Mast Cell Activation and KSHV Infection in Kaposi Sarcoma


Abstract

Purpose: Kaposi sarcoma (KS) is a vascular tumor initiated by infection of endothelial cells (ECs) with KS-associated herpesvirus (KSHV). KS is dependent on sustained proinflammatory signals provided by intralesional leukocytes and continued infection of new ECs. However, the sources of these cytokines and infectious virus within lesions are not fully understood. Here, mast cells (MCs) are identified as proinflammatory cells within KS lesions that are permissive for, and activated by, infection with KSHV.

Experimental Design: Three validated MC lines were used to assess permissivity of MCs to infection with KSHV and to evaluate MCs activation following infection. Biopsies from 31 AIDS-KS cases and 11 AIDS controls were evaluated by IHC for the presence of MCs in KS lesions and assessment of MC activation state and infection with KSHV. Plasma samples from 26 AIDS-KS, 13 classic KS, and 13 healthy adults were evaluated for levels of MC granule contents tryptase and histamine.

Results: In culture, MCs supported latent and lytic KSHV infection, and infection-induced MC degranulation. Within KS lesions, MCs were closely associated with spindle cells. Furthermore, MC activation was extensive within patients with KS, reflected by elevated circulating levels of tryptase and a histamine metabolite. One patient with clinical signs of extensive MC activation was treated with antagonists of MC proinflammatory mediators, which resulted in a rapid and durable regression of AIDS-KS lesions.

Conclusions: Using complimentary in vitro and in vivo studies we identify MCs as a potential long-lived reservoir for KSHV and a source of proinflammatory mediators within the KS lesional microenvironment. In addition, we identify MC antagonists as a promising novel therapeutic approach for KS.

Introduction

Kaposi sarcoma (KS) is a unique, highly inflammatory “hemorrhagic sarcoma” initiated by infection of endothelial cells (ECs) by KS-associated herpesvirus (KSHV; also known as human herpesvirus 8). There are four epidemiologic types of KS: Classic KS, which typically occurs in elderly men in Mediterranean regions and is relatively indolent; endemic KS, occurring in persons in sub-Saharan Africa; iatrogenic KS, occurring in immunosuppressed persons undergoing transplantation; and epidemic KS, occurring in patients with HIV/AIDS. Independent of form, histologically, lesions are characterized by proliferating spindle-shaped ECs and infiltrating leukocytes. Spindle cells carry latent KSHV, defined by limited viral gene expression and no progeny production, but cells explanted from KS lesions and grown in culture rapidly lose the KS genome. Thus, a source of infectious KSHV virions within lesions capable of infecting new ECs is thought to be essential. Furthermore, while latently infected ECs secrete some inflammatory mediators, the levels are insufficient to drive survival and growth of spindle cells in culture. Thus, paracrine inflammatory signaling is thought to be essential for KS lesion development. The interplay between infection and inflammation allows for the enhanced survival of spindle cells even prior to malignant transformation.

Previous reports have described mast cells (MCs) in KS lesions but their significance has not been established. MCs are long-lived, tissue-resident cells that, like ECs, develop from bone marrow–derived CD34+ progenitor cells. Consistent with their role in sensing pathogens and allergens, MCs are most prevalent in mucosal tissues where they interface directly with the external environment, as well as adjacent to microvessels where they monitor both blood and lymph circulation. As immune surveillance cells, they have essential roles in both acute and chronic inflammation. Given the shared ontogeny of ECs and MCs, as well as their close association within all vascularized tissues, it is not surprising that these cell types...
Translational Relevance

Inflammation is a key driver of oncogenesis and modulation of inflammation in the tumor microenvironment is emerging as a viable therapeutic approach. Kaposi sarcoma (KS), one of the most commonly diagnosed and aggressive cancers in sub-Saharan Africa, is an unusual cancer, dependent on infection with KS herpes virus (KSHV) and on inflammatory mediators. Despite extensive study the key cellular players and paracrine inflammatory mediators driving the disease are incompletely understood. Here we identify a previously unrecognized role for potent proinflammatory mast cells (MCs) in KS. MCs are localized, extensively activated, and infected by KSHV in KS tumors and their clinical importance is underscored by the successful treatment of a case of AIDS-KS with anti-MC therapy. These data strongly suggest a specific role for MCs in KSHV-driven oncogenesis and identify MCs as a promising novel, pathogenesis-targeted therapeutic approach to KS. Our work adds to the growing body of knowledge on the role of MCs in oncogenesis, and demonstrates the efficacy of using widely available MC-directed therapies for treatment of KS.

cross-regulate each other. For example, chemical mediators from MCs promote EC activation, which is required for the regulated recruitment of leukocytes to inflamed tissues (8, 9). There is evidence that MC activation of ECs is key to the pathophysiology of vascular compromise observed in severe dengue (10, 11) and influenza infections (12), and MCs are known to be permissible to infection by multiple viruses. Furthermore, there is accumulating evidence that MC activation is a promising target for adjunctive therapy in multiple cancer types. MCs are found in a wide range of malignancies, often associated with poor prognosis, increased metastasis, and reduced survival, including melanoma, prostate, pancreatic adenocarcinoma, and squamous cell carcinoma (reviewed in ref. 13). MC–cancer links are illustrated in the clinical observation that patients with mastocytosis, which is defined by pathologically increased numbers of MCs, have increased risk of developing both myeloid and lymphoid neoplasms (14). MCs are key effectors in the development and progression of small-bowel cancer (15), malignant pleural effusion (MPE; ref. 16), and primary cutaneous lymphoma (17), and are pathologically associated with several other B-cell cancers including Hodgkin lymphoma (18, 19), diffuse large B-cell lymphoma (20), and B-cell chronic lymphocytic leukemia (18).

