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Differences in Kaposi sarcoma-associated herpesvirus-specific and –nonspecific immune responses in classic Kaposi sarcoma cases and matched controls in Sicily

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SUMMARY

Kaposi sarcoma (KS) may develop because of incompetent immune responses, both nonspecifically and specifically against the KS-associated herpes virus (KSHV). Peripheral blood mononuclear cells from 15 classic (non-AIDS) KS cases, 13 KSHV seropositives (without KS), and 15 KSHV-seronegative controls were tested for interferon- γ T-cell (Elispot) responses to KSHV-LANA, KSHV-K8.1, and CMV/EBV peptide pools. The forearm and thigh of each participant also was tested for delayed-type hypersensitivity (DTH) against common recall antigens. Groups were compared with Fisher exact test and multinomial logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CI). KSHV Elispot response was detected in 10 (67%) classic KS cases, 11 (85%) KSHV seropositives (without KS), and 2 (13%) seronegative controls. All 4 cases with KSHV-LANA responses had current KS lesions, whereas 5 of 6 cases with KSHV-K8.1 responses had no lesions ($P=0.048$). No case responded to both LANA and K8.1. Compared to seronegative controls, risk for classic KS was inversely related to DTH in the thigh (OR 0.71, 95% CI 0.55–0.94, $P=0.01$), directly associated with DTH in the forearm (OR 1.35, 95% CI 1.02–1.80, $P=0.04$), and tended to be increased 5-fold per KSHV Elispot response (OR 5.13, 95% CI 0.86–30.77, $P=0.07$). Compared to KSHV seropositives (without KS), risk for classic KS, was reduced 5-fold (OR 0.20, CI 0.03–0.77, $P=0.04$) per KSHV response. CMV/EBV Elispot responses were irrelevant. Deficiency of both KSHV-specific and –nonspecific immunity is associated with classic KS. This may clarify why Kaposi sarcoma responds to immune reconstitution.

Keywords

Kaposi sarcoma; T-cell immunity; delayed-type hypersensitivity; case-control study; Italy

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INTRODUCTION

Intact cellular immunity is necessary for regulating herpesvirus infections and the diseases that they cause. Severe deficiency in cellular immunity, as with human immunodeficiency virus (HIV) and the acquired immunodeficiency syndrome (AIDS), results in dysregulation of the Kaposi sarcoma-associated herpesvirus (KSHV) and an extraordinarily high risk for Kaposi sarcoma (KS).^{1, 2} Which immune responses are required, and how they and other host factors regulate KSHV and the risk of KS, are not well defined.

Among HIV-infected men, detection of KSHV DNA in peripheral blood mononuclear cells (PBMC) is strongly predictive of subsequent AIDS-KS.^{1, 2} Correlates of the immune deficiency, HIV load in plasma and low CD4 lymphocyte count, are significantly related to the likelihood of detecting KSHV DNA in PBMC, with or without KS.^{3, 4} However, KSHV viremia can be detected in only about 15% of KSHV antibody-positive, AIDS-free adults.^{1, 2, 5} These findings imply that KSHV is generally well regulated and that KSHV regulation underlies, in part, the low risk for KS among immunocompetent people.⁶

Particular KSHV proteins may affect the risk of progression to KS. Like all herpesviruses, KSHV has a complex lifecycle.⁷ In the lytic phase, dozens of viral genes are expressed, producing infectious virions that disseminate the infection within and among individuals. In the latency phase, only a few genes are expressed, but they enable the viral genome to persist in the nucleus and replicate with host DNA during mitosis.⁸ Malignant spindle cells that express high levels of KSHV latency genes, especially the latency-associated nuclear antigen (LANA), comprise the bulk of a KS tumor.^{9, 10} However, KS tumors also have scattered cells expressing KSHV lytic genes that have been postulated to initiate and propagate the malignancy.¹¹

Patients with KS generally have blunted cell-mediated responses against KSHV latency or lytic phase genes, as determined by interferon- γ enzyme-linked immunospot (Elispot) assays.^{12–16} Effectively controlling HIV with combination antiretroviral therapy (cART), in addition to markedly reducing the risk of AIDS-KS and inducing remissions of early-stage AIDS-KS,^{17, 18} can also lead to modest recovery of KSHV T-cell responses in some patients.^{12, 13, 19} Moreover, one study reported that 12 of 23 HIV-infected, KSHV antibody-positive people had Elispot responses against KSHV peptides.¹⁴ However, cART-induced Elispot responses have not been associated with contemporaneous clearance of KSHV viremia, and many months on cART have been required to detect an improvement in AIDS-KS lesions.²⁰ This suggests that other host factors, in addition to KSHV-specific responses, may play major roles in regulating KSHV, as well as the appearance and remission of KS.

