfa, Fas, FasL |
| Cytokines/Chemokines/Receptors | 6Ckine/CCL21, CTACK/CCL27, DRS, ENA-78/CXCL5, EOTAXIN-1 (CCL11), EOTAXIN-2/CCL24/MPF-2, EOTAXIN-3/CCL26, FLLT3, Fractalkine, GCP-2/CCL6/LIX, G-CSF, GM-CSF, GROα, HCC-1/CCL14a, I-309/CCL1, IFNγ, IL-1α, IL-1β, IL-3, IL-5, IL-7, IL-8, IL-12p70, IL-12p40, IL-15, IL-17a, IL-17b, IL-28b, IL-29, IL-33, NF-HEV(mat), IP-10, I-TAC/CXCL11, IL-37, Lymphotactin, MCP-1-4, M-CSF, MDC, MIF, MIP-1α, MIP-1β, MIP-1β/MP-5/CCL15, MIP-3α/CCL20, MIP-3β/CCL19, MIP-4, MPO, NAP-2/CCL2, RANTES, sCD40L, SCF, SDF-1a/b/CXCL12, sIL-1RI, sIL-4R, sIL-6R, sRAGE, sTNFα, sTNFβ, TARC/CCL17, TGFα, TGFβ, TPO, TRAIL/TNFα, TSLP |
| Adhesion Molecules | E-Selectin, Fibronectin (FN), sCAM1, sCAM2 |
| MMPs/TIMPs | MMP-1-3, 7-9, 12,13, TIMP-1-4 |
| Bone Factors | Osteoprotegerin (OPG), Osteocalcin (OC), Osteopontin (OPN), RANKL |
| Apolipoproteins | Apo A1, Apo CII, Apo E |
| Acute Phase Proteins | α1-Antitrypsin (A1A), α2-Macroglobulin, Complement C3 (C3), Complement C4, Complement Factor H (CFH), Fibrinogen (FBN), Haptoglobin (HPN), human serum albumin (HSA), transthyretin (TTR), serum amyloid A (SAA), serum amyloid P (SAP) |
| Adipokines | Adiponectin, aPAl-1, Leptin, Resistin, sPIAl-1 |
| Other | HSP 70, Involucrin, pan-Keratin (1,10,11), Keratin-6, LPS, MICA, PEDF |

doi:10.1371/journal.pone.0099144.t002

Correlation with HIV viral load

Biomarker measurements from the A-NHL and control groups were combined in order to identify correlations between specific biomarkers and HIV viral load. Among the complete panel of 161 biomarkers, a total of 17 biomarkers exhibited significant correlations with HIV viral load (data not shown). Most notably, 11 of the significantly correlated biomarkers were among the consensus biomarkers described above, and are presented in Table 5. With the notable exception of MMP-9, each of the significant correlations among the consensus biomarkers was consistent with HIV progression, i.e., higher biomarker concentrations were correlated with higher HIV viral load.

A-NHL subtype specific biomarker alterations

Each of the 17 consensus biomarkers was evaluated in case subsets including subjects diagnosed with B-cell diffuse, CNS, or Burkitt’s Lymphoma (BL/BL-like NHL) using pair-wise one-way ANOVA. As this analysis included cases only, biomarker levels were normalized to CD4 counts prior to ANOVA to account for variations in disease severity. In a comparison of BL/BL-like and CNS NHL cases, OC and Timp-1 were significantly different with p<0.05. In the comparison of CNS and B-cell diffuse NHL cases, three significant alterations were observed including OC, SAP and Timp-1 (data not shown). In each comparison, significantly

mean values, respectively, and slightly different numbers of subjects. The application of the Bonferroni Step-down (Holm) correction to our parametric analyses yielded a restricted list of three biomarkers (IL-11, CXCL11, IL-29) which were observed to be highly significant (Table 4). A comparison of the non-parametric and parametric analyses prior to Bonferroni correction (overlap between tables 3 and 4) yielded a consensus list of 17 serum biomarkers significantly altered in the comparison of the A-NHL and control groups. The consensus list included cytokines/chemokines (CCL19, CXCL11, MCP-2, MIP-1β, IFNγ, IL-11, IL-29, IP-10, M-CSF, sIL-1RI1, acute phase proteins (C3, HPN, SAA, SAP), tissue remodeling factors (MMP-9, TIMP-1), and the bone metabolic factor OC.

