Kaposi’s Sarcoma-Associated Herpesvirus Infection of Endothelial Cells Inhibits Neutrophil Recruitment through an Interleukin-6-Dependent Mechanism: a New Paradigm for Viral Immune Evasion

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi’s sarcoma (KS), an endothelial cell (EC) neoplasm characterized by dysregulated angiogenesis and inflammation. KSHV infection of EC causes production of proinflammatory mediators, regarded as possible initiators of the substantial mononuclear leukocyte recruitment seen in KS. Conversely, KSHV immune evasion strategies exist, such as degradation of EC leukocyte adhesion receptors by viral proteins. Here, we report the effects of KSHV infection of primary EC on recruitment of flowing leukocytes. Infection did not initiate adhesion of any leukocyte subset per se. However, on cytokine-stimulated EC, KSHV specifically inhibited neutrophil, but not PBL or monocyte, transmigration, an observation consistent with the inflammatory cell profile found in KS lesions in vivo. This inhibition could be recapitulated on uninfected EC using supernatant from infected cultures. These supernatants contained elevated levels of human interleukin 6 (hIL-6), and both the KSHV- and the supernatant-induced inhibitions of neutrophil transmigration were abrogated in the presence of a hIL-6 neutralizing antibody. Furthermore, preconditioning of EC with hIL-6 mimicked the effect of KSHV. Using RNA interference (RNAi), we show that upregulation of suppressor of cytokine signaling 3 (SOCS3) was necessary for this effect of hIL-6. These studies reveal a novel paracrine mode of KSHV immune evasion, resulting in reduced recruitment of neutrophils, a cell type whose antiviral and antitumor roles are becoming increasingly appreciated. Moreover, the findings have implications for our understanding of the contribution of hIL-6 to the pathogenesis of other inflammatory disorders and tumors in which this cytokine is abundant.

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EC with the cytokine tumor necrosis factor alpha (TNF-α) or interleukin-1 (IL-1), neutrophil transmigration was significantly and specifically inhibited when EC were infected with KSHV. This process depends on human IL-6 (hIL-6)-mediated upregulation of suppressor of cytokine signaling 3 (SOCS3). We thus propose a model in which KSHV elicits increased hIL-6 expression which results in SOCS3 expression and subsequent inhibition of cytokine-stimulated pathways necessary for neutrophil, but not mononuclear cell, recruitment in response to inflammatory mediators. To our knowledge, this is the first report of viral exploitation of paracrine signaling to modify both infected and uninfected neighboring cells for the purpose of immune evasion. Given the abundance of hIL-6 found in many types of cancer, our findings have wider relevance in the context of tumor immune surveillance.

**MATERIALS AND METHODS**

**KSHV production.** rKSHV.219 (KSHV) was produced from the latently infected Vero line (71), which expresses red fluorescent protein (RFP) from the cytomegalovirus (CMV) immediate-early promoter, and a puromycin resistance gene as a selectable marker. The method of KSHV production in this system was as described by the originators, Vieira and O’Hearn (71). Briefly, Vero cells were maintained in Eagle’s minimal essential medium (MEM), 2.2 g/liter NaHCO3, 10% fetal calf serum, and pyrloom (5 μg/ml) (all from Sigma-Aldrich, Poole, United Kingdom) and penicillin and streptomycin (10 μg/ml; Gibco, Invitrogen). Vero cells were infected with BacK50 and treated with 1.25 mM sodium butyrate (Sigma) to induce lytic replication. BacK50 is a recombinant baculovirus engineered to express the KSHV lytic switch replication and transcription activator (RTA) protein, encoded by ORF50 (71). BacK50 was produced using insect Sf9 cells grown in Grace’s insect medium (Gibco) supplied with 10% fetal bovine serum (FBS) at 28°C in a 5% CO2 incubator. Three days after BacK50 infection, the baculovirus-containing supernatant was separated from Sf9 cells by centrifugation at 450 x g for 10 min. Supernatants were passed through a 0.45-μm filter. After 48 h of incubation, supernatant from the reactivated Vero cells was removed, centrifuged at 500 x g for 15 min to remove cell debris, and then ultracentrifuged at 65,000 x g for 4 h. The resultant pellet was resuspended overnight in EB2 medium (Lonza, Lon, Lon, Lon, Lon). The infective titers were determined by infecting HEK293 cells with serial dilutions of rKSHV.219 and counting the number of GFP-positive cells at 48 h postinfection, using a fluorescence microscope. One GFP-positive cell was assumed to result from infection by one infectious unit of virus; indeed, IFA staining confirmed that the majority of GFP-positive cells contained a single latency-associated nuclear antigen (LANA) dot. In some experiments, rKSHV.219 was UV inactivated prior to EC infection.