We recently noted that classic, non-AIDS KS patients and KSHV seropositives without KS had distinctly lower delayed-type hypersensitivity (DTH) responses.²¹ Herein, we examined whether this finding was due to, or independent of, differences in KSHV Elispot responses.

MATERIALS AND METHODS

Research participants and KSHV serology

As reported elsewhere in detail,²¹ for a study of DTH responses in the forearm and thigh during 2009, we recruited 44 subjects [15 with cKS, 14 KSHV seropositives without cKS (KSHV⁺/cKS⁻), and 15 KSHV seronegative controls] from the area of Palermo, Sicily, who had participated in a 2002–2006 population-based cKS case-control study.²² For the current study, cKS cases were classified as “active” (one or more current KS lesions) or “inactive” (history of previous KS removed by surgery). As reported in detail,²² seropositive subjects had antibodies detected by immunofluorescence assay (IFA) against KSHV LANA or by

enzyme immunoassay (EIA) against the KSHV K8.1 antigen, a structural lytic-phase protein; seronegative subjects were non-reactive against KSHV LANA and lytic antigens by IFA and against LANA and K8.1 antigen by EIA. Subjects with indeterminate KSHV serology²² were excluded from the current study. The study was approved by institutional review boards at the University of Palermo and at the National Cancer Institute in the USA.

DTH and data collection

Following informed consent and an examination for cKS lesions, one dermatologist (EV) identified disease-free areas on the volar surface of the forearm and the medial surface of the thigh. Using the conventional Mantoux method, six wheals were raised by intradermal injection of 100 µL of each antigen solution [tetanus toxoid (Imovax Tetano, Sanofi Pasteur MSD S.p.a., Rome); PPD (5 tuberculin units); and *C. albicans* (Candin; Allermid Laboratories Inc., San Diego, CA)]. DTH was performed far (more than 20 cm) from KS lesions.

Approximately 48 hours after administration, the same dermatologist examined each injection site on the forearm and thigh. The perpendicular diameters of palpable induration were determined using the ball-point pen method,²³ measured with calipers, and tabulated. Induration with a minimum diameter ≥5mm was categorized as positive, else negative. Cross-sectional area (the product of the perpendicular diameters) was calculated for indurations with minimum diameter ≥2mm.

KSHV-specific IFN-γ Elispot assay

Interferon-γ (IFN-γ) enzyme-linked immunospot (Elispot) experiments for the detection of KSHV-specific immune responses, against either lytic or latent viral antigens, were performed as already described.^{15,16} Briefly, patients' peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Lymphoprep density gradient separation (Axis-Shield PoC AS) and resuspended in RPMI1640/fetal calf serum 10% (Invitrogen) and cultured at 37°C in humidified 5% CO₂ atmosphere in a 96-well polyvinylidene difluoride-bached plate coated with anti-IFN-γ monoclonal antibody (Mabtech). A total of 1×10⁵ cells/well were stimulated for 16 hours with either orfK8.1 (gp35/37) or ORF73 (LANA) full-length recombinant proteins (10 µg/mL), previously produced in *E. coli* or baculovirus-infected insect cells, respectively, or with a pool of CMV/EBV gB and gH lytic proteins.¹⁵ Unstimulated PBMCs (medium alone) were used as negative controls, whereas the anti-CD3 monoclonal antibody and CEF peptide pool were separately added to positive control wells, according to manufacturer's instructions (Mabtech). All the assays were performed by one of the authors (PB), blinded to the patients' clinical histories and personal identifiers, using an automated Elispot counter (AID-GmbH, Strassberg, Germany). The number of KSHV-specific or CMV/EBV-specific T cells, expressed as spot-forming cells (SFC) per 1×10⁶ PBMCs, was calculated after the subtraction of negative control values. All test conditions were carried out in triplicate. Results were considered positive if there were at least 20 spots, if the number of SFC/10⁶ PBMC in KSHV-stimulated wells was 2-fold higher than that in unstimulated control wells, and if there were at least 50 SFC/10⁶ PBMC.

