Similar Immunological Profiles Between African Endemic and Human Immunodeficiency Virus Type 1–Associated Epidemic Kaposi Sarcoma (KS) Patients Reveal the Primary Role of KS-Associated Herpesvirus in KS Pathogenesis

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Background. Kaposi sarcoma (KS)–associated herpesvirus (KSHV) is etiologically linked to all KS forms, but mechanisms underlying KS development are unclear. The incidence of KS in human immunodeficiency virus type 1–infected (HIV-1⁺) individuals implicates immune dysregulation; however, the lack of characterization of KSHV immune responses in endemic KS makes the role of HIV-1 unclear. The study objective was to investigate the HIV-1 and KSHV roles in viral nucleic acid detection, antibody responses, and cytokine responses in polymerase chain reaction–confirmed epidemic KS and endemic KS patients and non-cancer controls from sub-Saharan Africa.

Methods. KSHV viral DNA (vDNA), total anti-KSHV antibody, KSHV neutralizing antibody (nAb), and cytokines were quantified.

Results. KSHV vDNA was detectable in tumors but variably in plasma and peripheral blood mononuclear cells. Consistent with elevated antibody-associated cytokines (interleukin [IL] 6, IL-5, and IL-10), nAb titers were higher in epidemic KS and endemic KS patients than in controls ($P < .05$). Despite HIV-1 coinfection in epidemic KS, nAb titers were similar between epidemic KS and endemic KS patients ($P = 0.3$).

Conclusions. Similarities in antibody and cytokine responses between epidemic and endemic KS patients suggest that KSHV drives KS pathogenesis, whereas HIV-1 exacerbates it.

Keywords. Kaposi sarcoma; neutralizing antibody; cytokines; KSHV; sub-Saharan Africa.

Kaposi sarcoma–associated herpesvirus (KSHV) is etiologically linked to all forms of Kaposi sarcoma (KS) [1, 2]. In sub-Saharan Africa, KSHV prevalence ranges from 30% to 90%, with higher levels in human immunodeficiency virus type 1 (HIV-1)–coinfected individuals [3–5]. Before the HIV-1/AIDS epidemic, African endemic KS comprised an estimated 4%–10% of African adult cancers [6–8]. However, with the HIV-1/AIDS epidemic, epidemic KS has become one of the most common tumors in sub-Saharan Africa [9–12].

The incidence of KS observed in HIV-1–coinfected individuals implicates immune dysregulation as an underlying mechanism [9–11]. The precise nature of this dysregulation and how it perpetrates KS remains poorly understood. During acute viral infections, production of neutralizing antibody (nAb) is often a correlate of protection or control, whereas viruses like Epstein-Barr virus and KSHV induce immune responses where high antibody (Ab) titers associate with disease [13, 14]. We previously reported higher prevalence and titers of KSHV nAb in Zambian epidemic KS patients vs asymptomatic controls [15]. Antibody responses in endemic KS patients are incompletely characterized. One study suggested higher levels of total anti-KSHV Ab in endemic KS patients vs HIV-1–seronegative patients with cancers other than KS [16]; however, nAb responses were not assessed.

Host cytokine alterations, particularly interleukin (IL) 6, have also been suggested to drive KS development in epidemic KS patients [17–19]. Our recent RNA-seq comparison of transcripts between KS tumors and normal tissues from the same

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individuals revealed elevated expression of CXCL-9, CXCL-10, CXCL-11, and transforming growth factor beta (TGF- β) in epidemic KS tumors [20]. HIV-1 coinfection and the presence of HIV-1 Tat protein in epidemic KS patients has also been proposed to contribute to cytokine dysregulations that promote KSHV reactivation and pathogenesis in vitro [21]; however, we did not detect *tat* transcripts in KS tumors [20]. Cytokine dysregulation and the role of innate immune mediators have not been studied in endemic KS patients.