DOI: 10.1371/journal.pone.0099144.t002
Table 3. Serum biomarker levels in AIDS-NHL subjects and controls evaluated by Non-parametric Statistics.

| Biomarker        | A-NHL (n = 47) | Control (n = 48) | A-NHL vs. Control |
|------------------|----------------|-----------------|------------------|
|                  | Median | IQR      | Median | IQR      | MWU p value (trend) |
| CCL19/MIP-3β     | 238.0  | 133.0    | 197.0  | 112.0    | 6.89E-03 (I)         |
| C3β              | 111.0  | 53.7     | 80.9   | 33.0     | 1.52E-03 (I)         |
| CXCL11/TAC       | 266.0  | 393.0    | 132.0  | 128.0    | 9.31E-05 (I)         |
| FBNβ             | 161.0  | 151.0    | 84.6   | 95.1     | 8.20E-04 (I)         |
| HPNβ             | 4054.0 | 3284.0   | 3041.0 | 2546.0   | 8.23E-03 (I)         |
| IFN-α            | 34.1   | 43.6     | 20.5   | 13.9     | 1.89E-03 (I)         |
| IL-11            | 278.0  | 163.0    | 146.0  | 145.0    | 9.97E-06 (I)         |
| IL-29/IFN-γ1     | 284.0  | 193.0    | 200.0  | 100.0    | 6.97E-04 (I)         |
| IP-10            | 98.2   | 96.0     | 60.2   | 53.4     | 1.48E-03 (I)         |
| MCP-2            | 71.3   | 56.1     | 53.5   | 35.8     | 8.32E-03 (I)         |
| M-CSF            | 317.0  | 554.0    | 86.8   | 250.0    | 2.45E-03 (I)         |
| MIP-1α           | 3720.0 | 2267.0   | 2816.0 | 1941.0   | 8.05E-03 (I)         |
| MPP-9β           | 81.0   | 76.5     | 128.0  | 133.0    | 5.88E-03 (D)         |
| OC               | 3811.0 | 2644.0   | 4536.0 | 2808.0   | 7.79E-03 (D)         |
| SAAβ             | 591.0  | 3361.0   | 295.0  | 462.0    | 9.69E-03 (I)         |
| SAPβ             | 4934.0 | 1962.0   | 3647.0 | 1207.0   | 2.69E-04 (I)         |
| sIL-1R1          | 26.8   | 15.7     | 21.1   | 8.10     | 7.37E-03 (D)         |
| TIMP-1β          | 160.0  | 50.6     | 137.0  | 33.8     | 4.57E-03 (I)         |
| A1α              | 1769.0 | 2124.0   | 1062.0 | 810.0    | 3.28E-03 (I)         |

IQR – interquartile range; MWU - Mann Whitney U test with 5% False Discovery Rate; Biomarkers in bold represent consensus markers also selected by parametric statistics; Values expressed in ng/ml, all others expressed in pg/ml; Trends: I – increased in A-NHL group, D – decreased in A-NHL group.
doi:10.1371/journal.pone.0099144.t003

Discussion

A diverse analysis of serum proteins in samples collected just before or after the time of A-NHL diagnosis identified a consensus list of potential A-NHL biomarkers, assembled through rigorous statistical analysis, which included various inflammatory mediators, acute phase proteins and tissue remodeling agents. Although our panel of candidate biomarkers was considerably large and composed primarily of inflammatory and immune-modulating factors, a relatively small subset was observed to be altered, suggestive of some level of specificity for A-NHL with respect to HIV+ status or other AIDS defining illness. Increased levels of a number of circulating proteins have previously been reported up to 3–5 years prior to the diagnosis of A-NHL, including IL-6, IL-10, sCD23, sCD27, sCD44, sCD27, CRP, CXCL13, and IgE. An extensive array of interactions within and among the two lists of biomarkers was identified (Figure 1). With the exceptions of MIP-1α, IFN-γα, and OC, each of the newly identified biomarkers was found to interact with at least one previously identified biomarker. MMP-9 demonstrated the most extensive network of interactions including six interactions with other newly identified biomarkers and four interactions with previously identified biomarkers. Other newly identified biomarkers demonstrating more than five interactions included IP-10 (7 new/2 previous), M-CSF (3 new/4 previous), sIL-1R1 (4 new/2 previous), TIMP-1 (3 new/3 previous), C3 (3 new/3 previous), and SAP (2 new/4 previous). Many of the previously identified biomarkers also displayed numerous interactions within and among the groups.

elevated levels of each biomarker were observed in CNS NHL relative to the other subtypes.