**Endothelial cell culture.** Human umbilical vein endothelial cells (EC) were isolated from umbilical cords as previously described (19) and maintained in medium 199 (M199; Invitrogen, Paisley, United Kingdom) containing 20% fetal calf serum, 28 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 1 mg/ml epidermal growth factor, and 1 μg/ml hydrocortisone (all from Sigma) until confluent. Primary cultures were dissociated with trypsin-EDTA (Sigma) and passages into tissue culture multwells plates (Falcon; Becton Dickinson Labware, NJ), as described previously (11), or into prefabricated channel slides (μ-Slide VI, ibidi, GmbH, Martinsried, Germany). Seeding density yielded confluent monolayers for infection within 24 h.

**Infection of endothelial cells and treatment with cytokines.** rKSHV.219 was diluted in EB2 medium to 0.025% (twolence/ml prior to infection in multwell plates) to a multiplicity of infection (MOI) of 2.5, or 0.1% and centrifuged at 450 x g for 30 min (uninfected samples were treated in an identical manner, except there was no addition of rKSHV.219). EC were incubated for a further 90 min at 37°C 5% CO2 before the KSHV-containing medium was replaced with M199 and supplements, and culture continued for between 4 h and 10 days. In some experiments, supernatants from uninfected or KSHV-infected EC (KSHV-EC) were harvested at various time points and stored at −80°C until required. EC monolayers remained viable over extended culture times and maintained full responses to cytokine treatment, as we have previously reported (10). In some experiments, the same protocol was used to infect EC with cytomegalovirus (CMV; Merlin strain; gift from Gavin Wilkinson and Paul Moss). CMV titer was determined from the assessment of fibroblast cytopathic effect upon infection with serial dilutions of virus. CMV was used at an MOI of 0.3, which yielded a level of infection similar to that yielded by KSHV when used at an MOI of 10. In some experiments, recombinant hIL-6 (50 ng/ml; Immunotools, Friesoythe, Germany) or KSHV-EC-conditioned supernatant (24-h culture) was added to untreated EC for 24 h. After the above-described treatments, either TNF-α (0, 10, or 100 U/ml, equivalent to 0, 30, 300 pg/ml; Sigma) or IL-1 (5 ng/ml; R&D Systems, Abingdon, United Kingdom) was added to EC for 4 h prior to the leukocyte adhesion assay. When desired, neutralizing antibody against hIL-6 (clone 6708; 5 μg/ml; R&D) or IL-8 (clone 6219; 5 μg/ml; R&D) was included in the medium 30 min prior to the neutrophil supernatant-treated, or infected EC for 24 h prior to TNF- or IL-1 treatment. The antibody against hIL-6 did not cross-react with viral IL-6 (vIL-6) (data not shown). Each experiment used first- or second-passage EC from a different donor.

**Treatment of endothelial cells with siRNA against SOCS3.** Human umbilical vein endothelial cells (HUVEC) were plated out in 6-well plates in M199 (supplemented as described above, but without antibiotics) to reach around 70% confluence the following day. A pool of 3 target specific commercial prevalidated SOCS3 small interfering RNAs (siRNAs), or an appropriate scrambled control (all Santa Cruz Biotechnology), were transfected using Lipofectamine (RNAiMAX, Invitrogen) reagent per the manufacturer’s instructions. Approximately 8 h later, EC were infected with KSHV, as described above. Treatment did not affect the subsequent level of GFP-positive EC. Knockdown was confirmed by Western blotting for protein at 24 h postinfection (see below).