We hypothesized that the prevalence and titer of nAbs and cytokines would correlate with high KSHV viral DNA (vDNA), but due to HIV-1 coinfection, the magnitude or breadth of responses would differ between the 2 types of KS. To test these hypotheses, Abs, nAbs, and cytokines were compared in 51 newly diagnosed KS patients and 80 noncancer controls from a region where both KSHV and HIV-1 are prevalent. The study highlights similarities between both types of KS while also revealing responses specific to each form. This comparison further refines our appreciation of the impact of KSHV and HIV-1 in the distinct clinical presentations of KS.

MATERIALS AND METHODS

Study Design, Subjects, and Samples

As part of larger ongoing projects, this cross-sectional study recruited 131 subjects (51 KS patients and 80 noncancer subjects) between 2016 and 2018 from Tanzania ($n = 27$) and Zambia ($n = 104$). Participants were aged >18 years and of both sexes. KS patients were newly diagnosed, and KS was histologically and polymerase chain reaction (PCR) confirmed. Due to reported 35% misdiagnosis of KS in the region following clinically based and/or histopathology-based (hematoxylin and eosin [H&E]) KS diagnosis [22, 23], we only included KS patients who were demonstrated to be vDNA positive by PCR from DNA extracted from tumor biopsies. KSHV and HIV-1 serology were the only criteria used to group the noncancer subjects.

Written consent was obtained from all study subjects. Peripheral blood samples (~ 10 mL) were collected in K2 ethylenediaminetetraacetic acid vacutainers, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (StemCell Technologies). Plasma and viable PBMCs were stored at -80°C . KS tumor biopsy samples (4 mm) were preserved in RNAlater solution (ThermoFisher) and frozen at -80°C . This study was approved by the review boards of the Tanzania National Institute for Medical Research, Ocean Road Cancer Institute, University of Zambia Biomedical Research Ethics Committee, and the University of Nebraska Medical Center and University of Nebraska–Lincoln.

HIV-1 Serology and Plasma Viral Load Quantification by Real-time PCR

Initial HIV-1 diagnosis was made according to Tanzania HIV Rapid Test Algorithm [24] and Alere Determine HIV-1/2 Ag/Ab Combo test in Zambia. HIV-1 serology results were

verified using HIV-1–2.0 First Response kit (Premier Medical Corporation Ltd). HIV-1 viral RNA was extracted from plasma according to the QIAamp viral RNA extraction protocol (Qiagen). To quantify plasma HIV-1 copies, the RNA Ultra-Sense One-Step quantitative real-time PCR (qPCR) system (Applied Biosystems) was used as previously published [25]. Reaction composition and cycling parameters are listed in the Supplementary Materials.

DNA Extraction and PCR Detection of KSHV

Thawed plasma samples (400 μL) were centrifuged at 8000g at room temperature for 10 minutes to remove residual cells. Fifteen microliters of DNase-I (Qiagen) was added and then incubated (2 hours at room temperature). DNase-I was inactivated for 20 minutes at 65°C and virion-associated DNA was extracted according to the QIAamp DNA mini kit (Qiagen). The completeness of DNase-I treatment was demonstrated through a negative PCR result for the human β -actin gene. Viral DNA was detected by nested PCR using an open reading frame 26 (ORF26) amplicon. The TaqMan Universal PCR master mix (Applied Biosystems) was used to quantify KSHV copies by qPCR using a KSHV-ORF26 plasmid standard under the same conditions. Reaction composition and cycling parameters are listed in the Supplementary Materials.

Frozen biopsy tissue was cryopulverized in liquid nitrogen using a mortar and a pestle, and then extracted using a DNA purification kit (Qiagen). Amplification of human β -actin gene from the extracted DNA verified genomic DNA quality. The DNA from 8E5, a human T-lymphoblastoid cell line containing a single defective HIV-1 proviral genome, was used as standard for β -globin copies in qPCR. KSHV and human β -globin copies were quantified from equivalent amount of genomic DNA template (50 ng) in triplicate under the same conditions. Copies of β -globin were used to estimate cellular genome equivalents for quantifying KSHV copies per cell. Detection of KSHV vDNA was as described above.

rKSHV.219 Production

Vero.219 cells stably expressing green fluorescent protein from the KSHV genome during latency were used to generate recombinant KSHV (rKSHV.219), as previously described [26]. The rKSHV.219 was titered on HEK293T, and 10^5 50% Tissue culture Infective Dose (TCID_{50})/mL were used to test plasma for neutralization on the same cell line.