Pathway Analysis

The IPA software package was first used to analyze the consensus list of 17 serum biomarkers identified in this study. The top biological functions associated with the consensus list included Cellular Movement (14/17 molecules, p = 1.6 x 10^-14–1.9 x 10^-6), Cell-to-Cell Signaling and Interaction (13/17 molecules, p = 1.4 x 10^-19–1.9 x 10^-3), Hematological System Development and Function (14/17 molecules, p = 1.6 x 10^-14–1.9 x 10^-3), and Immune Cell Trafficking (14/17 molecules, p = 1.6 x 10^-14–1.9 x 10^-3). Interactions between molecules were then examined for each biomarker included in the consensus list and several biomarkers examined in association with A-NHL in previous studies including IL-6, sCD30, IL-10, sCD23, sCD44, sCD27, CRP, CXCL13, and IgE. An extensive array of interactions within and among the two lists of biomarkers was identified (Figure 1). With the exceptions of MIP-1α, IFN-γα, and OC, each of the newly identified biomarkers was found to interact with at least one previously identified biomarker. MMP-9 demonstrated the most extensive network of interactions including six interactions with other newly identified biomarkers and four interactions with previously identified biomarkers. Other newly identified biomarkers demonstrating more than five interactions included IP-10 (7 new/2 previous), M-CSF (3 new/4 previous), sIL-1R1 (4 new/2 previous), TIMP-1 (3 new/3 previous), C3 (3 new/3 previous), and SAP (2 new/4 previous). Many of the previously identified biomarkers also displayed numerous interactions within and among the groups.

The conservative use of the Bonferroni correction as part of our parametric statistical analysis suggested that serum levels of IL-11, CXCL11 and IL-29 demonstrated the strongest associations with A-NHL in our study. These findings further agree with the hypothesized immune activating origins of this disease. IL-11, an important mediator of hematopoiesis, has been investigated as an
CXCL11 is a chemokine known to mediate the migration of activated T-cells, and has been described as an immunomodulatory target of miRNA in primary lymphoma and has also demonstrated potent antitumor activity in an animal model [38,39]. The type III interferon IL-29 is the focus of considerable interest in the setting of hepatitis C viral infection and was recently shown to impede HIV-1 infection in T-cells [40,41]. A role in the development of A-NHL for IL-29 has not yet been described.

A number of additional proteins with varying biological functions but unifying roles in inflammation and immune activation were also associated with A-NHL in univariate analyses in the current study, but were not statistically significant after Bonferroni correction. In such a broad survey, this is not unexpected, but the data does confirm previous reports of some of these proteins being associated with inflammatory processes around the time of A-NHL or NHL diagnosis. Several of the chemoattractant proteins (CCL19/MIP-3β, MCP-2, CXCL10/
B-cell lymphoma within 0–5 years [44]. Elevated pretreatment marker. In two recent studies, elevated levels of IP-10 in the sera of patients with AIDS-related non-Hodgkin lymphoma (A-NHL) have been reported to have an association with various forms of leukemia and lymphoma [42,43], 41. Of particular interest for A-NHL is IP-10, a leukocyte chemoattractant currently under investigation as a potential therapeutic agent in lymphoma, including A-NHL, stemming from its ability to mediate apoptosis in malignant cells [52–54].

Many of the biomarkers found to be significantly altered in A-NHL subjects appear to be positively associated with HIV disease progression, as indicated by their observed correlation with HIV viral load (Table 5). Each of the biomarkers found to be positively correlated with viral load were also observed to be elevated in the sera of A-NHL cases in comparison to the controls. As CD4 count was included in our matching criteria and was not significantly different between the two experimental groups, we propose that varying degrees of control of HIV (or lack thereof) within each experimental group, as opposed to among the groups, led to our observations. These observations are in line with the hypothesis that increases in HIV viral burden result in elevated levels of immune stimulation and inflammation which then promotes NHL pathogenesis. Based on these findings, we may also hypothesize that HIV-driven alterations in immune response are augmented in A-NHL, a phenomenon that warrants additional investigation. In contrast to other biomarkers, higher MMP-9 levels correlated with lower viral load and control (non-malignancy) status. A direct relationship between increased MMP-9 activity and immune competence and/or protection from lymphoma has not been described, leading us to speculate that the relationship between MMP-9 and A-NHL may be a protective one which warrants further exploration.

When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found. When A-NHL subjects of differing disease subtypes were evaluated separately, relatively few significant alterations were found. Several biomarkers including OC, SAP, and Timp-1 were evaluated separately, relatively few significant alterations were found.