Here we provide the first evidence of a central role of MCs in the pathogenesis of KS. Specifically, we demonstrate that MCs are permissive for KSHV infection and that infection leads to activation and degranulation of MCs. We demonstrate also that MCs are abundant within KS lesions, that they express both latent and lytic viral proteins, and that they are predominantly activated. We also demonstrate the potential of anti-MC medications as a promising novel therapeutic approach for KS. Collectively, our data identify MCs as potentially central mediators of KS pathophysiology, and further evaluation of the therapeutic potential of MC-targeted treatment is warranted.

Methods

**Cell culture**

BCBL-1 PEL cells were maintained in RPMI medium 1640 containing 10% FBS, 1% penicillin–streptomycin, and 0.05 mol/L β-mercaptoethanol. LAD2 (a kind gift from Arnold Kirshenbaum, NIH, Bethesda, MD) and LL/JVA (a kind gift from John Steinke, University of Virginia, Charlottesville, VA) MCs were cultured in serum-free media (StemPro-34 SFM, Life Technologies). HMC-1 clone 5C6 MCs were cultured in Iscove medium (Gibco) and authenticated by the University of Arizona genomics core by Promega PowerPlex assay and short tandem repeat results compared with genomic databases. Because LAD2 and LL/JVA cell sequences are not currently available, these cells were authenticated by flow cytometry using MC-specific receptor staining. CD117 (Thermo Fisher Scientific, clone 104D2) and FcRβ1 (Thermo Fisher Scientific, clone AER-37). MC media were supplemented with 2 mmol/L glutamine, 100 μ/mL penicillin, 50 μg/mL streptomycin. A total of 100 ng/mL SCF was added to LAD2 cultures. LAD2 cells were fed by hemidepletion of media twice a week. Primary human umbilical vein endothelial (HUVEC) cells were purchased from Lonza. Cultures were expanded in EBM-2 media (Lonza) supplemented with the EGM-2 bullet kit in 6-well tissue culture plates coated with 0.1% (w/v) gelatin in PBS and used between passage 2 and 5 for experiments. Cells were maintained at 37°C in 5% CO₂. All cell lines were tested every 1 to 2 months for Mycoplasma contamination using the MycoProbe Mycoplasma detection kit (R&D Systems). Cell lines were thawed and used for approximately 2 months before being discarded and a fresh aliquot thawed for use in experiments with the exception of LAD2 cells that were cultured continuously as per instructions.

**Virus production and quantification**

KSHV was obtained from cultures of BCBL-1 cells that harbor latent KSHV. Lytic reactivation of 5 x 10⁶ BCBL-1 cells/mL was induced by addition of 0.4 mmol/L Valproic acid (Sigma) to cultures for 7 days. On day 7, cell-free virus-containing supernatant was harvested from cultures and concentrated by centrifugation at 10,000 x g for 2 hours. The viral pellet was resuspended in EBM-2 media, aliquoted, and frozen at –80°C. To determine the viral titer, viral preparations were treated with DNAse 1 (Invitrogen). KSHV DNA was extracted (QIAEN QiAamp DNA Mini Kit) and copy number quantified by real-time DNA PCR using primers that amplify the latency-associated nuclear antigen (LANA, also known as ORF 73 gene, see Supplementary Table S1 for sequence). The KSHV ORF 73 gene cloned in the pCR2.1-TOPO vector (Invitrogen) was used for the external standard. Known amounts of ORF 73 plasmid (10², 10⁵, 10⁶, 10⁷, 10⁸, 10⁹, and 1 copies) were used to generate a standard curve. Reaction conditions for two-step PCR were performed on a Bio-Rad iCycler, using 42 cycles of 95°C for 10 seconds, then 60°C for 30 seconds. The C_(t) values were used to plot the standard graph and to calculate the copy numbers of viral DNA.

**Titration of KSHV**

Quantification of infectious virus was assessed on 1 x 10⁶ cells/well HUVEC monolayers plated on gelatin-coated 6-well plates with glass coverslips. The next day, monolayers were inoculated with KSHV at different dilutions in the presence of 8 μg/mL Polybrene (Sigma) for 2 hours at 37°C followed by spinoculation...
at 2,000 rpm for 15 minutes. Forty-eight hours postinfection (pi), HUVECs were washed with PBS, fixed in 4% paraformaldehyde for 10 minutes at room temperature, and stained with a rabbit anti-LANA (1:1,000) antibody, and visualized with goat anti-rabbit 488 (Molecular Probes) as described previously (21). Infectious units were determined by counting the number of LANA dots/cell in five fields of view with one LANA dot equivalent to one infectious viral particle, as described previously (22).

**In vitro KSHV infection of MCs**

A total of 4 × 10^6 HMC-1 or LAD2 MCs were infected with KSHV (MOI 1–3) for 3 hours at 37°C. Cells were mixed gently by hand every 20 minutes during the 3-hour incubation time. Postinfection, cells were washed twice with 100 volumes of warm media to remove any unbound virus, set up at 5 × 10^5 cells/mL, and cultured for indicated times. For some experiments, UV inactivation of virus was carried out. For ultraviolet inactivation (UV), KSHV was exposed to 5 mJ/cm^2 for 10 minutes at room temperature, and stained with a rabbit anti-LANA (1:1,000) antibody, and visualized with goat anti-rabbit 488 (Molecular Probes) as described previously (21). Infectious units were determined by counting the number of LANA dots/cell in five fields of view with one LANA dot equivalent to one infectious viral particle, as described previously (22).

**qPCR**

Total RNA from KSHV-infected MC cultures was isolated using the RNasy Mini Kit (QIAGEN) with DNase treatment performed on column with the Qiagen RNase-free DNase according to the manufacturer's protocols. The quality and concentration of RNA was assessed using the ND1000 spectrophotometer (Nanodrop). A total of 0.5 to 1 μg of RNA was reverse transcribed using a QIAGEN QuantiTect Reverse Transcription kit according to the manufacturer's instructions. All samples had a no RT control and NTC to control for DNA contamination. Each "#" represents an experiment executed independently, representing a different RNA sample.