**Leukocyte isolation.** Blood was collected from healthy volunteers into K, EDTA (Sarstedt, Leicester, United Kingdom) and used within 2 h. Neutrophils or peripheral blood lymphocytes (PBLS) were isolated by centrifuging the whole blood over a 2-step density gradient consisting of equal quantities of Histopaque-1119 and -1077 (Sigma) as described previously (12). The neutrophil or PBLS layer was aspirated from the density interface and washed twice in phosphate-buffered saline (PBS) containing 1 mM Ca2++, 0.5 Mg2++, and 0.15% cations-adjusted bovine serum albumin (BSA; Sigma). Sequential washout step preparations were over 95% pure, as assessed by staining with Diff-Quick to assess nuclear morphology. The PBLS were cultured on plastic to deplete contaminating monocytes prior to the wash stage. We have previously reported that lymphocyte preparations performed using this method are typically 95% ≤ ± 4% pure (68). Monocytes were isolated as described previously (68); 1 ml dextran (molecular mass, 500 kDa; 8% wt/vol in PBS; Sigma) was added to 10 ml whole blood, the tube was placed at a 45° angle from the vertical orientation, and red cells were allowed to sediment for 1 h. The leukocyte-rich supernatant was retrieved, placed on top of 3 ml Nycoprep 1068 medium (Amersham Biosciences, Uppsala, Sweden), and centrifuged at 4,000 x g for 15 min. The middle layer, rich in monocytes, was decanted. The monocytes were washed twice in EDTA-BSA and resuspended in PBS-BSA. We have previously reported that monocyte preparations are typically ≥ 88% ± 2% pure with the use of this method (68). Neutrophils, monocytes, or PBLS were counted using a Coulter counter (Coulter Electronics, Harpenden, United Kingdom), adjusted at 1 x 109/ml for neutrophils or PBLS, respectively, in PBS-BSA, and used within 2 h.

**Leukocyte recruitment assay.** Assays were performed using a system similar to that previously described (8). Channel slides containing confluent HUVEC were mounted on a microscope stage and viewed by phase-contrast video microscopy. One end of a channel was attached to a Harvard withdrawal syringe pump (Harvard Apparatus, South Natic, MA), to the desired flow rate. The other end of the channel was attached to an electronic valve (Lee Products, Gerards Cross, United Kingdom), allowing smooth transition between reservoirs containing leukocyte suspension or cell-free buffer. PBS-BSA was perfused across the HUVEC to remove residual TNF-α or IL-1. A 4-min bolus of neutrophils, PBLS, or monocytes was perfused through the microslide, followed by washout with PBS-BSA, at all a flow rate equivalent to a wall shear stress of 0.05 Pa. Video-microscopic recordings of leukocyte-HUVEC interactions were made for 15 s in 10 separate fields along the centerline of the channel following 6 min of washout. These were analyzed off-line using a computerized image analysis system (ImagePro; DataCell, Finchampstead, United Kingdom). Leukocytes were classified as (i) phase-bright cells rolling slowly over the EC surface (velocity, ~5 to 10 μm/s), (ii) phase-bright, activated cells, apparently stationary but actively migrating on the surface, or (iii) transmigrated phase-dark cells that were highly spread and migrating under the EC monolayer. Adherent cells were easily distinguished from nonadherent flowing cells, which were visible only as faint streaks. Adhesion data were calculated by counting all cells interacting with the monolayer and were expressed either as numbers of cells per mm2 or per 106 cells perfused or as numbers of cells relative to the level for control uninfected EC. The proportion of the adherent cells which were below the monolayer was calculated and transmigration expressed as a percentage of the total adherent.
RESULTS

KSHV infects primary EC and induces morphological changes. Early-passage EC were infected with recombinant rKSHV.219 (KSHV), which expresses the green fluorescent protein (GFP) from the EF-1α promoter, indicating latent infection, and the red fluorescent protein (RFP) from the KSHV lytic PAN promoter (71). The EC monolayer remained intact and viable when infected by spinoculation with KSHV at a multiplicity of infection (MOI) of 10 (Fig. 1A). Spindle-shaped morphology changes were apparent as early as 24 h postinfection and were always accompanied by GFP expression (Fig. 1A). Around 30% of treated EC expressed GFP (depending on the EC donor) by 24 h postinfection, and this level progressively increased to a maximum of around 75% by 10 days postinfection (Fig. 1B). GFP-expressing cells also showed typical punctate immunofluorescent staining for the latency-associated nuclear antigen (LANA). RFP could occasionally be detected in a minority of EC at 24 h and was undetectable after 5 days, demonstrating minimal lytic replication.