KSHV Serological Assays

KSHV serological status and Ab titer was determined by immunofluorescence assay (IFA) as described [27]. Slides were independently assessed by 2 readers. Neutralization assays were performed in triplicate as previously described [15]. In brief, heat-inactivated plasma (56°C for 60 minutes) was incubated at 1:50 dilution with rKSHV.219 at 37°C for 1 hour. HEK293T cells in 96-well plates were infected with virus–plasma mixture,

centrifuged (400g for 20 minutes) and then incubated (37°C for 72 hours). Infection was quantified by flow cytometry at 72 hours postinfection. Plasma samples demonstrating >50% inhibition of infection compared to a negative control plasma were categorized as neutralizing. Samples that were KSHV nAb positive at the 1:50 dilution were reassayed in 2-fold dilutions of plasma from 1:50 to 1:800 to define the 50% inhibitory concentration.

Multiplex Bead-Based Immunoassay

Cytokines/chemokines in plasma were quantified using Becton Dickinson Cytometric Bead Array Flex Set kits. The standard sensitivity array included IL-4, chemokine CXCL10 (IP-10), IL-5, and TGF- β in picograms/mL. The enhanced sensitivity array included interferon gamma (IFN- γ), IL-1 β , IL-6, IL-12p70, IL-17A, IL-10, and tumor necrosis factor alpha (TNF- α) in femtograms/mL. Raw data were collected and quantified on an Accuri C6 Plus cytometer (BD Biosciences) and analyzed with FlowJo version 10 software (TreeStar).

Flow Cytometry

Due to incomplete clinical records of CD4⁺ T-cell counts, flow cytometry was performed to calculate CD4⁺:CD8⁺ T-cell ratios and gauge immunosuppression in HIV-1-infected subjects. Ratios of 2 $\pm$ 0.8 are normal, and lower ratios indicate CD4⁺ T-cell suppression. Viable PBMCs were thawed, washed, and stained (10 minutes, at room temperature in the dark) with the following antibodies: hCD3-PerCP.Cy5.5, hCD4-PE, and hCD8-FITC (BD Biosciences). Raw data were quantified by DXP10 (Cytex Biosciences) and analyzed with FlowJo version 10 software.

Statistical Analysis

We have compared viral nucleic acid detection, antibody, and cytokine responses between epidemic and endemic KS patients. To determine cancer-specific changes, we compared HIV-1⁺KSHV⁺ vs epidemic KS patients and HIV-1⁺KSHV⁺ vs endemic KS patients. To determine whether there are statistically significant differences between groups, we used one-way analysis of variance. A nonparametric Mann-Whitney *U* test was used to assess differences between comparison groups. Correlation of biological parameters between groups was done by nonparametric Spearman correlation analysis. GraphPad Prism 5 and SAS version 9.2 (SAS Institute) software was used for statistical analyses. All tests were 2-tailed, and *P* values < .05 were considered significant.

RESULTS

Characteristics of the Study Cohort

The study cohort was comprised of 131 subjects: 51 KS patients and 80 noncancer control subjects. KS patients included 34 epidemic KS and 17 endemic KS patients. Noncancer controls were comprised of 20 HIV-1⁺KSHV⁻, 27 HIV-1⁺KSHV⁺, 11 HIV-1⁺KSHV⁻, and 22 HIV-1⁺KSHV⁺ patients (Table 1). While the

