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Opposing roles of RNA receptors TLR3 and RIG-I in the inflammatory response to double-stranded RNA in a Kaposi's sarcoma cell line

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Abstract

Kaposi's sarcoma (KS) is strongly associated with KS herpes virus infection, and inflammation plays an important role in this disease. We have shown that human KS biopsy-derived SLK cells, which are of endothelial origin and form KS-like tumors in nude mice, express the viral RNA pattern recognition receptors Toll-like receptor 3 (TLR3), retinoic acid-inducible gene-I (RIG-I), and melanoma-differentiation-associated gene 5 (MDA5). Furthermore, SLK cells have enhanced release of IL-6, IL-8 (CXCL8), RANTES (CCL5), and IP-10 (CXCL10) proteins in response to the synthetic viral RNA analog poly(I:C). SiRNA knockdowns demonstrated that TLR3 mediates this inflammatory response to poly(I:C) in SLK cells. Furthermore, knockdown of the RNA receptor RIG-I resulted in enhanced chemokine release, in a TLR3 pathway-dependent manner. Thus, exposure of KS cells to viral RNA ligands can result in a TLR3-mediated increase in the secretion of inflammatory proteins associated with KS cell growth that may contribute to disease.

Keywords

Kaposi's sarcoma; poly(I:C); TLR3; RIG-I; MDA5; Toll-like receptor; pattern recognition receptor

1. Introduction

Kaposi's sarcoma (KS) is a neoplasm comprised of spindle-shaped endothelial cells, angiogenic blood vessels, and immune cells, that is more common in individuals with HIV infection or that are immune suppressed. Inflammation plays an important role in Kaposi's sarcoma development [1]. Kaposi's sarcoma herpes virus (KSHV), also known as human herpes virus 8 (HHV-8), is strongly associated with this disease in both HIV-related and classical (HIV-negative) forms. KSHV viral proteins contribute to KS pathogenesis, and these include vGPCRs, vFLIP, vIL-6 and others [2]. In HIV-associated KS, it has been shown that the HIV tat protein binds to the VEGF receptor and is a survival factor for KS cells [3,4]. Numerous cytokines have also been shown to affect the growth of KS tumor-derived cells, including IL-6, IL-8, bFGF, and VEGF [5–8].

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Pattern recognition receptors recognize and respond to pathogen-associated molecular patterns (PAMPs). Pattern recognition receptors that recognize viral RNA include Toll-like receptors (TLRs) 3, 7, and 8, as well as retinoic acid-inducible gene I (RIG-I)-like RNA helicases such as RIG-I and melanoma-differentiation-associated gene-5 (MDA5) [9]. Ligands for these receptors include single-stranded (ss) or double-stranded (ds) RNA which are components of some viruses and can also be viral replication intermediates. Downstream signaling effects mediated by these pattern recognition receptors may include activation of NF- κ B and its transcriptional targets, which include pro-inflammatory genes, as well the induction of Type I interferon and interferon response genes [10].

In addition to the presence of KSHV, additional viral infection is often present in KS patients, most commonly HIV infection. Therefore, KS cells are exposed to a variety of viral ligands that may activate viral pattern recognition receptors, which could potentially lead to upregulation of inflammatory cytokines or chemokines implicated in KS cell growth. Our goal was to investigate the function of pattern recognition receptors in KS cells. These studies were conducted in the human KS biopsy-derived SLK cell line. SLK cells are KSHV negative, diploid, of endothelial origin, and form KS-like tumors in nude mice [11,12]. Since KS is a virus-associated disease, we were particularly interested in analyzing any functional viral pattern recognition receptors. We found that SLK cells express the viral pattern recognition receptors TLR3, RIG-I, and MDA5. We further characterized the inflammatory response of SLK cells to the synthetic viral RNA analog poly(I:C), and showed that it is mediated by TLR3. Furthermore, we found that siRNA-mediated knockdown of the RNA receptor RIG-I caused a significant, TLR3 pathway-dependent enhancement of the inflammatory response to poly (I:C) in SLK cells.