De novo KSHV infection of EC does not promote leukocyte recruitment. To determine whether KSHV infection of EC might induce an inflammatory response, we compared leukocyte recruitment from flow to the level for either untreated EC, tumor necrosis factor-α (TNF-α; 100 U/ml)-treated EC, or EC treated with KSHV (KSHV-EC; MOI = 10). To model early infection events, experiments were performed 24 h after KSHV inoculation. As expected, TNF-α stimulation robustly increased the adhesion of neutrophils (Fig. 2A), peripheral blood lymphocytes (PBLs) (Fig. 2B), and monocytes (Fig. 2C). KSHV-EC did not increase adhesion of any type of leukocyte above the low background level seen on unstimulated EC (Fig. 2A, B, and C). The same was true when EC were maintained in culture for 10 days prior to the assay (data not shown). KSHV infection of EC specifically inhibits neutrophil transmigration in response to TNF-α stimulation. As de novo KSHV infection did not induce leukocyte recruitment to EC per se, we investigated whether KSHV infection of EC modified recruitment in response to the inflammatory cytokine TNF-α. The numbers of neutrophils, PBLs, or monocytes adhering to TNF-α-stimulated EC were not affected by KSHV infection (Fig. 3A). However, transmigration of adherent neutrophils across the EC monolayer was strongly inhibited by KSHV infection (MOI of 10) (Fig. 3B), while PBL and monocyte transmigration remained unaffected. Photomicrographs show how surface-adherent and transmigrated neutrophils could be distinguished (Fig. 3C) and how KSHV-EC support lower levels of neutrophil transmigration than the untreated controls (Fig. 3D). For reference, the untreated controls supported 41.7% ± 4.1% neutrophil transmigration (means ± standard errors of the means [SEM] from 27 experiments). To investigate whether the extent of the KSHV-mediated...
Inhibition of neutrophil transmigration was influenced by the amount of input virus, infection of the EC was performed at various MOIs. Neutrophil adhesion was unaffected by KSHV infection, regardless of MOI (Fig. 4A). Neutrophil transmigration was significantly inhibited at an MOI of 5 or 10, but not 2 (Fig. 4B), although a downward trend was apparent. To determine whether the response to KSHV depended upon virus gene expression, we UV inactivated KSHV prior to infection at the highest MOI tested (10). EC infected with UV-inactivated KSHV failed to express GFP and were negative for LANA by an immunofluorescence assay. UV-inactivated virus had no effect on neutrophil transmigration, compared to the level for the uninfected control.

Neutrophil transmigration was significantly and similarly inhibited from 24 h after KSHV inoculation onwards (Fig. 5B). KSHV did not inhibit neutrophil transmigration at the earlier time points tested, although a trend was apparent at 12 h postinfection (Fig. 5B). Analysis of neutrophil behavior revealed that those that had not transmigrated had a tendency to roll on KSHV-EC, rather than become firmly adherent (Fig. 5C), compared to the level for the control.

Neutrophil transmigration in response to IL-1 is inhibited, but not to the extent seen with TNF-α stimulation. In order to test whether the inhibition in neutrophil transmigration caused by KSHV was cytokine specific, we used interleukin-1 (IL-1) as a comparison to TNF-α. Similarly to TNF-α, neutrophil adhesion in response to IL-1 stimulation was not affected by KSHV infection (Fig. 6A), while neutrophil transmigration was significantly inhibited (Fig. 6B). This inhibition was considerably weaker than that observed when TNF-α was the stimulus.