Table 1. Characteristics of the Study Cohort

Variable	Noncancer Controls				Kaposi Sarcoma Patients		P Values		
	HIV-1-KSHV ⁻ (n = 20)	HIV-1 ⁺ KSHV ⁺ (n = 27)	HIV-1 ⁺ KSHV ⁻ (n = 11)	HIV-1 ⁺ KSHV ⁺ (n = 22)	Endemic KS (n = 17)	Epidemic KS (n = 34)	HIV-1 ⁺ KSHV ⁺ vs Epidemic KS	HIV-1 ⁺ KSHV ⁻ vs Epidemic KS	Endemic KS vs Epidemic KS
Median age, y (range)	35 (21–52)	28 (21–60)	40 (24–50)	38 (29–68)	51 (24–87)	37 (21–60)	.5	.0009	.05
Male sex, No. (%)	7 (35)	13 (48.1)	5 (45.5)	11 (50)	16 (94.1)	19 (55.9)	.7	.002	.006
ART treatment, No. (%)			9 (81.8)	13 (59.1)		30 (88.2)	.8		.01
Median ART duration, mo (range)			4 (0.25–144)	9 (0.1–48)		5 (0.1–120)			
Median CD4 ⁺ :CD8 ⁺ T-cell ratio	1.58	1.56	0.23	0.20	1.27	0.18	.2	.4	< .0001
Individuals with plasma HIV-1 detection (range in copies/mL)			3 (<50–1.7 $\times$ 10 ⁶)	10 (<50–1.4 $\times$ 10 ⁷)		14 (<50–1.7 $\times$ 10 ⁶)	.8		
Median KS duration, mo (range)					12 (3–108)	5 (5–96)			.003
Median self-reported HIV-1 duration, mo (range)			12 (1–140)	12 (0.5–120)		4 (0.3–120)	.8		

“–” denotes negative and “+” denotes positive. Values in bold type indicate significant differences.

Abbreviations: ART, antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; KS, Kaposi sarcoma; KSHV, Kaposi sarcoma-associated herpesvirus.

age distributions of the noncancer control groups and epidemic KS patients were similar (Table 1), the endemic KS patients were significantly older than both ($P = .05$ and $P = .0009$, respectively). The majority of the endemic KS patients (94.1%) were male, consistent with previously published reports of male predominance [28]. A greater proportion of epidemic KS patients were on antiretroviral therapy (ART) at KS diagnosis compared with HIV-1⁺KSHV⁺ controls (88.2% vs 59.1%, respectively; $P = .01$). Despite the high rate of ART use in epidemic KS patients, CD4⁺ T-cell reconstitution was suboptimal as evidenced by a significantly lower median CD4⁺:CD8⁺ T-cell ratio vs endemic KS patients or HIV-1⁻KSHV⁻ controls ($P < .0001$). Median CD4⁺:CD8⁺ T-cell ratios were not significantly different between epidemic KS patients and HIV-1⁺KSHV⁺ controls ($P = .3$) or between endemic KS patients, and HIV-1⁻KSHV⁺ or HIV-1⁻KSHV⁻ controls ($P = .4$). Epidemic KS patients self-reported shorter KS duration prior to seeking medical attention than endemic KS patients (5 vs 12 months, respectively; $P = .003$; Table 1). No statistically significant difference in any of the studied parameters was evident between subjects from the 2 countries.

Comparison of KSHV Viral DNA Detection in Epidemic KS and Endemic KS Patients

To investigate whether KSHV was being differentially produced or distinctly compartmentalized between epidemic KS and endemic KS patients, KSHV vDNA levels were compared in KS lesions, plasma, and PBMCs by PCR for KSHV-ORF26. As anticipated, vDNA was detected in all tumor tissues from both epidemic KS and endemic KS patients (Figure 1A). However, vDNA detection was significantly higher in PBMCs from epidemic KS vs endemic KS patients ($P = .02$), with a similar trend in plasma ($P = .08$). Surprisingly, HIV-1 plasma viremia in epidemic KS patients was not associated with KSHV-vDNA detection in any sampled compartment/tissue. Despite detectable vDNA in PBMCs and in plasma by nested PCR, KSHV copies were below the qPCR detection limit of 50 copies for several of the samples (Figure 1B). To ensure detection of authentic virion-associated DNA, we treated plasma with DNase I to remove vDNA from degraded infected cells before extraction. We verified this treatment by showing the extracted plasma to be devoid of amplifiable cellular DNA (Figure 1C). Consistent with ART treatment, most epidemic KS patients had undetectable plasma HIV-1 viral load. Overall, we observed a variable pattern of KSHV vDNA detection in plasma and PBMCs from both epidemic KS and endemic KS patients, highlighting inconsistent vDNA detection in these compartments (Figure 1D). Our results suggest that sampling KS tumor is the most reliable method for KSHV vDNA detection.