2. Materials and Methods

2.1. Cells and reagents

The human KS-derived SLK cell line [11,12] was obtained from Donald Ganem's laboratory (UCSF) and cultured in DMEM-High Glucose supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μ g/ml). Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex (Walkersville, MD) and cultured in EGM media (Cambrex). HUVECs were used within passage 4–6. Human blood samples were obtained from the San Diego Blood Bank. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia). Poly(I:C) from Invivogen (San Diego, CA) was resuspended in sterile, endotoxin-free buffered H₂O supplied by the manufacturer, and the buffered H₂O was used as a control. I κ B- α antibody (sc-371) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and β -actin antibody was obtained from Sigma (St. Louis, MO).

2.2. Cell stimulation experiments

Cells were plated the day prior to stimulation at subconfluent densities. To reduce the background cytokine and chemokine levels, media was replaced with fresh media and TLR agonist or control was added. For cytokine and chemokine analysis, supernatants were collected approximately 24 hours post-treatment, and retained at -20°C for subsequent immunoassays. For analysis of signaling events by western blot or changes in mRNA levels, protein or RNA was isolated at various timepoints.

2.3. Western blot analysis

Cells were washed once with PBS and lysed in Phosphosafe (Novagen, Madison, WI) according to the manufacturer's instructions. Protein concentrations were determined by Bradford analysis using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and

equivalent amounts of each sample were resolved on 4–12% Bis-Tris Novex gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon-FL membranes (Millipore, Bedford, MA). Antibody staining was performed using primary antibodies previously described and fluorescent-labeled secondary antibodies. Gels were scanned with an Odyssey gel scanner (Licor Bioscience, Lincoln, NE). β -actin levels were also determined as an additional control for equivalent protein loading.

2.4. Cytokine and chemokine analysis

Human IL-8 ELISAs were carried out according to the manufacturer's instructions using antibody pairs from BD Biosciences (San Jose, CA). Luminex multiplex cytokine analysis was conducted using the Beadlyte Human Multicytokine Flex Kit from Upstate Cell Signaling Solutions (Lake Placid, NY) according to the manufacturer's instructions.

2.5. RNA isolation, RT-PCR, and real-time quantitative PCR analysis

Cells were homogenized in Trizol reagent from Invitrogen (Carlsbad, CA) and stored at -80°C . For RNA preparation, Trizol homogenates were chloroform precipitated, ethanol was added, and the remaining RNA isolation was carried out using RNeasy spin columns from Qiagen (Valencia, CA). RNAs were treated with DNase I from Roche (Indianapolis, IN) to remove contaminating genomic DNA. Superscript II from Invitrogen was used with random hexamers for cDNA synthesis according to the manufacturer's instructions.

For PCR reactions, the quantity of cDNA synthesis reactions used was equivalent to 50–100 ng of input RNA. Primers sets used are listed in Table 1 of Supplementary Materials. PCR was carried out using AmpliTaq Gold DNA polymerase from Roche (Indianapolis, IN) according to manufacturer's instructions with the following cycling conditions: 95°C for 5 min (1 cycle); 94°C for 30 sec, 50°C for 30 sec, 72°C for 60 sec (30–35 cycles); 72°C for 8 min (1 cycle). Products were resolved on 1–2% agarose gels and visualized by ethidium bromide staining.

Real-time quantitative PCR (QPCR) reactions were carried out using a BioRad iCycler with Taqman probes (Integrated DNA Technologies, Coralville, IA) or Universal Probe Library probes (Roche, Indianapolis, IN), primer sets (Integrated DNA Technologies, Coralville, IA), and Taqman Universal PCR Master Mix (Roche, Indianapolis, IN) according to the manufacturer's instructions. Primer sets and probes used are listed in Table 2 of Supplementary Materials. For QPCR reactions, the quantity of cDNA synthesis reaction used was equivalent to 50 ng of input RNA. The cycling conditions were 50°C , 2 min (1 cycle); 95°C , 10 min (1 cycle); and 95°C for 15 sec, 60°C for 60 sec (40–50 cycles). Unless otherwise stated, analyses were done using the standard curve method. To generate standard curves for relative quantitation of levels of each gene, cDNA from peripheral blood mononuclear cells, which express Toll-like receptors 1–10, RIG-I, and MDA5, was used. Data is shown as normalized gene of interest level divided by normalized 18S rRNA level. For the analysis of RNA receptor mRNA levels from siRNA transfection optimization experiments, the comparative Ct method was used.