**RT-PCR**

Total RNA was isolated and converted into cDNA as described in the qPCR section. The primer sequences used in the RT-PCR analyses are listed in Supplementary Table S1. For RT-PCR, cDNA was diluted 1:4 and 2 μL used in subsequent PCR reactions with KSHV gene-specific primers. All samples had a no RT (reverse transcriptase) control and NTC to control for DNA contamination. BCBL-1 cells induced with 0.4 mmol/L Valproic acid for 2 days served as a positive control for lytic viral gene expression. PCR was carried out using Promega Go-TaqGreen Master mix in a Bio-Rad thermocycler. PCR products were run on 2% agarose gels and viewed on a Bio-Rad geldoc.

**Limiting dilution qPCR**

To determine the frequency of cells carrying the KSHV genome limiting dilution qPCR was performed on serial dilutions of MCs infected with KSHV at 24 hpi using previously published methods (24). At 24 hpi, MCs were washed twice with 10 and 100× volume of 5% FCS Iscove’s before being resuspended in isosotic buffer [150 mmol/L KCl, 10 mmol/L Tris–HCl (pH 7.5), 1.5 mmol/L MgCl_2]. Serial 10-fold and twofold dilutions of MCs were made in an isosotic buffer ranging from 1,000 test cells to one test cell per PCR. Ten to 12 PCRs were analyzed per cell concentration. Five-microliter cell dilutions were added to PCR tubes containing 5 μL of lysis buffer [10 mmol/L Tris–HCl (pH 8.5), 1.5 mmol/L MgCl_2, 1% Nonidet P-40, 1% Tween 20, 0.2 mg/mL protease K] and lysed overnight at 56°C. Proteinase K was inactivated at 95°C for 15 minutes, and 15 μL of Bio-Rad iQ SYBR Green Supermix Master Mix containing LANA primers were added directly to each cell lysate and run on a Bio-Rad IQ5 cycler, using a two-step program of 95/60 for 45 cycles. Negative uninfected cells and positive BCBL-1 controls were included for each set of PCRs performed. Poisson distribution was used to calculate the percentage of MCs infected.

**KSHV infection assay**

HMC-1 cells were infected with KSHV at MOI 1–3 as described above for 24 to 72 hours. Following inoculation, MCs were washed twice with 100 volumes of 5% FCS Iscove’s and set up at 5 × 10^5 cells/mL. At 48 hpi, cell-free supernatants were harvested and used directly to infect semiconfluent monolayers of primary HUVECs as described above. Cell-free supernatants from mock-infected or UV-inactivated virus inoculated cultures were used as controls for LANA-specific staining. At 48 hours posttreatment, KSHV infection was assessed by LANA microscopy as described above (21).

**β-hexosaminidase release assay (degranulation assay)**

LAD2 and LLUVA cells (1 × 10^6 cells/mL) were incubated for 60 minutes at 37°C in the absence or presence of increasing concentrations of live or UV-inactivated KSHV. Mock-treated cultures were used to indicate baseline β-hexosaminidase release. Cultures were centrifuged at 200 × g for 10 minutes at 4°C. After collection of supernatants, the pellets were resuspended at 1 × 10^6 cells/mL in buffer and disrupted by three rounds of snap freeze-thaw. Supernatants and pellets were frozen at −80°C for future analysis. β-hexosaminidase assay was carried out as described previously (25). The net percent β-hexosaminidase release was calculated as follows: ([β-hexosaminidase in supernatant]−[β-hexosaminidase in supernatant + β-hexosaminidase in pellet]) × 100.

**Histology**

Deidentified tissue from 31 cases of AIDS-KS (20 dermal, one oral mucosa, six lymph node, two lung, and two intestinal) and 10 control non-KS samples from 10 HIV+ cases (two dermal, two lung, three lymph node, and three intestinal) were obtained from the AIDS and Cancer Specimen Resource (ACSR/NCI) and the Tissue Archives, Department of Pathology, College of Medicine, The Ohio State University (Columbus, OH). Dermal biopsies from one patient with AIDS-KS was obtained as part of routine clinical care in the Infectious Diseases clinic at SUNY Upstate Medical University. Hematoxylin and eosin (H&E) and Prussian Blue Iron (potassium ferrocyanide 2%) stains were performed using standard protocols at the Pathology laboratory of Ohio State University or at SUNY Upstate (New York, NY). IHC following stepwise citrate antigen retrieval was performed to identify HHV-8 LANA (Novoceastra, clone 13B10 at 1:100 dilution); tryptase (Dako, clone AA1 at 1:100 dilution); HHV-8 K8.1a (Advanced Biotechnologies, clone 228 at 1:500 dilution); histamine (Lifespan Biosciences, polyclonal at 1:2,000 dilution) and CD117 (Dako, clone PD-1 at 1:2,000 dilution). Stained sections were digitized at 20× using Aperio XT digital imaging system (Aperio) and reviewed by Leona Ayers, in the Department of Pathology, Ohio State University (Columbus, OH), or by Ethel...
Cesarman at Weill Cornel Medical College (New York, NY). Representative images were selected for publication presentation.

**Tryptase and N-methylhistamine ELISA**

Total MC tryptase (Cusabio) and the histamine metabolite N-methylhistamine (IBI International) were measured by ELISA on archived plasma samples from 25 patients with HIV+ and 13 patients with HIV- KS obtained from the ACSR. Control samples were obtained from 13 healthy adults from the university community at SUNY Upstate (New York, NY). The assays were carried out according to the manufacturer’s instructions.