Statistical analysis

Analyses were specified in advance. Responses to both of a pair of peptide pools (e.g., both KSHV-LANA and KSHV-K8.1, or KSHV-LANA and CMV/EBV, or KSHV-K8.1 and CMV/EBV) were coded as “2”; response to only one pool in each pair was coded as “1”; and response to neither pool in a pair was coded as “0”. This 2,1,0 classification was then used in contingency table analyses with two-sided exact tests to compare categorical frequencies of detecting Elispot responses to the different peptide pools (KSHV-LANA, KSHV-K8.1, and CMV/EBV). KSHV seropositives and cKS cases were compared in a

logistic regression model with number of Elispot responses to KSHV-LANA, KSHV-K8.1 or both (coded 0,1,2 as above) as an independent ordinal variable to calculate odds ratios (OR) and 95% confidence intervals (CI). Similarly, multinomial logistic regression was used, with KSHV seronegatives as the referent group, to calculate the OR and CI for cKS and for KSHV seropositivity associated with three independent variables: number of KSHV Elispot responses (0,1,2 ordinal scale) and sum of the square-root transformed areas of DTH responses in the thigh and forearm.²¹ Two-sided P-value ≤ 0.05 was deemed to be statistically significant.

RESULTS

KSHV-specific immune responses

Of 44 participants in the DTH study, 43 (98%) had satisfactory T-cell responses detected by Elispot [median 2000 (range 400–2000) SFC/ 10^6 PBMC with anti-CD3]. One KSHV⁺/cKS[−] control who had <300 SFC/ 10^6 PBMC with anti-CD3 was excluded from analysis.

As shown in Table 1 and Figure 1, ten of the 15 cKS cases had anti-KSHV T-cell responses against either the latent (LANA) or the lytic (K8.1) viral antigens, but none of the cases had responses against both LANA and K8.1. Four cases with “active” KS (including two of three with approximately 20 lesions), had responses against LANA [median 145 (range 120–220) SFC/ 10^6 PBMC], but not against K8.1. Six cases (including one with 2 lesions and five with “inactive” disease) had responses against K8.1 [median 113 (range 70–220) SFC/ 10^6 PBMC], but not against LANA. Thus, response against LANA versus K8.1 appeared to differ by disease activity (2-sided exact $P=0.048$). Irrespective of lesions, nine of the cKS cases had responses against the CMV/EBV peptide pool [median 140 (range 60–2000) SFC/ 10^6 PBMC].

In contrast to the cKS cases, anti-KSHV T-cell responses were detected in most (11/13; 85%) of the KSHV seropositive controls (KSHV⁺/cKS[−]). Moreover, five of the KSHV⁺/cKS[−] controls had robust responses against both LANA and K8.1 [median 380 (range 160–640) SFC/ 10^6 cells]. Three KSHV⁺/cKS[−] controls had responses against only LANA [median 65 (range 50–120) SFC/ 10^6 cells], and three other KSHV⁺/cKS[−] controls had responses against only against K8.1 [median 130 (range 50–130) SFC/ 10^6 cells; Table 1 and Figure 1].

Two KSHV seronegatives had Elispot responses to both K8.1 and LANA (Table 1, Figure 1); the other 13 KSHV seronegatives had no responses to the KSHV peptide pools. Magnitude of the Elispot responses did not appear to differ between the groups (Table 1, Figure 1).

As breadth of response may affect risk of cKS, we tested the effect of response to CMV/EBV plus either K8.1 or LANA. The cKS cases and KSHV⁺/cKS[−] controls did not differ in likelihood of response against CMV/EBV, K8.1 or both (2-sided exact $P=0.32$) nor against CMV/EBV, LANA or both ($P=0.14$). Notably, cases were less likely than KSHV⁺/cKS[−] controls to respond to K8.1, LANA or both ($P=0.04$). Compared to KSHV⁺/cKS[−] controls, the risk for cKS was 5-fold lower (OR 0.20, 95% CI 0.03–0.77, $P=0.04$) per response to a KSHV peptide pool.

Elispot and DTH responses

As noted previously,²¹ the KSHV seronegatives tended to have stronger DTH responses, particularly in the thigh, compared to the cKS cases and KSHV⁺/cKS[−] controls. As shown in Table 2, compared to the KSHV seronegatives, cKS was associated with weaker DTH response in the thigh (OR 0.71, 95% CI 0.55–0.94, $P=0.01$), with stronger DTH in the

forearm (OR 1.35, 95% CI 1.02–1.80, $P=0.04$), and with the number of KSHV Elispot responses to K8.1, LANA or both (OR 5.13, 95% CI 0.86–30.77, $P=0.07$). Compared to KSHV seronegatives, the associations for KSHV⁺/cKS⁻ controls resembled those for cKS (Table 1). The odds of Elispot responses to KSHV peptides were 3.7-fold higher in KSHV⁺/cKS⁻ controls (OR 18.77) than in cKS cases (OR 5.13), but this difference was not statistically significant given the small sample size and very wide confidence intervals (Table 1).