2.6. Small interfering RNA (siRNA) experiments

The following small interfering RNAs (siRNAs) were purchased from Dharmacon RNA Technologies (Lafayette, CO): TLR3 (D-007745-01), RIG-I (D-012511-01), MDA5 (D-013041-01), non-targeting siRNA #2 (D-001210-02), and siGLO Green Transfection Indicator (D-001630-01). An initial transfection experiment was conducted to confirm and optimize the transfection of SLK cells. SLK cells were transfected with varying concentrations of FITC-labeled siGLO siRNA combined with varying concentrations of Lipofectamine RNAiMAX from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. The extent of siRNA delivery into cells was determined 24 hours later by flow cytometry using a

FACS Calibur (Becton Dickinson, San Jose, CA). For all the transfection conditions tested, the majority of cells were FITC-positive compared to controls. However, the highest concentration of transfection reagent tested (3 μ l transfection reagent per 1 ml media) resulted in the greatest fluorescent signal and was therefore used in subsequent experiments. In an initial experiment the optimal conditions were used with the siRNAs of interest and the extent of knockdown was confirmed by QPCR (as described previously) to be 85% compared to levels in non-targeting siRNA treated cells. In all the siRNA experiments shown, cells were plated one day prior to transfection. Media was changed 4–6 hours post-transfection and poly(I:C) treatments were done 48 hours post-siRNA transfection.

2.7 Statistical analysis

Graphpad Prism software version 4.0b (San Diego, CA) was used for graphing data and statistical analyses. P values were determined by unpaired two-tailed t tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Human Kaposi's sarcoma-derived SLK cells and umbilical vein endothelial cells have similar, but distinct, Toll-like receptor mRNA constitutive expression patterns

The constitutive mRNA levels of human TLRs 1–10 were determined in SLK cells (human Kaposi's sarcoma-derived cells of endothelial origin) and HUVECs (normal primary human umbilical vein endothelial cells) for comparison. Human peripheral blood mononuclear cells (PBMC), which were found to express all 10 TLRs, were used as a positive control. Endpoint PCR showed that both SLK cells and HUVECs express TLR3 and TLR6, and HUVECs also express TLR1 and TLR4 (Fig. 1A). The TLR mRNA expression pattern by PCR was similar between HUVECs obtained from two different sources (data not shown). QPCR results also showed SLK cells express TLR3 and TLR6 mRNAs (Fig. 1B). Both PCR and QPCR showed that HUVECs express TLR3, TLR4, and TLR6 mRNA, however HUVECs appeared to have low levels of TLR9 and barely detectable TLR1 by QPCR (Fig. 1B) whereas by PCR there appeared to be significant TLR1 mRNA (Fig. 1A). These studies are the first to show that KS-derived SLK cells express TLR3 and TLR6 mRNAs. Prior studies have shown that HUVECs express TLR1, TLR2, TLR3, and TLR4 mRNA [13,14].