**Statistical analysis**

Difference in percent β-hexosaminidase release was analyzed by using one-way ANOVA, followed by Bonferroni multiple comparison test. Differences in tryptase and N-methylhistamine levels between patients with KS and healthy comparators were assessed by one-way ANOVA, followed by Mann–Whitney two-tailed nonparametric test. To test the relationship between HIV VL and CD4+ T-cell count and MC activation products, Spearman nonparametric correlation analysis was performed. Value was considered significant if \( P < 0.05 \). All statistical analyses were performed with Prism 6 software (GraphPad).

**Study approval**

Human studies were approved a priori by the Institutional Review Board at SUNY Upstate Medical University (New York, NY). All tissues and plasma samples were obtained for testing following signed Material Transfer Agreements based on SUNY Upstate Medical University and Ohio State IRB approvals. Normal comparators were obtained following signed consent and SUNY Upstate Medical University approval. The case study patient was included upon signed consent at the Infectious Disease clinic, SUNY Upstate Medical University.

**Results**

**MCs are permissive to KSHV infection in vitro and in vivo**

Because MCs are permissive to infection by other viruses, we assessed the ability of two extensively validated human MC lines, HMC-1 SC6 and LAD2, for permissivity to infection with KSHV. MCs were inoculated at an MOI of 1–3, and RNA was then isolated at 6, 24, 48, and 72 hpi. We assessed expression of KSHV latent genes (ORF71, ORF72, ORF73, and K12) and lytic genes of all three temporal classes (including immediate early, ORF50; early, ORF27, K2, K4, K5, K6; and late, ORF64 and K8.1) and found robust expression at all time points analyzed, suggesting that MCs support KSHV gene expression (Fig. 1A). No viral gene expression was observed in mock- or UV-irradiated virus treated MCs or in the no RT controls. Valproic acid–treated BCBL-1 cells served as a positive control for KSHV lytic gene expression. Overall, these data demonstrate MCs support both latent and lytic KSHV gene expression.

KSHV-infected ECs lose the viral episome as they divide and eventually die (2, 26). To evaluate the ability of MCs to maintain KSHV infection over a longer time period and to evaluate the levels of latent ORF73/LANA quantitatively, we measured gene expression in cultured MCs for up to 15 days postinfection by qPCR. Expression of ORF73 was robust throughout the culture period in both HMC-1 and LAD2 MCs (Fig. 1B), suggesting maintenance of viral infection long-term. To evaluate the number of infected cells in our cultures, we carried out limiting dilution qPCR. Poisson distribution demonstrated 14% of HMC-1 (Fig. 1C) and 11% of LAD2 (Fig. 1D) MCs in culture harbored LANA+, KSHV genomes at 24 hpi suggesting both LAD2 and HMC-1 cells supported similar levels of KSHV infection. To investigate whether MCs support infection in KS lesions, biopsies of AIDS-KS were examined by double staining for MC-specific tryptase and either the KSHV latent LANA/ORF73 (nuclear) or lytic K8.1A (cytoplasmic) proteins. We observed subpopulations of MCs that expressed LANA (Fig. 1E and F) or K8.1A protein (Fig. 1G). Known structures of the KSHV latency program demonstrates that the KSHV latency program was established in these MCs and, as expected, within their companion plasma cell in vivo. To quantify the number of LANA+ MCs, we counted five high-powered fields of view from five different dermal lesions and found 12% ± 3% (mean ± SD) of MCs were LANA+. We also identified MCs expressing cytoplasmic lytic K8.1A protein in LNs, consistent with our in vitro infection data; Fig. 1G, left inset box demonstrates tryptase ‘K8.1A’ MC seen paired with K8.1A+ plasma cell, while the right inset box shows a single tryptase ‘K8.1A’ MC. The strongly cytoplasmic localization of late lytic K8.1A suggests that these cells clearly support lytic infection in vivo. K8.1A was not assessed in all lesion locations and is currently under investigation. The close association of MCs with lytic infected plasma cells presents a potential in vivo mechanism for KSHV infection of MCs within the lesion microenvironment. We were unable to visualize both lytic K8.1A and latent LANA expression in tryptase ‘MCs due to technical constraints that prevented us from determining the ratio of latent to lytic MCs within KS lesions. Taken together, our data clearly demonstrated that MCs are permissive for KSHV infection and maintain viral gene expression long-term, positioning MCs as possible KSHV reservoirs.

**MCs support productive infection with KSHV**

Because we observed lytic gene expression from all temporal classes in MC cultures up to 72 hpi, we evaluated whether MCs complete the lytic cycle and produce progeny virus. To test this, HMC-1 cells were infected with live and UV-irradiated KSHV and cell-free supernatants obtained at various times postinfection were evaluated for the presence of encapsidated virus particles. Supernatants were treated with DNase to digest any naked viral DNA, proteinase K treated to remove the DNAse, and then DNA was extracted. Quantitative PCR to detect KSHV LANA-positive genomes demonstrated approximately 10^7 particles/mL in culture medium isolated from cells infected with live KSHV as early as 24 hours postinfection (Fig. 1H). Cultures treated with UV-inactivated virus had approximately 10^3 particles/mL controlling for amount of residual virus left after extensive wash. The data suggest that MCs can produce encapsidated viral genomes.