In a model (not shown) that considered K8.1 and LANA Elispot responses separately, the odds of cKS were higher with K8.1 than LANA (OR 12.74 and 2.82, respectively), as were the odds of KSHV⁺/cKS⁻ (OR 45.46 and 10.51 with K8.1 and LANA, respectively); the associations with forearm and thigh DTH in both cKS cases and KSHV⁺/cKS⁻ controls in this model were almost identical to those presented in Table 1. In other models (not shown), neither cKS nor KSHV⁺/cKS⁻ was related to Elispot response to the CMV/EBV peptide pool, sex, or history of smoking, corticosteroid use, or diabetes; and inclusion of these did not substantially alter the associations presented in Table 1.

DISCUSSION

In this study, KSHV-specific cytotoxic T cells against both lytic (K8.1) and latent (LANA) viral antigens were frequent in KSHV seropositives who have never developed KS tumors, which is consistent with the accepted notion that robust and broad anti-KSHV immunity is required to prevent KS occurrence in KSHV⁺ elderly individuals, as well as in KSHV⁺/HIV⁺ patients and in KSHV⁺ organ recipients.^{14,15,28} However, among cKS patients, we unexpectedly observed that LANA-specific T-cell responses were detected in patients with “active” cKS tumors, while always lacking in “inactive” cKS cases, treated with surgical removal of skin lesions. Conversely, K8.1-specific T-cell responses were virtually absent in active cKS patients, while frequently associated with inactive cKS. Statistical analysis supported the observation that KSHV-specific responses differed by the presence or absence of active KS lesions.

Active KS disease is classically characterized by neoplastic spindle cells that manifest high-level expression of KSHV latent genes (especially LANA), but the expression of the viral lytic program is likely to contribute to KS proliferation.^{11,30} In the setting of iatrogenic KS and AIDS-KS, several reports have shown that LANA-specific T cells were often undetectable at KS presentation but then readily detected with tumor remission, indicating a correlation between anti-tumor immunity and regression of KS.^{14–16,29} In contrast, the current study found that virus-specific T-cell responses against LANA were associated with the persistence of active cKS disease, suggesting that a “LANA-only” response may be merely a marker of disease activity and not sufficient to eradicate cKS. The importance of K8.1-specific T-cell responses for the avoidance or regression of disease has been suggested by studies of transplant KS and iatrogenic KS.^{14–16,29} The results of the current study support this model. We found that Elispot responses against K8.1 peptides were inversely correlated with KS occurrence, that is, frequently detected in KSHV⁺/cKS⁻ individuals, absent during active cKS disease, and then detected after surgical KS treatment.

Considering *in vivo* responses, both the cKS cases and the KSHV⁺/cKS⁻ controls, compared to KSHV seronegatives, had reduced DTH in the thigh against conventional antigens (tetanus, tuberculin and candida). These differences in DTH responsiveness were independent of the KSHV Elispot responses. Elispot responses to CMV/EBV were unrelated to DTH and also did not differ between the groups.

In addition to supporting the well established association between deficient cellular immunity and KS, these results illustrate the complexity of this association. Adjusted for DTH in the thigh and forearm, risk of cKS was increased 5-fold (albeit with marginal statistical significance, $P=0.07$) with fewer KSHV-specific *in vitro* responses. And adjusted for the *in vitro* responses, cKS risk was significantly associated with lower DTH in the thigh and higher DTH in the forearm. This anatomic discordance in DTH with cKS is plausible, because cKS most often originates on the leg or foot. The DTH associations were adjusted for KSHV-specific responses. This supports the concept that non-viral host factors affect the likelihood that KS will develop. Male gender, non-smoking, corticosteroid use, and diabetes have been associated with an increased risk of cKS,²² but these non-viral factors did not substantially alter the DTH and KSHV Elispot associations with cKS in the current study.

