



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

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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.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data have been deposited with the Center for Analysis and Management of Multicenter AIDS Cohort Study (CAMACS) and may be accessed by contacting the CAMACS administrators <http://www.statepi.jhsph.edu/macsc/macsc.html>.

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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 TNF α [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 diagnosis 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 Luminex 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

Table 1. Characteristics of Study Population.

	A-NHL Cases (n = 47)	Controls (n = 48)
Age (years), range (median)	25–56 (42)	26–56 (40)
Race, n (%)		
White	46 (98)	47 (98)
Black	1 (2)	1 (2)
CD4+ T Cell Count (cells/mm ³), range (mean)	7–788 (146)	6–794 (193)
Receiving HAART [31], n (%)		
Current	6 (13)	5 (10)
Former	1 (2)	1 (2)
Never	38 (81)	42 (87.5)
Unknown	2 (4)	0 (0)
HBV Serology, n (%)		
Positive	41 (87)	42 (87.5)
Negative	3 (6)	6 (12.5)
Unknown	3 (6)	0 (0)
HHV-8 Serology, n (%)		
Positive	28 (57)	35 (73)
Negative	9 (19)	9 (19)
Unknown/Indiscriminate	10 (21)	4 (8)
NHL Subtype, n (%)		
B-cell Diffuse	20 (43)	
CNS	11 (23)	
BL, BL-like	7 (15)	
NHL, NOS	6 (13)	
Other	3 (6)	

HAART – highly active antiretroviral therapy; HBV/HHV-8 status obtained at time of blood draw; CNS – central nervous system; BL – Burkitt's lymphoma; NOS – not otherwise specified.

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targeting ErbB2, EGFR, Cytokeratin 19(Cyfra 21-1), mesothelin (MSLN), AFP, CA 72-4, MICA, SCC, HSP 70, 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-1 α , IL-1 β , IL-3, IL-5, IL-7, IL-8, IL-12p40, IL-15, IL-17, TNF- α , IFN α , GM-CSF, MCP-1, MCP-3, MIP-1 α , MIP-1 β , IP-10, Eotaxin (CCL11), RANTES, DR5, EGF, FGFb, G-CSF, HGF, VEGF, and GRO α were obtained from Life Technologies/Biosource (Grand Island, NY). All other assays were obtained from Merck/Millipore (Darmstadt, 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 regards 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

Table 2. Complete List of Evaluated Biomarkers.

Tumor Markers	AFP, CA 15-3, CA 19-9, CA125, CA72-4, CEA, EPCAM, HE4, Kallikrein-10 (Kik10), Mesothelin (MSLN), PSA, squamous cell carcinoma antigen (SCC), tissue transglutaminase (TgII)
Growth/Angiogenesis Factors	Angiostatin (AS), EGF, EGFR, Endostatin (ES), ErbB2, FGFb, HGF, IGFBP 1-7, NGF, PDGF-BB, SCGF-B, sVEGF-1, sVEGFR2-3, TGF α , Thrombospondin (TSP)
Hormones	ACTH, Cortisol, FSH, GH, Insulin, LH, Prolactin, PTH, TSH
Apoptosis Factors	Cyfra 21-1, Fas, FasL
Cytokines/Chemokines/Receptors	6CKine/CCL21, CTACK/CCL27, DR5, ENA-78/CXCL5, EOTAXIN-1 (CCL11), EOTAXIN-2/CC124/MPIF-2, EOTAXIN-3/CCL26, FLT3, Fractalkine, GCP-2/CXCL6/LIX, G-CSF, GM-CSF, GRO α , HCC-1/CCL14a, I-309/CCL1, IFN α , IL-1 α , IL-1 β , IL-3,5,7,8,11, IL-12p70, IL-12p40, IL-15-17,20, IL-28 α , IL-29, IL-33/NF-HEV(mat), IP-10, I-TAC/CXCL11, LIF, Lymphotactin, MCP-1-4, M-CSF, MDC, MIF, MIP-1 α , MIP-1 β , MIP-1 δ /MIP-5/CCL15, MIP-3 α /CCL20, MIP-3 β /CCL19, MIP-4, MPO, NAP-2/CXCL7, RANTES, sCD40L, SCF, SDF-1a+b/CXCL12, sIL-1RII, sIL-4R, sIL-6R, sRAGE, sTNF –RI, sTNF-RII, TARC/CCL17, TNF α , TNF β , TPO, TRAIL/TNFSF10, TSLP
Adhesion Molecules	E-Selectin, Fibronectin (FN), sICAM, sVCAM
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 CIII, Apo E
Acute Phase Proteins	α 1-Antitrypsin (A1A), α 2-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, aPAI-1, Leptin, Resistin, tPAI-1
Other	HSP 70, Involucrin, pan-Keratin (1,10,11), Keratin-6, LPS, MICA, PEDF

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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 inputted into the software and did not utilize biomarker concentrations.