### Table 5. Spearman Correlations of Consensus Biomarkers and HIV Load among all subjects.*

| Biomarker | Spearman r | p     |
|-----------|------------|-------|
| CCL19/Mip-3β | 0.67       | 0.0001|
| CXCL11/TAC  | 0.52       | 0.0001|
| IL-11      | 0.50       | 0.0001|
| IP-10      | 0.46       | 0.0003|
| MCP-2      | 0.42       | 0.001 |
| IFN-α      | 0.42       | 0.0011|
| M-CSF      | 0.41       | 0.0013|
| SAP        | 0.40       | 0.0019|
| MMP-9      | −0.38      | 0.0029|

*Analysis included all subjects with available HIV load measurement (n = 58).

doi:10.1371/journal.pone.0099144.t005

Immunological Biomarkers in AIDS-Related Non-Hodgkin Lymphoma
In the current study, an analysis of a broad array of circulating proteins yielded a relatively small but robust collection of inflammatory mediators associated with the development of A-NHL in comparison to controls. Many of the identified biomarkers were positively correlated to HIV viral load and indicate heightened immune responses in A-NHL with respect to HIV infection and/or AIDS in the absence of lymphoma. A minority of the subjects included in the current study were former or current recipients of HAART. Although the distribution of HAART recipients was similar among cases and controls, we cannot rule out some effect of treatment on our biomarker findings. Our findings further support the hypothesis that A-NHL develops in response to persistent immune activation and inflammation and adds new points of focus for further investigation. Ongoing analysis into the precise role of each of the biomarkers identified in our report should enhance our ability to diagnose, monitor and treat this disease.

![A-NHL biomarker pathway analysis](https://example.com/image.png)

**Figure 1. A-NHL biomarker pathway analysis.** The Ingenuity Pathway Analysis (IPA) software package was utilized to identify pathways and specific interactions associated with the serum biomarkers identified in the current and previous reports. Biomarkers evaluated from the current study (rectangles) included CCL19, CXCL11, MCP-2, MIP-1α, IFN-α, IL-11, IL-29, IP-10, M-CSF, sIL-1R1, C3, HPN, SAA, SAP, MMP-9, TIMP-1 and OC. Biomarkers evaluated based on previous findings (ovals) included IL-6, sCD23, IL-10, sCD23, sCD44, sCD27, CRP, BCA-1/CXCL13 and IgE. Solid lines indicate direct interactions, dashed lines indicate indirect interactions. doi:10.1371/journal.pone.0099144.g001
Table S2 Percentage of samples classified as out of range low according to the Bioplex Manager software.

Author Contributions

Conceived and designed the experiments: BMN ECB LAK CRR AEL. Performed the experiments: BMN AEL. Analyzed the data: BMN ECB IFJ LAK CRR AEL. Contributed reagents/materials/analysis tools: ECB JHB CRR AEL. Contributed to the writing of the manuscript: BMN ECB IFJ LAK CRR AEL.
47. Citak EC, Oguiz A, Karadeniz C, Akyurek N (2008) Role of gelatinases (MMP-2 and MMP-9), TIMP-1, vascular endothelial growth factor (VEGF), and microvessel density on the clinicopathological behavior of childhood non-Hodgkin lymphoma. Pediatr Hematol Oncol 25: 53–66.

48. Hottinger AF, Isamoto FM, Karimi S, Riedel E, Dantis J, et al. (2011) YKL-40 and MMP-9 as serum markers for patients with primary central nervous system lymphoma. Ann Neurol 70: 163–169.

49. Tarantul VZ, Nikolaev AI, Martyrezko A, Hennig H, Hunsmann G, et al. (2000) Differential gene expression in B-cell non-Hodgkin’s lymphoma of SIV-infected monkey. AIDS Res Hum Retroviruses 16: 173–179.

50. Bassig BA, Zheng T, Zhang Y, Berndt SI, Holford TR, et al. (2012) Polymorphisms in complement system genes and risk of non-Hodgkin lymphoma. Environ Mol Mutagen 53: 144–151.

51. Marquart HV, Olesen EH, Johnson AA, Damgaard G, Leslie RG (1997) A comparative study of normal B cells and the EBV-positive Burkitt’s lymphoma cell line, Raji, as activators of the complement system. Scand J Immunol 46: 246–253.

52. Harrington WJ Jr, Cabral L, Cai JP, Chan ASS, Wood C (1996) Azothymidine and interferon-alpha are active in AIDS-associated small non-cleaved cell lymphoma but not large-cell lymphoma. Lancet 348: 833.

53. Lee RK, Cai JP, Deyev V, Gill PS, Cabral L, et al. (1999) Azidothymidine and interferon-alpha induce apoptosis in herpesvirus-associated lymphomas. Cancer Res 59: 5514–5520.

54. Toomey NL, Deyev VV, Wood C, Boise LH, Scott D, et al. (2001) Induction of a TRAIL-mediated suicide program by interferon alpha in primary effusion lymphoma. Oncogene 20: 7029–7040.