Herpesviruses are known to produce defective, noninfectious progeny (27); therefore, we evaluated whether MC-derived KSHV virions were infectious to ECs using cell-free supernatants harvested from infected MCs at 24 hpi. LANA-positive ECs are evident 48 hours postinfection by antibody-specific staining indicating...
MCs support KSHV infection in vitro and in patient lesions. A–D, HMC-1 and LAD2 cells were infected with KSHV and cultured for indicated times. A, MCs express abundant KSHV genes. GAPDH was used as a loading control. Data are representative of two separate experiments. B, qRT-PCR gene expression indicated MCs express LANA in growing cultures over the entire culture period. Data are expressed as \( C_t \) values normalized to GAPDH. Data are representative of three independent experiments performed in triplicate. C and D, Limiting dilution qPCR analysis of infected HMC-1 (C) and LAD2 (D) demonstrated approximately one in seven MCs were KSHV genome\(^+\) at 24 hpi. Data are expressed as mean ± SEM, \( n = 3 \). E–G, HIV/KS tissue double stained for KSHV LANA (nucleus, brown) and MC-specific tryptase (cytoplasm, red) demonstrate: LN shows (top upper inset; E) two tryptase\(^+\) MCs, one LANA\(^-\) (blue nucleus, arrow head) and the other LANA\(^+\) (brown nucleus, black arrow) paired with a LANA\(^+\) plasma cell (brown nucleus); dermal lesion shows tryptase\(^+\) MCs (black arrows) and tryptase\(^+\) LANA\(^+\) MCs (arrow heads; F). G, HIV-KS LN double stained for MC-specific tryptase (cytoplasm, red) and KSHV lytic antigen K8.1a (cytoplasm, brown) shows (left top inset) a tryptase\(^+\) K8.1a\(^+\) MC paired with a KSHV K8.1a\(^+\) stained plasma cell cytoplasm and (right bottom inset) a solo tryptase\(^+\) K8.1a\(^+\) MC cytoplasm (brown). Scale bar, 25 \( \mu m \) (inset scale bar, 10 \( \mu m \)). H–I, HMC-1 cells were infected as described in the Materials and Methods. H, KSHV-infected MCs produce encapsulated, DNAse resistant, virus during infection. Data are from two independent experiments with three experimental replicates and two technical replicates each and expressed as mean ± SEM. Mock infected cultures gave no CTs. I, Mast cell-derived-KSHV establishes latency in primary human endothelial cells. Cell-free supernatants were isolated from MCs uninfected or infected with KSHV at 24 hpi and used to treat primary human ECs. Forty-eight hours posttreatment, primary ECs were fixed and stained as indicated in the Materials and Methods; LANA\(^+\) nuclei-localized staining demonstrated establishment of latency. DAPI was used to visualize nuclei. Magnification \( \times630 \). Data are representative of three independent experiments.
that ECs were infected and had established latency with the encapsidated cell-free virus present in MC cultures (Fig. 1I, top). No staining was observed in mock-infected (Fig. 1I, bottom) or UV-treated cultures (data not shown). qRTPCR analysis of HUVEC cultures treated for 3 days with cell-free supernatants from MCs infected with live virus confirmed LANA expression in these cells. Two independent experiments, in triplicate and run in duplicate, gave an average of 12.69 ± 1.91 CTs for GAPDH expression and 30.50 ± 8.60 CTs for LANA expression (mean ± SD). UV virus–treated HUVECs, mock treated, and no RT controls gave no amplification. Together, our in vitro and in vivo studies provide consistent evidence to affirm our hypothesis that MCs support KSHV infection and may represent a reservoir for latent KSHV.

**KSHV induces activation of MCs in vitro**

MCs mediate inflammation by releasing a wide variety of mediators including β-hexosaminidase, tryptase, histamine, and heparin. To determine whether KSHV activates MCs to release stored mediators, we evaluated β-hexosaminidase release by LILVA and LAD2 MCs. Within 1 hour of treatment KSHV induced a significant, dose-dependent release of β-hexosaminidase by both MC lines into cultures with up to 40% (LAD2, Fig. 2A) and 25% (LILVA, Fig. 2B) of total enzyme released at the highest

![Figure 2.](image-url)
concentration of virus compared with mock. Degranulation was dependent on live virus, as MCs infected with UV-inactivated virus remained quiescent. These data demonstrate robust activation with degranulation of MCs following infection with KSHV, suggesting that MCs are capable of modulating the local inflammatory environment in KS lesions through the release of preformed mediators.

**Activated MCs are associated with spindle cells in vivo**

MC location and activation state was assessed in 20 HIV+ KS dermal biopsies and were compared with two control HIV+ tissues without KS lesions. Compared with resting tissue MCs (Fig. 2C) in normal skin, in early patch stage lesions, activated MCs had pronounced tryptase+ cytoplasmic granules and enlarged nuclei (Fig. 2D) consistent with an activated phenotype. MCs were found to align directionally with infected ECs, vacuolate, and release granules in the direction of the enlarged, infected LANA+ ECs even before full tissue invasion occurs (Fig. 2E). In more advanced KS nodular lesions showing increased tissue KS spindle cell density, massive (28) degranulation of MCs was observed with discrete tryptase-filled granules flooding the infected spindle cells (Fig. 2F, red granules, 100 75 50 50 40 30 20 10 0 HIV+ KS+ HIV+ KS+ HIV- KS+ HIV- KS+ HCHC Tryptase (ng/mL) N-Methylhistamine (pg/mL) 5,000 4,000 3,000 2,000 1,500 1,000 500 0 A – D, HIV+ patients with KS herpesvirus (KSHV) lesions double stained for MC-specific tryptase (cytoplasm, red) and KSHV LANA (nucleus, brown). A, Skin-KS lesions with abundant LANA+ ECs and activated tryptase+ MCs. A subset of activated MCs are LANA+ (right inset box). B, Lung-LANA+ spindle cell nuclei and associated tryptase+ MCs. C, Lymph node- LANA+ spindle cell proliferation and associated tryptase+ activated, enlarged, MCs, and D, gut with LANA+ spindle cell nuclei and abundant activated MCs with tryptase degranulation. E–H, Control HIV+ KS-negative tissues show small dendritic-shaped MCs with tryptase+ cytoplasm. Note the broad distribution of MCs and their obscure appearance in control HIV+ tissues. Scale bar, 50 μm. I–J, Elevated mast cell tryptase (I) and N-methylhistamine (J) in plasma samples from 26 patients with AIDS-associated HIV+ KS, 13 patients with classic HIV+ KS, and 13 healthy comparators (HC). Data are expressed as mean ± SEM. Differences in tryptase and N-methylhistamine levels between patients with KS and healthy comparators were assessed by one-way ANOVA followed by Mann-Whitney two-tailed nonparametric test (***, P < 0.001).
see inset box). In tumor tissues with confluent spindle cell density, partial and fully degranulated MCs were observed (Fig. 2G). The MC nuclei in some of the activated MC cells showed nuclear staining with anti-LANA antibody supporting KSHV infection in a subpopulation of these effector skin MCs. Degranulated "ghost" or "phantom" MCs were plasma membrane c-kit/CD117 positive (Fig. 2G, top inset box) showed nuclear staining with anti-LANA antibody supporting KSHV infection in a subpopulation of these effector skin MCs. Degranulated "ghost" or anti-LANA antibody supporting KSHV infection in a subpopulation of these effector skin MCs. Degranulated "ghost" or "phantom" MCs were plasma membrane c-kit/CD117+/C0+ (prototypical marker of MCs, data not shown) confirming the presence of a granule-depleted MCs. In later-stage KS lesions, MC degranulation was less obvious. However, the presence of significant extravasated erythrocytes and hemosiderin deposition suggested ongoing local heparin anticoagulation and bleeding related to release of heparin-stabilized tryptase (Fig. 2H). Images representative of single antibody staining for both KSHV LANA and the MC markers tryptase, histamine, and CD117, are shown in Supplementary Fig. S1. MC association with LANA+/C0+ spindle cells as well as increased density, activation, and degranulation were observed in all cutaneous KS lesions examined.