CMV does not inhibit neutrophil transmigration in response to TNF-α. To determine whether the inhibition of neutrophil transmigration was a mechanism specific to KSHV or a more general consequence of herpesvirus infection, we tested the effects of the endothelium-tropic human cytomegalovirus (hCMV; Merlin strain) on neutrophil transmigration. Around 30% of the EC were positive for CMV infection at 24 h post-inoculation, as measured by quantification of GFP expression and accompanying morphological changes. The early CMV antigen E1 could be detected by RT-PCR in infected EC (data not shown). When CMV-EC were stimulated with TNF-α, neither neutrophil adhesion nor transmigration was modified compared to the level for the uninfected control (Fig. 7A and B). Therefore, the inhibition of neutrophil recruitment seen with KSHV infection is not a generic effect of viral infection of EC.

KSHV modulates ICAM-1 expression. The EC adhesion molecule ICAM-1 is involved in leukocyte transmigration, and its expression has been reported to be modified by KSHV (67). To establish the effects of KSHV on ICAM-1, we measured surface expression, by flow cytometry, on resting and stimulated EC with and without KSHV infection. Constitutive ICAM-1 expression could be detected on untreated cultures (Fig. 8A), and levels were increased when EC were treated with 100 U/ml TNF-α (Fig. 8B). GFP-negative EC within KSHV-treated cultures constitutively expressed ICAM-1 (Fig. 8C and E), whereas GFP-positive cells within the KSHV-treated cultures were largely negative (Fig. 8C and E). Importantly, the GFP-negative EC within this culture showed expression levels similar to those shown by EC which had never been treated with virus (Fig. 8A and C). Thus, the reduction in ICAM-1 was a direct consequence of KSHV gene expression. When KSHV-treated EC were stimulated with TNF-α, both GFP-positive and GFP-negative EC showed an upregulation of ICAM-1 expression (Fig. 8D and F). Although expression levels on GFP-positive EC did not generally reach those seen on the GFP-negative EC (Fig. 8D and F) it is clear that infected EC were still able to upregulate ICAM-1 in response to TNF-α stimulation.

hIL-6 is responsible for the KSHV-mediated inhibition of neutrophil transmigration in response to TNF-α. Since suppression of migration did not require infection of all EC, we tested whether KSHV induced the release of the soluble fac-
A.  

B.  

C.  

D.  

FIG. 3. Effect of EC KSHV infection on neutrophil, PBL, or monocyte recruitment in response to TNF-α stimulation. EC were infected with KSHV at an MOI of 10, cultured for 24 h, and then treated with 100 U/ml TNF-α for 4 h before analysis of neutrophil, PBL, or monocyte adhesion (A) and transmigration (B) (relative to the level for the uninfected control). Data are means ± SEM from 3 to 7 independent experiments. *, *P < 0.01 for comparison to the uninfected control. (C) Image showing phase-bright surface-adherent neutrophils (1) and phase dark transmigrated neutrophils transmigrated below the EC layer (2). (D) Images showing neutrophil adhesion and transmigration to EC with and without TNF-α or KSHV treatment. KSHV infection significantly reduced the number of transmigrated (phase dark) neutrophils on TNF-α-stimulated EC.