Comparison of Total Anti-KSHV and nAb Responses in Epidemic KS and Endemic KS Patients

Because humoral responses correlate with levels of viral antigen in many viral infections, we examined whether vDNA correlated

with total and KSHV nAb and whether HIV-1 coinfection led to differentials in the magnitude of responses. Total anti-KSHV Abs were titrated by IFA on KSHV chronically infected BC-3 cells. Both epidemic KS and endemic KS patients had significantly higher anti-KSHV Ab titers than noncancer controls ($P < .05$; Figure 2A). As anticipated, higher anti-KSHV Ab titers were detected in both KS groups as compared to their cognate noncancer controls ($P < .05$; Figure 2A). Interestingly, despite HIV-1 coinfection in epidemic KS patients, the levels of anti-KSHV Ab titers were comparable between epidemic KS and endemic KS patients ($P = .8$) (Figure 2A). Nevertheless, among the epidemic KS patients, those with detectable plasma HIV-1 viremia presented significantly lower anti-KSHV Ab titers compared to HIV-1 aviremic patients ($P = .0004$) (Figure 2B). However, anti-KSHV Ab titer did not correlate with KSHV vDNA detection in plasma or in PBMCs of KS patients. We could not establish whether anti-KSHV Ab titer correlated with KSHV vDNA copies in plasma or in PBMCs as the vDNA quantity was below the qPCR detection limit for most samples.

A KSHV neutralization assay with flow cytometric read-out was used to quantify KSHV nAb responses and determine whether differences between epidemic KS and endemic KS patients exist. Endemic KS patients mounted high KSHV nAb responses similar to those in epidemic KS patients ($P = .3$). KS patient responses were higher than those in their cognate noncancer controls ($P < .05$; Figure 3A). The majority of KSHV-seropositive noncancer controls showed <50% KSHV neutralization at a 50-fold dilution. Importantly, epidemic KS patients with plasma HIV-1 viremia had significantly lower KSHV nAb titers compared with HIV-1 aviremic cases ($P = .0001$; Figure 3B). However, KSHV nAb titer did not correlate with plasma or PBMC vDNA detection. There was a significant correlation between total KSHV Ab titer and KSHV nAb titer in both epidemic KS and endemic KS patients ($r = 0.7384$, $P < .0001$; Figure 4). The concordance between KSHV nAb titer and total KSHV Ab suggests that the latter may be a prerequisite for the development or maturation of nAb. Together, our data suggest that endemic KS and epidemic KS patients mount similar anti-KSHV and KSHV nAb responses, despite HIV-1 coinfection in epidemic KS.

Comparison of Cytokine Expression in Epidemic KS and Endemic KS Patients

A dysregulated cytokine milieu has been suggested in epidemic KS development [17–19, 29, 30], but is also a hallmark of HIV-1 disease [31–33]. As their role in endemic KS has not been evaluated, we sought to quantify several cytokines and chemokines and compare those responses vs epidemic KS in an effort to differentiate effects due to HIV-1 from those associated with KS malignancy. A diverse panel of 11 cytokines/chemokines was selected based on reported association with KS, KSHV, HIV-1, and general viral infection (IL-17A, IFN- γ , IL-12p70, IL-1 β , and