Increased MC density, activation, and degranulation were not limited to cutaneous KS. We also examined 11 tissue sections from lung, gut, and lymph node KS along with 11 corresponding HIV+ control tissues. Similar to active KS lesions in the dermis (Fig. 3A), lung (Fig. 3B), lymph node (Fig. 3C), and gut (Fig. 3D) lesions demonstrated association of activated MCs with KSHV LANA+/C0+ spindle cells. These KS lesion-associated MCs demonstrated the same tryptase+/C0+ granulated, hypogranulated, and degranulated MC forms irrespective of KS lesion location. In overall comparison of KS lesion tissue to control HIV+ tissues (Fig. 3E–H), we consistently observed large and activated tryptase+/C0+ MCs within and around KS lesions that was not seen in our HIV+ KS+ control tissues. Together, these data provide in vivo evidence for the accumulation, infection, activation, and degranulation of MCs within KSHV-LANA+/C0+ in diverse tissues. These data suggest MCs are a key component of KS lesions capable of supporting infection and as a potent source of inflammatory mediators necessary to help drive the establishment and progression of lesions.

MC-specific tryptase and N-methylhistamine are elevated in the blood of patients with KS.

Our complementary in vitro and in vivo data suggest MCs are key cellular players in the KS lesion microenvironment, promoting and supporting disease in a multifaceted way including by intense...
activation and inflammation. In some diseases, MC activation is so widespread or intense that the response can be measured in blood. We hypothesized that the extensive KSHV-mediated activation of MCs in KS would result in circulating levels of MC-specific, granule-stored, mediators. Increased tryptase (Fig. 3I) and N-methylhistamine, a metabolite of histamine (Fig. 3J), were found in plasma samples obtained from 26 patients with extensive HIV-associated KS (HIV+ KS+) and in 13 HIV- Classic KS (HIV- KS-) patients as compared with 13 healthy comparators (HC). These data confirm significant in vivo MC activation and degranulation (29, 30). Corresponding patient characteristics and assay results are given in Table 1. The mean age of HIV+ KS+ patients was 71 years and 11 of 13 were male; for the HIV- KS- patients, the mean age was 36 years and 18 of 26 were male, with a mean CD4+ T-cell count of 205.8. Tryptase levels in classic HIV- KS-, AIDS-associated HIV+ KS+ versus HC were significantly elevated, 32.25 ± 8.24 versus 9.98 ± 1.83 versus 1.64 ± 0.69 ng/mL, P = 0.0053. Significant increases were also observed in N-methylhistamine levels in patients with KS, 1,578 ± 333.10 versus 723.10 ± 114.10 versus 101.10 ± 31.96 pg/mL, P < 0.001, mean ± SEM. Seven classic HIV+ KS+ patients (54%) and six AIDS-associated HIV+ KS+ patients (23%) had tryptase levels higher than 20 ng/mL. Levels observed in MC activation disease and in mastocytosis. No correlation was found between levels of MC activation products and either HIV viral load or CD4+ T-cell count (HIV VL vs. tryptase r = 0.359, P = 0.092; CD4+ T-cell count vs. tryptase r = −0.031, P = 0.886; HIV VL vs. N-methylhistamine r = −0.188, P = 0.391; CD4+ T-cell count vs. N-methylhistamine r = −0.061, P = 0.778). In addition, no correlation was found between age or sex of individual and levels of MC activation products tryptase (age vs. tryptase r = 0.297, P = 0.118; sex vs. tryptase r = 0.143, P = 0.451; age vs. N-methylhistamine r = 0.117, P = 0.484; sex vs. N-methylhistamine r = 0.005, P = 0.974). The corresponding significant increase in circulating histamine in patients with KS supports active MC degranulation in these patients that is independent of the degree of immune suppression, age, or sex.