Results

Biomarker alterations among A-NHL cases and controls

The non-parametric analysis of biomarker distributions among all cases and controls revealed that 19 out of the 161 biomarkers tested were significantly altered in the comparison of A-NHL cases and controls (Table 3). Among these 19 significant biomarkers, two (MMP-9, OC) were observed to be decreased in the A-NHL group in comparison to the controls, while the remaining 17 were increased. The parametric statistical analysis on 46 case-control pairs yielded a larger list of 37 significant biomarkers (Table 4) which included all but two biomarkers (FBN, A1A) identified in the non-parametric analysis. Among the 37 significant biomarkers in this analysis, four (OC, ApoA1, MMP-9, IGFBP-6) were observed to be decreased in the A-NHL subjects relative to the control groups, while the remaining 33 biomarkers were all increased. All trends in biomarker alterations between the cases and controls were consistent among the non-parametric and parametric analyses despite the differential use of median and

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-1 δ , IFN α , IL-11, IL-29, IP-10, M-CSF, sIL-1R1), acute phase proteins (C3, HPN, SAA, SAP), tissue remodeling factors (MMP-9, TIMP-1), and the bone metabolic factor OC.

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 were 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

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	133	197	112	6.89E-03	(I)
C3†	111	53.7	80.9	33.0	1.52E-03	(I)
CXCL11/I-TAC	266	393	132	128	9.31E-05	(I)
FBN †	161	151	84.6	95.1	8.20E-04	(I)
HPN†	4054	3284	3041	2546	8.23E-03	(I)
IFN-α	34.1	43.6	20.5	13.9	1.89E-03	(I)
IL-11	278	163	146	145	9.97E-06	(I)
IL-29/IFNλ1	284	193	200	100	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	554	86.8	250	2.45E-03	(I)
MIP-1δ	3720	2267	2816	1941	8.05E-03	(I)
MMP-9†	81.0	76.5	128	133	5.88E-03	(D)
OC	3811	2644	4536	2808	7.79E-03	(D)
SAA†	591	3361	295	462	9.69E-03	(I)
SAP†	4934	1962	3647	1207	2.69E-04	(I)
sIL-1R1	26.8	15.7	21.1	8.10	7.37E-03	(I)
TIMP-1†	160	50.6	137	33.8	4.57E-03	(I)
A1A †	1769	2124	1062	810	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.

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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, $p = 1.6 \times 10^{-14}$ – 1.9×10^{-3}), Cell-to-Cell Signaling and Interaction (13/17 molecules, $p = 1.4 \times 10^{-10}$ – 1.9×10^{-3}), Hematological System Development and Function (14/17 molecules, $p = 1.6 \times 10^{-14}$ – 1.9×10^{-3}), and Immune Cell Trafficking (14/17 molecules, $p = 1.6 \times 10^{-14}$ – 1.9×10^{-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-1 δ , IFN α , 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.

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, sCD30, sCD44, IgE, CRP, and BCA-1/CXCL13 [21–24,26–28,30,35,36]. While the current study did not include a direct examination of these biomarkers, an analysis of biomarker interactions revealed a number of mechanistic links between and among those biomarkers identified both previously and currently (Figure 1). The most recent longitudinal study of A-NHL risk in HIV+ persons identified IP-10 and TNF α , in addition to several others, as potential biomarkers [22]. The current cross-sectional analysis evaluated both of these biomarkers but found only IP-10 to be significantly altered.

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

Table 4. Conditional Logistic Regression Analyses with Bonferroni Step-down Adjustment of Serum Biomarker Levels of AIDS-NHL and control subjects.