tor(s) that modulated endothelial responses. When supernatant from KSHV-EC was used to condition uninfected EC for 24 h prior to TNF-α stimulation, neutrophil transmigration was inhibited, whereas supernatant from uninfected EC had no affect (Fig. 9A). In agreement with previous reports (3, 75), analysis showed that there was high concentration of human IL-6 (hIL-6) in supernatants from KSHV-EC, compared to the level for the uninfected controls (41.8 ng/ml versus 3.2 ng/ml, respectively; mean from 2 experiments). To determine whether hIL-6 was responsible for the inhibition of neutrophil transmigration, a neutralizing antibody against hIL-6 was added to the supernatant during the 24-h conditioning. Neutralization of hIL-6 abolished the inhibitory effect of the supernatant (Fig. 9A). Furthermore, the KSHV-mediated inhibition of neutrophil transmigration was also abolished when the hIL-6 neutralizing antibody was present during the 24-h infection prior to TNF-α treatment (Fig. 9B). As there were also elevated levels of hIL-8 in supernatants from KSHV-EC, compared to the level for the uninfected control (fold increase = 2.1 ± 0.3; means ± SEM for 3 experiments; *P < 0.05 for comparison by an unpaired t test), we used hIL-8 neutralizing antibodies to determine whether there was any contribution of IL-8 to the inhibition of neutrophil transmigration. In the presence of this neutralizing antibody, there was no effect on the inhibition of neutrophil migration induced by KSHV, showing that there was no role for IL-8 (Fig. 9B). In order to confirm that hIL-6 was capable of inhibition of neutrophil transmigration, EC were conditioned with 50 ng/ml recombinant hIL-6 (a concentration similar to that found in KSHV-EC supernatant) for 24 h prior to addition of TNF-α. Neutrophil transmigration
was indeed inhibited at this concentration (Fig. 9C). When 1 or 10 ng/ml hIL-6 was used to precondition EC, there was no effect on subsequent neutrophil transmigration (89.6% ± 5.0% or 87.4% ± 15.8%, respectively, relative to the level for the untreated control; means ± SEM from 3 experiments). Consistent with our previous observations using KSHV-infected EC, PBL transmigration was not inhibited by this preconditioning of the EC with 50 ng/ml hIL-6 (Fig. 9C). Furthermore, IL-6 preconditioning significantly inhibited neutrophil transmigration in response to IL-1 stimulation of EC (Fig. 9C), but not to the extent seen when TNF-α was used, mirroring the results obtained from the whole-virus studies. To determine when hIL-6 secretion was induced by KSHV infection, we analyzed supernatants from untreated EC or KSHV-EC. There was a significant increase in hIL-6 in supernatants from KSHV-EC, compared to the level for untreated EC, by 8 h postinfection (Fig. 9D). This is consistent with the time frame in which inhibition of neutrophil recruitment is observed. Taken together, these results suggest that hIL-6 acts to inhibit elements of the inflammatory pathways initiated by TNF-α and, to a lesser extent, IL-1.

**Upregulation of SOCS3 is required for KSHV-mediated inhibition of neutrophil transmigration in response to TNF-α.**

SOCS3, a negative regulator of the JAK/STAT signaling pathway, is induced upon hIL-6 signaling (2). As high levels of IL-6 were expressed with infection, we hypothesized that KSHV infection would result in expression of SOCS3. Indeed, a robust upregulation of SOCS3 mRNA was observed by qRT-PCR in KSHV-infected EC at 24 h postinfection, compared to the level for control uninfected EC (fold increase = 20.8 ± 5.1; means ± SEM for 4 experiments; *P* < 0.01 for comparison by a paired *t* test). To determine whether SOCS3 had a role in the KSHV-mediated inhibition of neutrophil transmigration, we inhibited its KSHV-induced expression using siRNA, prior to TNF-α treatment. Western blotting confirmed that SOCS3 protein was upregulated by KSHV infection (Fig. 10A), and that it could be knocked down using siRNA against SOCS3,
but was unaffected by the scrambled control (Fig. 10A). KSHV-infected EC treated with SOCS3 siRNA prior to infection supported neutrophil transmigration to the same level as that seen with uninfected control EC (Fig. 10B). KSHV-induced inhibition of neutrophil transmigration was still present for KSHV-EC treated with a nonspecific scrambled siRNA control (Fig. 10B). Thus, SOCS3 is required for KSHV-mediated inhibition of neutrophil recruitment.

**DISCUSSION**

EC regulate leukocyte recruitment from the circulation into the tissue. In the present study, we have described, for the first time, how this process is modified by KSHV infection. *De novo* KSHV infection of EC did not induce the recruitment of neutrophils, monocytes, or PBLs at any examined time point postinfection. KSHV reduced the proportion of adherent neutrophils transmigrating through EC in response to TNF-α and, to a lesser extent, IL-1. This effect was seen from 24 h postinfection and was still present when infected cells were maintained in culture for 10 days prior to the assay. CMV, another endothelium-tropic herpesvirus, did not affect neutrophil transmigration in this way, showing this is not a generic consequence of herpesvirus infection. Neither PBL nor monocyte transmigration was affected by KSHV infection under any conditions tested. When uninfected EC were conditioned with KSHV-EC supernatant, the inhibitory effect of KSHV infection on neutrophil transmigration was recapitulated, indicating a role for a soluble mediator. Using functional blocking antibodies, we have demonstrated that hIL-6 is the key regulator of this inhibition via the activity of SOCS3, an IL-6-inducible gene product that is a negative regulator of the JAK/STAT signaling pathway. To our knowledge, this is the first report of a paracrine mechanism of KSHV immune evasion. It introduces the novel concept that viral modification of not only infected cells but also the uninfected neighbors contributes to the immune evasion strategy.