3.2. Human Kaposi's sarcoma SLK cells produce IL-8 in response to the synthetic double-stranded RNA analog poly(I:C) and express multiple RNA receptors

In some instances, cells that have barely detectable mRNA levels of a Toll-like receptor still have a functional receptor that responds to the appropriate ligand(s), which may be mediated by an increase in receptor level upon stimulation with ligand. Therefore, to determine if SLK cells have functional Toll-like receptors, these cells were stimulated for 24 hours with the ligands for Toll-like receptors 1–9 and supernatants were assayed by ELISA for secreted human IL-8, an inflammatory chemokine that is released by many different cell types in response to TLR activation. The synthetic double-stranded RNA analog poly(I:C) was the only ligand that led to significant release of IL-8 from SLK cells (Fig. 2A, left panel). Poly(I:C) also induced significant IL-8 release from HUVECs (Fig. 2A, right panel), consistent with previous studies [15]. Dose titration of poly(I:C) with concentrations ranging from 1–100 μ g/ml showed that approximately 25 μ g/ml poly(I:C) resulted in maximal IL-8 release from SLK cells (data not shown), and this concentration was used in all subsequent experiments.

Poly(I:C), a synthetic RNA analog that is used as a viral RNA mimic, is a known ligand for TLR3. However, additional RNA receptors exist that might also mediate the response to poly(I:C). These include the RIG-I-like RNA helicases RIG-I and MDA5. We determined that SLK cells express mRNA for RIG-I and MDA5 by endpoint PCR (Fig. 2B) and QPCR (Fig. 2C).

We also observed RIG-I mRNA expression in HUVECs and a very low level of MDA5 mRNA (Fig. 2B, 2C). It has been shown previously that HUVECs express RIG-I and MDA5 mRNA in response to viral infection [16].

3.3. Poly(I:C) stimulation of SLK cells results in I κ B- α degradation and increased levels of inflammatory cytokines and chemokines

Stimulation of the RNA receptors TLR3, RIG-I, or MDA5 can lead to NF- κ B activation, for which I κ B- α degradation is a precursor. Poly(I:C) treatment of SLK cells resulted in I κ B- α degradation (Fig. 3A). I κ B- α degradation was detected at the 3 hour timepoint, at which point an increase in IL-8 and IL-6 mRNA levels was also observed (Fig. 3B).

In order to have a broader view of the potential effects of poly(I:C) stimulation on SLK cells, a screen was conducted to determine what additional cytokines, chemokines, and growth factors are released in response to poly(I:C) stimulation. Supernatants from poly(I:C)-stimulated cells were analyzed by Luminex multiplex cytokine analysis. Secreted IP-10 (CXCL10), RANTES (CCL5), and IL-6 protein were all significantly increased in poly(I:C) treated cell supernatants versus control supernatants (Fig. 3C–E). The increased IL-6 release from SLK cells is consistent with previous studies showing that cells from KS biopsies secrete IL-6 in response to poly(I:C) [17].

3.4. Toll-like receptor 3 mediates the response to poly(I:C) in SLK cells

Poly(I:C) is a known TLR3 ligand. However, as noted earlier, SLK cells also express mRNA for RIG-I like RNA helicases RIG-I and MDA5 which are additional RNA receptors that could mediate the response to poly(I:C). Therefore, siRNA-mediated silencing of each of these genes was conducted to determine what receptor(s) mediate the response to poly(I:C) in SLK cells. RT-PCR analysis of mRNA levels of each gene was conducted on samples from both control and poly(I:C) treated cells. Poly(I:C) treatment led to increased levels of each RNA receptor compared to control buffer treated cells (Fig. 4A). The levels of TLR3, RIG-I, and MDA5 in poly(I:C) treated cells were significantly reduced by transfection with the appropriate gene-specific siRNA as compared to the non-targeting siRNA control (Fig. 4A). In order to determine whether the siRNAs, which are short double-stranded RNAs, elicit RNA receptor activation the levels of secreted IL-8 were determined by ELISA 24 hours post-siRNA transfection. Secreted IL-8 levels were not significantly increased by siRNA transfection as compared to control (no siRNA) transfection (data not shown). To assess the functional consequences of siRNA-mediated reduction of RNA receptor mRNAs, 48 hours post-siRNA transfection SLK cells were treated with poly(I:C) or buffer (negative control) for 24 hours and supernatants were collected and assayed for levels of secreted inflammatory proteins. TLR3 siRNA-transfected SLK cells had significantly lower levels of secreted IL-8 in response to poly(I:C) as compared to non-targeting siRNA-transfected controls (Fig. 4B). Furthermore, IP-10 and IL-6 levels were also significantly reduced compared to the same control (Fig. 4C & 4E). These results were obtained using 40 nM siRNA; however, a similar reduction of secreted IL-8 was obtained with 20 nM siRNA (data not shown). MDA5 siRNA-transfected SLK cells had secreted IL-8 levels greater than the non-targeting siRNA control (Fig. 4B), and increased RANTES (Fig. 4D). IP-10 levels were not significantly different (Fig. 4C). Secreted IL-6 from MDA5 siRNA transfected cells was significantly lower compared to the non-targeting siRNA-transfected cells but not significantly different from the transfection only control cells.