Antagonism of MC inflammatory mediators is associated with durable regression of AIDS-KS lesions

A 46-year-old HIV+ man with acutely progressing KS presented to establish care in our Infectious Diseases clinic. Since his HIV diagnosis 14 years prior, he had been intermittently adherent to his antiretroviral medications (ARVs). At diagnosis, he had KS lesions on the distal lower extremities bilaterally, but his lesions had been stable in number, size, and color for years. Five weeks prior to presentation to our clinic, he underwent an emergency appendectomy. Following surgery, he developed worsening of long-standing, but previously unrecognized, symptoms consistent with MC activation (31), including intermittent vertigo, headaches, gastroesophageal reflux (GERD), nausea, watery diarrhea, arthralgias, myalgias, and fatigue. Coincident with worsening of these symptoms, he noted development of significant bilateral lower extremity edema, bilateral tinnitus (also symptoms of significant MC activation), and an increase in the size and a darkening pigmentation of all lower extremity lesions, in particular the lesions on his left foot. In addition, he noted that a new lesion developed on his abdomen. Biopsies of the lower extremity and abdominal lesions were obtained. H&E staining demonstrated abnormal microvascular structures with extravasated erythrocytes and significant infiltration of mononuclear cells (Fig. 4A). IHC staining for the KSHV-specific LANA (brown) confirmed that the lesions were KS (Fig. 4B). Serial sections stained for MC markers tryptase (Fig. 4C, brown) and CD117 (Fig. 4D, red) demonstrated extensive MC infiltration. Notably, the distribution of tryptase in many cells was extracellular, indicating that the potent proinflammatory effects of MC degranulation were active in the patient’s tumors.

![Image](image_url)
We hypothesized that his worsening constitutional symptoms and progression of his previously stable KS both resulted from extensive MC activation triggered by his recent inflammatory appendicitis and abdominal surgery. Appendicitis is characterized by enhanced MC localization and activation (32). We sought to interrupt the proinflammatory response driven by ongoing MC degranulation with agents that block the target effects of MC products. Therefore, we prescribed cetirizine (a type 1 histamine receptor antagonist, 20 mg daily), ranitidine (a type 2 histamine receptor antagonist, 300 mg twice a day), montelukast (a leukotriene receptor antagonist, 10 mg daily), and vitamin C (a MC stabilizer, 1 g daily).

At a follow-up appointment two weeks later, his weight was down 4.5 kg, and he demonstrated significantly reduced lower extremity edema. In addition, he reported resolution of many of his worsening constitutional symptoms, including fatigue, GERD, diarrhea, tinnitus, and vertigo. Notably, he thought that the KS lesions on his legs and abdomen had lightened and shrunk. Upon examination, his lesions had become lighter in color and were visibly smaller (Fig. 4E). The top three images are of the right leg, the bottom three images are of the left foot; both series demonstrate dramatic reduction in lesion pigmentation and size over the course of treatment. Over the course of 2 months of treatment targeting MC-specific mediators we observed that his lesions continued to shrink, becoming smaller and macular; the lesion on his abdomen had fully resolved (not shown). The patient also reported feeling the best he had in more than 10 years.

These dramatic changes occurred in the absence of HIV control. At presentation to our clinic he reported variable adherence to his previous ARVs, including Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir. His HIV viral load was 40,121 RNA copies/mL and previously prescribed Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir. His HIV viral load was 40,121 RNA copies/mL and was in fact resistant to Elvitegravir, Emtricitabine, and Tenofovir. Viral resistance testing was sent at the initial visit and subsequently demonstrated that his viral isolate was in fact resistant to Elvitegravir, Emtricitabine, and Tenofovir. It is significant, therefore, that his dramatic regression of KS lesions was associated with initiation of anti-MC medications rather than suppression of his viral load. When his resistance testing was available, his ARVs were changed to Duranavir, Ritonavir, Dolutegravir, and Etravirine. One month after changing his ARV regimen, his viral load was 162 and CD4\(^+\) T-cell count was 577. Thus, viral suppression was achieved after KS lesion regression was observed.

Discussion

Progression of KS lesions is dependent on the expression of both latent and lytic viral genes, repeated rounds of new infection, and secretion of proinflammatory cytokines. Given that the majority of ECs within the KS lesional microenvironment are latently infected, and explanted KS cells rapidly lose viral infection upon culture, non-EC cell types are likely involved in the production of progeny virus. Likewise, explanted KS cells require the provision of exogenous inflammatory cytokines for growth. Therefore, the known autocrine and paracrine signaling pathways active in KS cells are not sufficient to drive tumor progression. On the basis of our complementary in vitro and in vivo observations, we propose that MCs may play a role in KS pathogenesis by acting as a long-lived reservoir for KSHV and by chronic release of proinflammatory compounds. A proposed model for the role of MCs in KS is shown in Fig. 5.

MCs support productive infection

Prior to lesion development, KSHV infection of target cells takes place (Fig. 5A). Like ECs, MCs are fully permissive to KSHV infection in vitro (Fig. 1). Expression of viral transcripts was rapid and sustained following infection of MC lines. MCs expressed viral transcripts by 6 hpi, similar to what was observed following primary EC infections (Fig. 1A; ref. 33). In contrast to the rapid establishment of latency in ECs following infection, however, MCs maintained expression of lytic transcripts at 72 hpi, while previous data suggest that virtually all lytic genes declined or were

Figure 5.