Figure 1. A, Kaposi sarcoma (KS)–associated herpesvirus (KSHV) prevalence in KS tumor biopsies, plasma, and peripheral blood mononuclear cells (PBMCs) of KS patients (34 epidemic KS [human immunodeficiency virus type 1 {HIV-1}–positive Kaposi sarcoma] and 17 endemic KS [HIV-1–negative Kaposi sarcoma]), as determined by nested polymerase chain reaction (PCR) of KSHV open reading frame 26 (ORF26) DNA. B, Viral DNA (vDNA) copies in KS tumors, PBMCs, and plasma of KS patients as determined by real-time quantitative PCR (qPCR) of KSHV-ORF26. In some of the vDNA detectable PBMCs and plasma samples, vDNA copies were below the qPCR detection limit of 50 copies. Dotted line indicates qPCR detection limit. C, Human β -actin and KSHV-ORF26 (600 bp and 170 bp, respectively) duplex PCR products in 1.5% agarose gel. Forty nanograms of DNA extracted with or without DNase-I treatment of plasma was used as template for PCR reactions. Lane 1, sample number 3122 (40 ng); lane 2, sample number 3129 (40 ng); lane 3, sample number 3136; (+), positive control, BC-3 DNA (40 ng); L, 100 bp DNA ladder. D, vDNA detection patterns in KS tumor biopsies, plasma, and PBMCs of KS patients (34 epidemic KS and 17 endemic KS), as determined by nested PCR of KSHV-ORF26 DNA. Four epidemic KS samples and 1 endemic KS PBMC sample were not available. * $P = .02$. Abbreviations: HIV-1, human immunodeficiency virus type 1; KS, Kaposi sarcoma; KSHV, Kaposi sarcoma–associated herpesvirus; NTC, no template control; ORF26, open reading frame 26; PBMC, peripheral blood mononuclear cell.

TNF- α) [18, 34]. We included CXCL10 and TGF- β as they were reported in other cancer conditions and were highly expressed in our recent RNA-seq analysis of epidemic KS tumors [20,

35]. Also, due to observed high antibody responses in both forms of KS, antibody-associated cytokines were also included (IL-5, IL-4, IL-6, and IL-10) [36–39]. Consistent with previous

Figure 2. Immunofluorescence assay for total anti-Kaposi sarcoma (KS)-associated herpesvirus (KSHV) antibody from plasma of KS patients and noncancer control subjects (reciprocal endpoint plasma dilution). *A*, Anti-KSHV antibody titer in plasma of KS patients and noncancer control subjects. *B*, Anti-KSHV antibody titer in human immunodeficiency virus type 1 plasma viremic and aviremic epidemic KS patients. Abbreviations: HIV-1, human immunodeficiency virus type 1; KSHV, Kaposi sarcoma-associated herpesvirus.

reports, our data showed that IL-6 was elevated in both forms of KS compared to noncancer controls ($P < .05$; Figure 5A). This elevation was also significantly higher in epidemic KS than endemic KS patients ($P = .001$). Consistent with a role for IL-6 in B-cell proliferation and differentiation [39], we observed elevated anti-KSHV Ab responses in epidemic KS and endemic KS patients. We also observed a near-significant trend toward increased IL-6 in KSHV-seropositive noncancer controls, suggesting a KSHV-specific response ($P = .05$) (Figure 5A). Similarly, IL-10, an immunoregulatory cytokine that also associates with increased Ab responses [36, 37], was elevated in both epidemic KS and endemic KS patients compared with noncancer controls ($P < .05$; Figure 5B). However, despite similar KSHV Ab responses between epidemic KS and endemic KS patients, IL-10 was significantly higher in epidemic KS than endemic KS patients ($P = .01$). Like IL-6, IL-10 levels appeared to be KSHV associated, as they were also significantly elevated in KSHV-seropositive noncancer controls ($P < .05$; Figure 5B).

Furthermore, IL-5, a hallmark of antibody-associated Th2 effector cell function [38], was similarly elevated in both epidemic KS and endemic KS patients compared with noncancer controls ($P < .05$; Figure 5C). However, IL-5 exhibited dichotomous expression within KS patient and noncancer control groups, suggesting the possibility of other stimuli, such as parasitic infections. Unlike other analytes, the levels of chemokine CXCL10 were found to associate with HIV-1 infection. CXCL10 was elevated specifically in HIV-1-infected noncancer controls and epidemic KS patients (Figure 5D), and particularly in patients with detectable HIV-1 plasma viremia, consistent with previous reports [33]. Levels of CXCL10 in endemic KS patients were similar to those of noncancer controls. Similar to our recent RNA-seq analysis of epidemic KS tumor tissues, CXCL-10 and TGF- β were significantly elevated in plasma of epidemic KS [20] (Figure 5D and Supplementary Figure 1A). As some of the cytokines in the panel were known to associate with increased Ab responses, we performed correlation analyses to