3.5. siRNA knockdown of RIG-I enhances cytokine and chemokine production from SLK cells in a TLR3 pathway-dependent manner

In contrast to the TLR3 siRNA-transfected cells, which had reduced cytokine and chemokine release after poly(I:C) stimulation, RIG-I siRNA-transfected cells had significantly higher

levels of secreted IL-8, IP-10, RANTES, and IL-6 protein, approximately 12-fold, 4-fold, 8-fold, and 2-fold respectively as compared to the non-targeting siRNA control (Fig. 4B–E). To determine if the enhanced chemokine release from the RIG-I knockdown cells was dependent on the TLR3 pathway, silencing of each RNA receptor individually as well as a TLR3/RIG-I double knockdown was carried out. Knockdown was confirmed by RT-PCR (Fig. 5A). SiRNA-mediated silencing of RIG-I and TLR3 in combination significantly reduced the IL-8 release in response to poly(I:C) compared to that observed in the RIG-I single knockdown (Fig. 5B). These results suggest that the enhanced IL-8 chemokine release from RIG-I knockdown cells occurs in a TLR3 pathway-dependent manner.

4. Discussion

Kaposi's sarcoma is strongly associated with KSHV infection, and is more prevalent in HIV-infected individuals. Understanding the effects of viral ligands on KS cells is useful in studying the pathogenesis of KS. Treatment with the double-stranded RNA analog poly(I:C) is used to simulate aspects of viral infection. It was previously reported that Kaposi's sarcoma cells from patient biopsies produce IL-6 and upregulate adhesion molecules ICAM-1, VCAM-1, and E-selectin, and NF- κ B binding activity in response to poly(I:C) [17,18], however the receptors mediating the effect were not identified.

The majority of the tumor cells of Kaposi's sarcoma are spindle cells, which are endothelial cells that have undergone a morphology change from a cuboidal to a spindle shape. KSHV infection of endothelial cells *in vitro* can cause their conversion to a spindle shape [19,20] and this phenotype can be recapitulated by expression of the KSHV viral protein vFLIP [21]. During the course of these studies it was observed that poly(I:C) treatment can also cause this morphology change in HUVECs (data not shown), and this was also observed previously [22]. Thus exposure of endothelial cells to viral RNA ligands can also induce a spindle-cell conversion that is associated with Kaposi's sarcoma development.

Double-stranded RNA can lead to the induction of genes associated with inflammation and an antiviral response, including NF- κ B target genes, Type-I interferons, and interferon response genes [10]. Inflammation is associated with Kaposi's sarcoma development, and numerous inflammatory factors have been shown to affect KS cell growth *in vitro* and in animal models. In these studies, we found that IL-8, IL-6, IP-10, and RANTES were generated by SLK cells in response to poly(I:C). IL-6 and IL-8 are directly implicated in KS cell growth. Kaposi sarcoma-derived cells from HIV-infected individuals proliferate in response to IL-6 [5]. IL-8, a chemoattractant for neutrophils that has been shown to induce angiogenesis, has been shown to be an autocrine growth factor and a surrogate marker for Kaposi's sarcoma [6]. Serum levels of IL-8 are elevated in patients with KS compared to controls and inhibition of IL-8 with anti-human IL-8 monoclonal antibody leads to a reduction in growth of SLK tumor xenografts in mice [6]. IP-10 (CXCL10) is an interferon-inducible chemokine that is an immunostimulant and RANTES (CCL5) is an interferon-inducible chemokine that is associated with chronic inflammation. These cytokines and chemokines, found to be produced by SLK cells, are also known to be products of endothelial cells. Poly(I:C) was used to mimic viral infection in this *in vitro* system; however, it remains to be determined how Kaposi's sarcoma cells *in vivo* respond to exposure to different types of whole virus.