*In vitro* KSHV infection induces EC production of a number of proinflammatory factors, which are also present in KS lesions (14, 24, 66, 77). It has been assumed that such factors could initiate formation of the inflammatory milieu found in KS (74, 77). However, in our model system, KSHV infection alone did not prime EC to recruit leukocytes. On the contrary, we have demonstrated that KSHV infection of EC selectively inhibits neutrophil transmigration in response to cytokine stimulation. These data are consistent with the observation that neutrophils are sparse within KS lesions, where the inflammatory infiltrate is composed mainly of lymphocytic cells (21, 52, 56, 59). Recruitment of PBLs or monocytes was not affected in our system. Our study provides a possible explanation for the apparent underrepresentation of neutrophils in an otherwise inflammatory cell “hot spot.”

In our system, KSHV-EC were predominantly latently infected, where a minimal number of gene products are expressed (57). Most infected EC in KS lesions also exist in this state (64), although a small number will be undergoing lytic reactivation during which most viral proteins are expressed.
The KSHV protein K5 is of interest in the context of leukocyte recruitment, as it downregulates EC ICAM-1 (20, 33), which is critical for neutrophil transmigration (63, 79). Despite the classification of K5 as a lytic cycle protein, the latently infected KSHV-EC in our system have reduced ICAM-1 surface expression, in agreement with a previous study which reports a role for K5 in the reduction of ICAM-1 in latently infected EC (67). We found the reduction in the expression of ICAM-1 on KSHV-EC correlated with GFP intensity, and so presumably viral load, as previously reported (1).
It is unlikely, however, that changes in ICAM-1 expression explain the inhibition in neutrophil transmigration with infection. First, we observed that ICAM-1 expression on KSHV-infected EC was strongly upregulated upon TNF-α or IL-1 stimulation, the conditions under which neutrophil transmigration through EC was inhibited by the virus. This is consistent with previous studies which have shown that ICAM-1 is expressed on cells cultured from KS lesions (61), where they are exposed to high levels of inflammatory mediators. Second, transmigration was not impaired for lymphocytes, which also utilize ICAM-1 for transmigration (63). In addition, ICAM-1 reduction was restricted to infected cells. The inhibitory effect of KSHV was maximal after 24 h postinfection, when only

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(KSHV-EC), with or without the addition of neutralizing antibody (ab) against hIL-6, for 24 h prior to TNF-α treatment (data relative to the level for control EC in own medium) (A); EC infected with KSHV, with or without anti-IL-6 or anti-IL-8 neutralizing antibody for 24 h, prior to TNF-α treatment (data relative to the level for control cytokine-treated EC which were not conditioned with hIL-6) (C). (D) Relative levels of hIL-6 in supernatant from either untreated EC or KSHV-EC at various time points postinfection. Data are means ± SEM from 3 to 7 independent experiments. *, P < 0.01 for comparison to the control.

FIG. 9. Role of human IL-6 (hIL-6) in KSHV-mediated inhibition of neutrophil transmigration in response to TNF-α. Neutrophil or PBL transmigration efficiency was analyzed for the following: EC treated with supernatant from untreated EC (EC) or KSHV-infected EC (KSHV-EC), with or without prior treatment with siRNA against SOCS3, or a scrambled control. The loading control is β-actin. (B) EC were treated with or without siRNA against SOCS3 or a scrambled control. EC were infected with KSHV (MOI of 10), cultured for 24 h, and then treated with 100 U/ml TNF-α before analysis of neutrophil transmigration efficiency. Data are means ± SEM from 5 independent experiments. *, P < 0.05 for comparison to the uninfected control for each treatment.