Figure 3. Kaposi sarcoma (KS)-associated herpesvirus (KSHV) neutralizing antibody (nAb) titer in plasma of KS patients and noncancer control subjects, presented as reciprocal of 50% inhibitory concentration (IC₅₀). *A*, KSHV nAb titer in plasma of KS patients and noncancer control subjects. Plasma samples that were nAb positive at 1:50 dilution were reassayed in 2-fold dilutions of plasma from 1:50 to 1:800 to define the IC₅₀. The IC₅₀ values in reciprocal were used for plotting. All of the KSHV-seronegative samples were assigned a value of 1 in reciprocal IC₅₀ in graphical depictions, and KSHV-seropositive samples with <50% KSHV neutralization at 1:50 dilution were assigned a value of 30 in reciprocal IC₅₀ plots. *B*, KSHV nAb titer in human immunodeficiency virus type 1 plasma viremic and aviremic epidemic KS patients. Abbreviations: HIV-1, human immunodeficiency virus type 1; IC₅₀, 50% inhibitory concentration; KSHV, Kaposi sarcoma-associated herpesvirus; nAb, neutralizing antibody.

Figure 4. Correlation of plasma anti-Kaposi sarcoma (KS)-associated herpesvirus (KSHV) antibody and KSHV neutralizing antibody in epidemic KS, endemic KS, and noncancer controls by nonparametric Spearman correlation analysis. Abbreviations: IC₅₀, 50% inhibitory plasma concentration; KSHV, Kaposi sarcoma-associated herpesvirus; nAb, neutralizing antibody.

test for associations between cytokine responses and Ab production. IL-6, IL-10, and IL-5 were found to significantly correlate with KSHV nAb titers in KS patients ($r = 0.4707$, $P < .0001$; $r = 0.6193$, $P < .0001$; and $r = 0.2386$, $P = .007$, respectively; Figure 6A–C). Other tested analytes such as IL-4, IL-17A, IFN- γ , and TNF were below the detection limit for most samples, and IL-1 β and IL-12p70 demonstrated no significant difference between groups (Supplementary Figure 1B and 1C). Overall, our data suggest the existence of a similarly dysregulated circulating cytokine/chemokine environment in epidemic KS and endemic KS patients despite the presence of HIV-1 coinfection in epidemic KS patients.

DISCUSSION

We report here the first in-depth comparison of KSHV immune responses between epidemic KS, endemic KS patients, and

Figure 5. Levels of significantly different cytokines/chemokines in plasma of Kaposi sarcoma (KS) patients and noncancer control subjects. *A*, Interleukin (IL) 6. *B*, IL-10. *C*, IL-5. *D*, CXCL10. Abbreviations: Conc., concentration; HIV-1, human immunodeficiency virus type 1; IL, interleukin; CXCL10 (IP-10), C-X-C motif chemokine 10 (Interferon gamma-induced protein 10); KSHV, Kaposi sarcoma-associated herpesvirus.

noncancer controls from sub-Saharan Africa. Importantly, this comparative approach offered an opportunity to dissect the roles of KSHV and HIV-1 coinfection in KS pathogenesis in a way that could not be accomplished by studying epidemic KS alone. Our findings revealed remarkable similarities in KSHV vDNA detection and immune parameters between the epidemic and endemic forms of KS, but also highlighted some intriguing distinctions in cytokine responses that require further investigation.

In this study, KSHV was detectable in all KS tumors with comparable vDNA load despite longer self-reported duration of KS in endemic KS compared with epidemic KS patients ($P = .003$). This shorter KS duration in epidemic KS patients suggested increased awareness of opportunistic conditions in HIV-1 by clinicians and by individuals infected with HIV-1 visiting care and treatment centers. It is possible that a more aggressive disease course in epidemic KS led the patients to seek medical attention earlier compared to a more indolent disease

progression in endemic KS patients. Furthermore, the observed inconsistent KSHV-vDNA detection pattern in comparison to the 100% detection rate in KS tumor suggests that directly sampling of KS tumors for the presence of vDNA by PCR is the most reliable method. From the reported high rates of KS misdiagnosis from the standard diagnostic (clinical and H&E) methods in the region [22, 23], PCR detection of KSHV vDNA from the tumors should be considered as a complement to histological and immunohistochemical diagnosis of KS. On a cautionary note, we have found that most of the PCR-amplifiable KSHV vDNA in plasma is from degraded infected cells and not virion associated.