Compared to the KSHV seronegatives, the KSHV⁺/cKS⁻ controls and the cKS cases had remarkably similar associations with Elispot responses and DTH (Table 1). This largely reflects the characteristics of the KSHV seronegatives, who had infrequent KSHV-specific Elispot responses (Figure 1), as well as strong DTH in the thigh.²¹ Although KSHV is not clearly immune suppressive, KSHV-seropositives nonetheless appear to have differences in the distributions of both B-lymphocyte and $\gamma\delta$ T-lymphocyte subsets.^{24, 25} In addition, KSHV may suppress the generation of hematopoietic progenitor cells.²⁶

The major limitation of our study was its small size, resulting in low precision (wide confidence intervals), inability to assess subgroups, and possible Type 2 errors, especially for modification by variables such as sex, smoking and diabetes. Two strengths should be noted. The participants were drawn from a case-control study that represented the Palermo region of Sicily,²² and Elispot testing was performed with state-of-the-art methods by a highly experienced laboratory that was masked to the case-control classifications. KSHV-LANA and KSHV-K8.1 Elispot responses were highly concordant in the KSHV⁺/cKS⁻ and seronegative control groups.

In summary, we found that cKS was independently associated with deficiencies in cellular immunity, both *in vitro* against KSHV-specific antigens and *in vivo* against common microbial antigens. The presence or absence of T-cell responses against K8.1 antigens may represent a novel marker of immunological protection against cKS in KSHV seropositive people. Future studies that include both perspectives, and perhaps humoral and other measures of immunity,²⁷ would be needed to elucidate whether deficient DTH or skewed T-cell responses towards either K8.1 or LANA might be harnessed as tools to predict KS development, response to therapy, or disease relapse.

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Figure 1.

Presence and level of *in vitro* interferon- γ Elispot responses of peripheral blood mononuclear cells (PBMC) from cases of classic Kaposi sarcoma (KS), KS-associated herpesvirus (KSHV) seropositive controls, and KSHV seronegative controls against pools of peptides from the K8.1 and LANA antigens of KSHV. Data are spot-forming cells (SFC) per 10^6 PBMC. The cutoff for detection is 50 SFC/ 10^6 PBMC, as indicated by the dashed lines.

Table 1

Prevalence and magnitude of interferon- γ Elispot responses of cases of Sicilian classic Kaposi sarcoma (cKS), as well as KS-associated herpesvirus (KSHV) seropositive and seronegative Sicilian controls without KS, against pools of antigenic peptides from the K8.1 and LANA antigens of KSHV and from cytomegalovirus and Epstein-Barr virus (CMV/EBV).

Group	Number (row %) of positive subjects Median [range] of spot-forming cells/10 ⁶ PBMC			
	Peptide pool			
	K8.1	LANA	K8.1 and LANA	CMV/EBV
All cKS cases (n=15)	6 (40) 113 [70–220]	4 (27) 145 [120–220]	0 (0) 0 [0]	9 (60) 140 [60–2000]
- cKS cases without lesions (n=9)	5 (56) 130 [70–220]	0 (0) 0 [0]	0 (0) 0 [0]	5 (56) 340 [60–2000]
- cKS cases with 1–2 lesions (n=3)	1 (33) 90 [90]	2 (67) 170 [120–220]	0 (0) 0 [0]	3 (100) 110 [85–140]
- cKS cases with ~20 lesions (n=3)	0 (0) 0 [0]	2 (67) 145 [130–160]	0 (0) 0 [0]	1 (33) 200 [200]
KSHV seropositives, no KS (n=13)	8 (62) 140 [50–250]	8 (62) 93 [50–390]	5 (38.5) 380 [160–640]	9 (69) 240 [50–1200]
KSHV seronegatives, no KS (n=15)	2 (13) 135 [90–180]	2 (13) 90 [80–100]	2 (13) 225 [170–280]	7 (47) 200 [80–1100]

Table 2

Multinomial logistic regression model for the risk [odds ratio, 95% confidence interval (CI)] of classic Kaposi sarcoma (KS) and KS-associated herpesvirus seropositivity without KS (KSHV⁺/cKS⁻ controls), compared to KSHV seronegative controls.

Type of immune response	Classic KS		KSHV ⁺ /cKS ⁻ controls	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Number of K8.1 and/or LANA Elispot responses (0,1,2) [†]	5.13 (0.86 – 30.77)	0.07	18.77 (2.68 – 131.57)	<0.01
Delayed-type hypersensitivity, thigh [*]	0.72 (0.55 – 0.94)	0.01	0.73 (0.55 – 0.97)	0.02
Delayed-type hypersensitivity, forearm [*]	1.35 (1.02 – 1.80)	0.04	1.23 (0.90 – 1.66)	0.19

^{*} Square-root transformed sum of cross-sectional diameters (in mm²) of induration against tetanus, tuberculin and candida 48 hours after intradermal injection.

[†] Ordinal variable, with each participant coded as no Elispot response to K8.1 or LANA peptide pools (0), response to only one of these pools (1), or response to both of these pools (2).