Model for the role of MCs in KSHV-induced Kaposi sarcoma. MCs support both latent and lytic KSHV infection in vivo; in vitro our data suggest completion of the cycle with release infectious virus able to establish latency in primary infected ECs. In contrast, primary KSHV infection of EC results in the establishment of viral latency soon after infection and is characterized by very limited viral gene expression and no viral progeny production. Infection induces major EC actin cytoskeleton changes resulting in spindle formation. Spindle cells proliferate in response to local inflammatory mediators and during proliferation some ECs lose the viral episome and eventually die. KS lesion maintenance and expansion must involve both a source of infectious virus capable of “reseeding” the lesion, and a potent source of required paracrine inflammatory effectors. KSHV virus induces significant MC activation, both in culture and in vivo, with release of protumourigenic, proinflammatory, and proangiogenic granule contents, including the highly abundant tryptase, histamine, and heparin, and the lysosomal enzyme β-hexosaminidase, into the cellular environment promoting oncogenesis via proliferation and survival of the latently infected ECs that compose the bulk of the tumor. Furthermore, concomitant release of heparin upon release of tryptase likely induces the edema and hemorrhage that are prominent characteristics of KS lesions.
Mast Cell Activation in Kaposi Sarcoma

Masts accumulate in KS tissue

IHC analysis of KS biopsies taken from diverse tissues and from lesions at different stages of development consistently demonstrated an enrichment of MCs in KS lesions compared with normal tissue (Figs. 2 and 3). This accumulation is likely driven by both recruitment of bone marrow–derived CD34+ MCs and local proliferation of tissue-resident MCs. KS lesions are rich in MC chemotactic factors, including platelet-derived growth factor–AB, VEGF (which is also present in MC granules), basic fibroblast growth factor, stromal-derived factor-1 (SCF), and CCL5. Moreover, KSHV encodes a homolog of cellular CCL2, K4; CCL2 is essential for recruitment and activation of MCs in adenocarcinoma-associated malignant pleural effusion (16). Our data demonstrate that in vitro–infected MCs express K4 (Fig. 1A). In addition, lesions are rich in COX-2, known to contribute to histogenesis of myriad tumor types, including MPE, primary cutaneous lymphoma, papilloma virus–associated squamous cell carcinomas, and pancreatic β-cell tumors. Likewise, there is precedence for an antitumor response with agents that antagonize MC survival or degranulation. Sodium cromolyn, an MC stabilizer that inhibits degranulation, blocks maintenance, and progression of pancreatic β-cell tumors. Treatment of mice with the c-kit inhibitor imatinib mesylate significantly limited MPE formation.

Antagonism of MC granule contents has anti-KS effects

Our model of MC contribution to the establishment and progression of KS lesions is supported by our demonstration of elevated plasma levels of MC granule contents and the rapid and durable regression of KS lesions in a patient with AIDS-associated KS treated with MC antagonists (Fig. 4). While this is the first demonstration of an association between MCs and KS, MCs are known to contribute to the pathogenesis of myriad tumor types, including MPE, primary cutaneous lymphoma, papilloma virus–associated squamous cell carcinomas, and pancreatic β-cell tumors. Treatment of mice with the c-kit inhibitor imatinib mesylate significantly limited MPE formation. Along with granule content markers, the other prototypical marker for MCs is C-kit/CD117. SCF binding triggers CD117 signaling, which is critical for the differentiation, migration, maturation, and survival of MCs (51). C-kit/CD117 activity is the target for treatment with the c-kit tyrosine kinase inhibitor imatinib mesylate and is used in the treatment of patients with mastocytosis (52). Detection of CD117 staining identified both granulated MCs and "ghost" or granule-depleted MCs within KS.
lesions independent of granule content. Importantly, fully degranulated MCs remain viable, slowly regranulating over a period of days (53). These cells are then able to undergo the entire process of activation and degranulation again, making them particularly potent effectors. One may envisage a MC activation and degranulation response in KS disease, followed by regranulation and subsequent degranulation thereby providing a continuous source of paracrine inflammatory mediators that help drive KS development and maintenance.

Previous work demonstrated CD117 expression in vitro on latently infected dermal ECs (43, 54) and on cells present in large numbers in KS lesions, with 93% of CD117+ lesions LANA+. Clinical trials testing the efficacy of Gleevec/ imatinib mesylate in the treatment of KS showed promise, with demonstrated clinical and histologic regression of cutaneous KS lesions (56). Interestingly, all reports found no correlation with KSHV LANA and CD117/c-kit expression, suggesting that the main proliferating component of lesions, the KS LANA+ EC spindle cells were not the predominant source of the c-kit signal. In this study, we did not observe CD117+ staining on KS spindle cells using conditions that readily stained MCs. It is interesting to speculate that perhaps the reason why LANA positivity was not correlated with CD117 staining was that the CD117+ cells detected were MCs, a subset of which we have shown are KSHV LANA+. Activated MCs can have an elongated phenotype and therefore some of the c-kit-positive spindloid cells in KS biopsies may in fact be MCs rather than ECs. In comparison with the accumulated bundles of LANA+ EC spindle cells in later lesions, the signal from the CD117+ LANA+MC would be small.

Reconstitution of immune competence and suppression of viral load is the first goal of therapy for patients with KS. However, our study results suggest that modulation of MC activity could potently alter KS tumorigenesis irrespective of a significant immunologic and viremic response to ARVs. Safe, widely available MC-stabilizing agents that are used to treat MC activation and allergic disease could provide a cost-effective approach to reducing both lesion inflammation and angiogenesis. Thus, modifying MC activation may represent a therapeutic strategy amenable for use in KS–prevalent, resource-constrained settings like sub-Saharan Africa.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L.W. Ayers, D. Wang, R. Rochford, C.A. King Development of methodology: L.W. Ayers, A. Barbachano-Guerrero, C.A. King Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.W. Ayers, A. Barbachano-Guerrero, E. Asiago-Reddy, R.C. Barnett, J.N. Martin, C.A. King Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.W. Ayers, A. Barbachano-Guerrero, S.C. McMulliner, E. Cesaran, D. Wang, J.N. Martin, C.A. King Writing, review, and/or revision of the manuscript: L.W. Ayers, A. Barbachano-Guerrero, S.C. McMulliner, D. Wang, R. Rochford, J.N. Martin, C.A. King Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.W. Ayers, J.A. Ritches, C.A. King Study supervision: R. Rochford, C.A. King Other (performed KS tissue IHC and interpreted appropriate findings for MCs and viral antigens to support our hypothesis): L.W. Ayers

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References