FIG. 10. Effect of SOCS3 siRNA on KSHV-mediated inhibition of neutrophil transmigration in response to TNF-α. (A) SOCS3 expression in untreated EC or KSHV-EC, with or without prior treatment with siRNA against SOCS3, or a scrambled control. The loading control is β-actin. (B) EC were treated with or without siRNA against SOCS3 or a scrambled control. EC were infected with KSHV (MOI of 10), cultured for 24 h, and then treated with 100 U/ml TNF-α before analysis of neutrophil transmigration efficiency. Data are means ± SEM from 3 to 7 independent experiments. *, P < 0.01 for comparison to the control.
around 30% (compared to around 75% at later time points) of the EC were positive for KSHV. If ICAM-1 downregulation was responsible for the inhibition in neutrophil transmigration, greater inhibition one would have been expected at the later time points postinfection. An alternative explanation might be reduction in expression or presentation of neutrophil-specific CXC chemokines, which are essential for migration in this model (41). We checked mRNA levels for CXCL1, -5, and -8 following TNF-α stimulation, but they were not reduced by infection (data not shown). It is nevertheless possible that infection modified the surface glycoalyx, which is important for presentation of these chemokines, and this might be a fruitful area for further investigation.

We therefore hypothesized that there was a role for a paracrine mediator and indeed found that supernatant from KSHV-EC could recapitulate the effect of infection. This culture medium contained elevated hIL-6 levels, compared to that of uninfected EC, as has previously been reported (75). This difference was apparent from around 8 h postinfection, after which the elevated hIL-6 levels in supernatants from KSHV-EC appeared to plateau. The KSHV-encoded proteins LANA and vFLIP have been shown to induce hIL-6 expression through activation of the hIL-6 promoter (3, 4, 80). Both LANA and vFLIP are expressed during latent infection, thus making them likely candidates for the induction of hIL-6 seen in our model. In murine macrophages and human myelomonocytic cells, KSHV-encoded microRNA can also induce IL-6 (55). In addition, hIL-6 is one of the few cellular genes that escape KSHV-induc3ed host gene shutoff (28), but its function in KS pathogenesis thus far is unclear. It possibly plays a role in promoting cellular proliferation of KS-infected cells (7, 45), although others have found no role for the cytokine in this context (49). Interestingly, studies of HIV patients who were homozygous for an hIL-6 polymorphism within the promoter region associated with increased IL-6 production were overrepresented among those with KS lesions, providing further evidence of its importance in KS pathogenesis (26). Previously, the role of hIL-6 in inflammation has been unclear, some studies implicating a proinflammatory role (54), while others claimed it could act as an anti-inflammatory agent (76). The idea that IL-6 may have a dual role, favoring the resolution of the acute neutrophilic infiltrate and the initiation of the later component of neutrophil azurophil granules, can be highly toxic (34). Defensins, the most abundant component of neutrophil azurophil granules, can be highly toxic against myeloid leukemia cells (27, 40), and neutrophils can attack tumor cells by antibody-dependent cell-mediated cytotoxicity (30, 32, 36, 37, 60). The antiviral role of neutrophils is becoming increasingly appreciated. In mice, neutrophils reduced replication of a pathogenic recombinant influenza virus mice (69) and limited mouse hepatitis virus replication (81). In the context of herpesvirus infection, neutrophils were demonstrated to control herpes simplex virus 2 infection, also in a murine model (46). Thus, in the context of the KS lesion, inhibition of neutrophil recruitment would provide a survival advantage to both the virus and the transformed EC in which it resides. Intuitively, one may question why the virus has evolved to inhibit neutrophil but not PBL recruitment. Agents produced from infiltrating T-lymphocytes could play a role in the development of KS lesions (25), while KSHV-infected EC are protected from the antiviral activity of CD8 T-lymphocyte attack by virus-induced downregulation of cell surface major histocompatibility complex (MHC) class I expression (67).
In summary, this study reveals a previously unrecognized paracrine immunomodulatory strategy in which KSHV modiﬁes both infected and uninfected cells. This ﬁnding not only introduces a new paradigm for viral immune evasion but also suggests a mechanism by which IL-6 might modify neutrophil recruitment in inﬂammatory disorders and tumors where local concentrations of this cytokine are high.

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REFERENCES


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