Biomarker	A-NHL (n = 46)		Controls (n = 46)		Exact p-value	Bonferroni Adjusted p-value
	Mean	SD	Mean	SD		
ApoA1	1267	252	1374	213	0.0111	NS
CA 19-9	4.97	26.9	0.490	0.197	0.0013	NS
CCL19/MIP-3β	648	2777	196	77.5	0.0025	NS
CCL20/MIP-3 α	47.2	188	11.5	7.51	0.0056	NS
C3	111	47.3	81.9	30.3	0.0003	NS
CFH	559	169	473	94.8	0.0016	NS
CXCL11/I-TAC	436	535	175	151	0.0001	0.017
CXCL6/GCP-2	342	1362	114	51.7	0.0138	NS
CCL11	212	145	190	70.7	0.0061	NS
G-CSF	176	325	73.8	71.9	0.0023	NS
HPN[†]	5110	4037	3148	2284	0.0093	NS
I-309	11.3	29.9	3.39	3.52	0.0126	NS
IFNα	13365	87796	65.5	189	0.0407	NS
IGFBP-3 [†]	1524	248	1420	328	0.0085	NS
IGFBP-5 [†]	120	72.6	90.8	58.3	0.0124	NS
IGFBP-6 [†]	149	42.0	169	64.0	0.0274	NS
IL-11	5663	36435	176	97.9	<0.0001	<0.017
IL-20	115	253	56.8	34.3	0.0177	NS
IL-28A	555	3128	16.0	20.4	0.0249	NS
IL-29	11685	76491	232	127	0.0001	0.017
IL-7	49.7	146	18.4	7.37	0.0066	NS
IP-10	130	93.0	86.8	91.4	0.0173	NS
MCP-2	88.0	66.9	61.5	36.1	0.025	NS
M-CSF	15887	105262	277	405	0.0065	NS
MIF	964	2916	246	181	0.0402	NS
MIP-1δ	5617	7942	3295	2265	0.0056	NS
MIP-4 [†]	22.2	19.4	16.0	7.88	0.0205	NS
MMP-3 [†]	57.5	163	18.1	7.30	0.0453	NS
MMP-9[†]	108	94.1	154	103	0.0171	NS
OC	3996	2421	5375	3116	0.006	NS
PEDF [†]	1777	424	1604	339	0.0379	NS
SAA[†]	18574	67358	582	1020	0.0008	NS
SAP[†]	4759	1623	3867	1307	0.004	NS
SCGF-B [†]	60.3	49.4	45.9	15.7	0.0464	NS
sIL-1RI	45.2	106	24.3	8.43	0.0041	NS
sTNF-RII [†]	10.5	4.56	8.43	3.56	0.0172	NS
TIMP-1[†]	199	174	148	40.4	0.0096	NS

SD – standard deviation, NS - non-significant;

[†]concentration expressed as ng/ml, all others expressed in pg/ml; Biomarkers in bold represent consensus markers also selected by non-parametric statistics.

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experimental treatment for hematological malignancies [37]. 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-3 β , MCP-2, CXCL9/

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

Biomarker	Spearman <i>r</i>	<i>p</i>
CCL19/Mip-3 β	0.67	0.0001
CXCL11/I-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).
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MIG) 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 implicated in the development and progression of B-cell lymphoma. In two recent studies, elevated levels of IP-10 in the sera of HIV-positive individuals were associated with the development of B-cell lymphoma within 0–5 years²², [44]. Elevated pretreatment serum levels of IP-10 were also associated with increased likelihood of disease relapse and diminished survival in HIV-uninfected patients with diffuse large B-cell lymphoma [45]. The gelatinase MMP-9 and its inhibitor TIMP-1 mediate breakdown of the extracellular matrix during inflammatory and other responses, and one or both have been linked to lymphoma at the time of diagnosis [46] [47,48]. The acute phase protein, SAA has been shown to be overexpressed in B-cell NHL associated with SIV infection in monkeys [49], and complement components (C3 and CR2) may be involved in lymphomagenesis and/or complement activation [50], [51]. IFN α is 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].