Our study is the first to quantify KSHV nAb responses in African endemic KS patients. We found that both epidemic KS and endemic KS patients have similar KSHV nAb prevalence and titers and were higher than noncancer controls. Importantly, epidemic KS patients with detectable HIV-1 viremia and low median CD4⁺:CD8⁺ T-cell ratio had significantly

Figure 6. Correlation of plasma cytokine levels and Kaposi sarcoma (KS)-associated herpesvirus (KSHV) neutralizing antibody (nAb) titers in KS patients and noncancer control subjects by nonparametric Spearman correlation analysis. Interleukin 6 (A), interleukin 10 (B), and interleukin 5 (C) vs KSHV nAb. Abbreviations: Conc., concentration; IC_{50} , 50% inhibitory plasma concentration; IL, interleukin; nAb, neutralizing antibody.

lower anti-KSHV Ab and KSHV nAb titers compared with HIV-1 aviremic patients, suggesting a possible role of T-cell help in KSHV nAb maturation that needs to be further investigated. Also, as we have previously suggested for epidemic KS [15], it appeared that KSHV viremia was necessary to drive maturation of KSHV nAb-producing B cells specific to KSHV in both forms of KS patients. Nevertheless, humoral immune responses appear to lack protective efficacy against KS. Whether such responses, especially KSHV nAb, could be protective against KS if present prior to KSHV infection needs to be investigated.

Similar to several reports of elevated IL-6 in epidemic KS patients, we found that IL-6 was also elevated in endemic KS

patients compared with cognate controls ($P = .02$). Since IL-6 is known to enhance proliferation of KSHV-infected cells, it could contribute to development and maintenance of KS tumors. Our analyses of the immune responses in both KS groups suggest the presence of a robust humoral response that is induced or maintained by a plasma cytokine environment that appears to be skewed toward Ab production. This is supported by the elevation of other Ab-associated cytokines like IL-10 and IL-5 in both epidemic KS and endemic KS patients. Nevertheless, the cytokine environment does not seem to favor profound inflammation or cytotoxic T-lymphocyte responses. Unfortunately, it appears that generation of KSHV nAb, much like in the case of HIV-1 infection, appears too late in disease to be efficacious in preventing or limiting tumorigenesis. Furthermore, HIV-1 infection induces chronic inflammatory response with dysregulation of cytokines despite ART [40]. For instance, HIV-1 induces inflammation through increased CXCL10 production [33]. In epidemic KS patients and HIV-1⁺ noncancer controls, CXCL10 was elevated, particularly in plasma of HIV-1 viremic individuals, suggesting chronic immune activation by low level of HIV-1 replication in latent reservoirs [41, 42]. Together, our findings suggest that KS is largely due to chronic immune activation derived from KSHV infection or from KS tumorigenesis and that HIV-1 coinfection exacerbates the disease.

The unique strength of our study is the comparative investigation of immune responses in sub-Saharan Africa epidemic KS and endemic KS patients and noncancer controls. Previous studies have focused almost exclusively on epidemic KS or on classical KS. In a few instances, comparisons have been made between epidemic KS and classical KS, though typically in geographical regions with different KSHV and HIV-1 prevalence patterns than sub-Saharan Africa. Our study has begun the assessment of immune responses in endemic KS patients, but the limited recognition of endemic KS patients and the late disease presentation when they were identified have limited the depth of our investigations. Studies on KSHV cell-mediated responses, especially on endemic KS patients, are warranted to fully characterize the immune responses. Longitudinal analyses of the current cohort as they undergo KS- and HIV-1-specific treatment will help to illuminate additional potential immune markers of treatment response and long-term control.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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