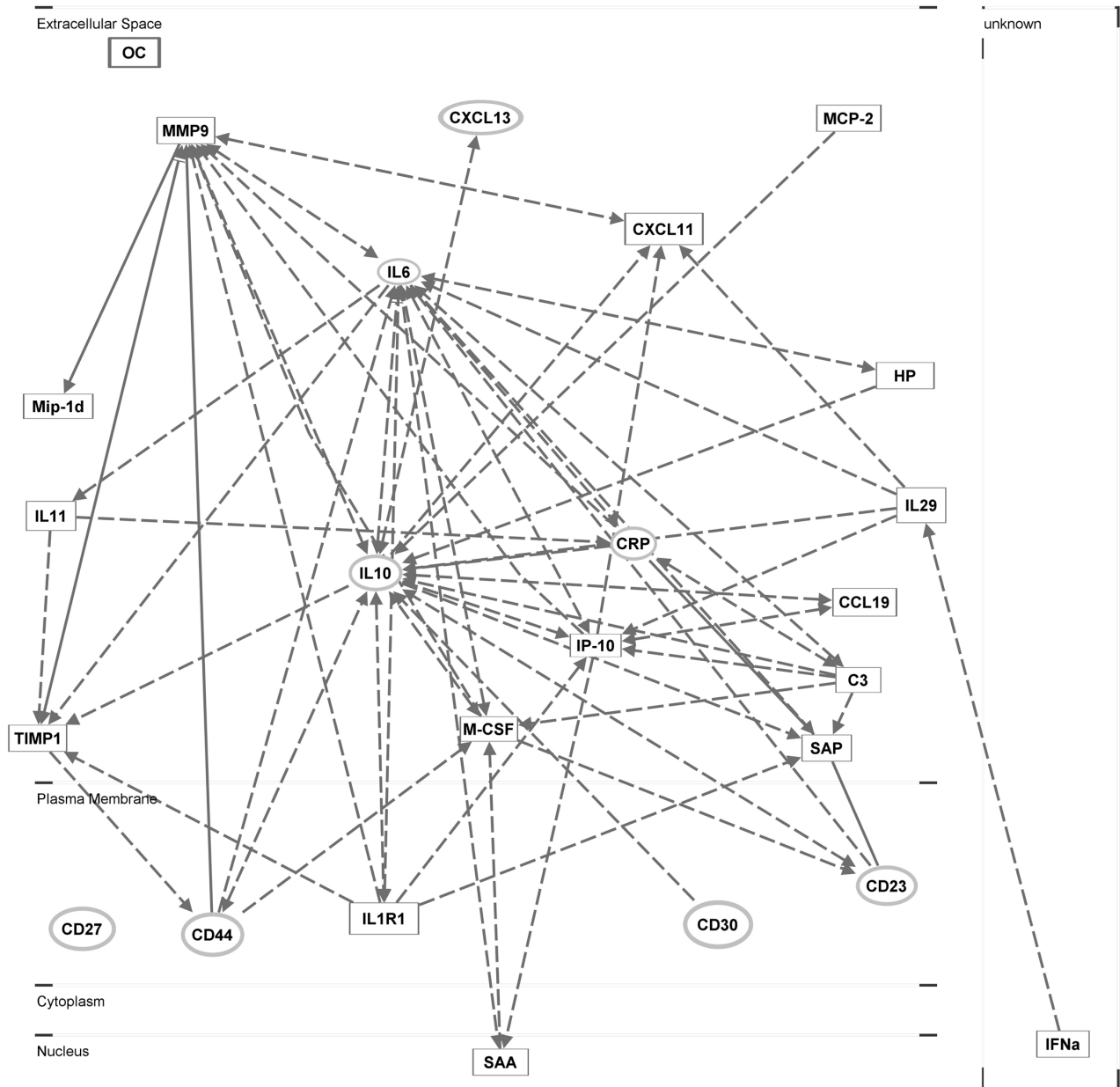
Biological roles related to the development of lymphoma have not been conclusively described for MIP-1 δ , M-CSF, sIL-1R1, HPN, SAP, and OC and, to the best of our knowledge, we are the first to report on these associations. Each of these proteins does possess functions relating them to inflammation and were linked mechanistically to other potential biomarkers in our pathway analysis. Further investigation into the precise activity of each of these proteins in lymphoma would be of great interest.

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 found to be significantly elevated in CNS NHL in comparison to other subtypes. To the best of our knowledge, we are the first to report on such associations. For the vast majority of the biomarkers examined, we were not able to detect any significant differences between NHL subtypes, although this may be due to the relatively small sample size of each subset and the amount of variability within both case and control groups. Our results are similar to the recent findings of Breen et al. in which no significant differences were observed when comparing biomarker levels of diffuse large B cell cases to BL cases; however, in that study all significantly increased biomarker levels in cases compared to controls were associated with the systemic form of NHL but not CNS NHL [21]. While utilizing many of the same MACS A-NHL cases, the current investigation in general examined different time points (closer to or after NHL diagnosis), and each study evaluated a distinct set of biomarkers.

The results of the pathway analysis indicate the existence of a complex network of interactions among the collective list of biomarkers identified in the current and previous investigations. An examination of this network suggests central roles for two previously identified biomarkers, IL-6 and IL-10 (Figure 1). Several newly identified biomarkers including MMP-9, IP-10, and M-CSF also appear to play central roles. Each of the three biomarkers demonstrating the strongest associations with A-NHL in the current study (IL-11, CXCL11, and IL-29) were found to interact with biomarkers from both the current and previous reports. In the course of further development of potential A-NHL biomarkers, many candidate proteins are likely to be eliminated based on technical and/or biological limitations. A more complete understanding of the network of interactions displayed in this analysis should enable us to more effectively identify potential alternatives to those biomarkers which work in close proximity within disease processes.



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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-1δ, 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, sCD30, 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

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.

Supporting Information

Table S1 Description of Biomarker assays utilized in the current study.

(DOCX)

Table S2 Percentage of samples classified as out of range low according to the Bioplex Manager software.

(DOCX)

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