The response to poly(I:C) varies among cell types, and in some instances leads to apoptotic cell death. For example, TLR3 activation by poly(I:C) treatment was shown to trigger apoptosis in human breast cancer cells [23]. Prior studies showed reduced proliferation of KS cells from patient biopsies after incubation with poly(I:C) [17]. We did not observe reduced proliferation of SLK cells in response to stimulation with poly(I:C) (data not shown), perhaps due to an underlying difference between primary KS isolates and the transformed KS cell line SLK.

Viral pattern recognition receptors include Toll-like receptors 3, 7, and 8 and cytoplasmic RNA helicases RIG-I and MDA5. These studies showed that SLK cells express mRNA for viral RNA receptors TLR3, RIG-I and MDA5, which they share in common with the related cell type primary human umbilical vein endothelial cells. RNA receptor activity is often influenced by the cell type and the type of RNA ligand. In some cases, the mode of delivery, for example transfection versus addition to media, also influences the response [24], which may be due to effects on localization of the ligand within the cell as well as the amount of ligand taken up. TLR3 is commonly located in endosomes and recognizes double-stranded RNA and its mimic poly(I:C) [25], and more recently it was shown to also recognize single-stranded poly(I) [26]. RIG-I and MDA5 are cytoplasmic RNA helicases originally identified as double-stranded RNA receptors [10]; however, RIG-I was shown to recognize single-stranded RNA with 5' triphosphates [27,28]. It has been shown in mice that MDA5 and RIG-I detect different types of dsRNAs, for example RIG-I recognizes *in vitro* transcribed dsRNAs and influenza virus, whereas MDA5 recognizes poly(I:C) and picornavirus [29]. Both RIG-I and MDA5 have been shown to respond to poly(I:C) *in vitro* [30].

Our studies showed that siRNA-mediated knockdown of TLR3 significantly reduces the levels of cytokines and chemokines released from SLK cells in response to poly(I:C). Therefore, the viral RNA receptor TLR3 mediates the response to poly(I:C) in SLK cells. The levels of secreted cytokines and chemokines were significantly, but not completely, reduced by knockdown of TLR3. This may be due to the fact that TLR3 mRNA is not completely knocked down and presumably TLR3 protein is only partially reduced, or because TLR3 is not the sole mediator of the poly(I:C) response in these cells. MDA5 knockdown in SLK cells had variable and modest effects on cytokine and chemokine release, suggesting that it does not strongly regulate the downstream signaling pathway activated by poly(I:C) in SLK cells.

SiRNA-mediated knockdown of RIG-I significantly enhanced the release of all cytokines and chemokines assayed: IL-8, IL-6, IP-10, and RANTES. It remains to be determined if this is a direct effect of RIG-I reduction, or due to secondary effects on downstream component(s) of the RIG-I pathway. Enhanced release of cytokines and chemokines from RIG-I silenced cells has also been recently reported in another cancer cell line. Liu et al. observed that RIG-I silenced A549 lung cancer cells had significantly higher levels of IP-10, RANTES (CCL-5), and ISG15 after 18 hours of RSV infection as compared to control cells [31]. We found that knockdown of TLR3 in addition to RIG-I reversed the significantly increased IL-8 release in response to poly(I:C) observed from the RIG-I knockdown cells. This suggests that the enhanced chemokine release observed in RIG-I knockdown cells is mediated by the TLR3 pathway. Our findings regarding the effects of RIG-I silencing on the TLR3-mediated poly(I:C) response suggest that these RNA receptors and their downstream signaling pathways do not function independently. Rather, there is some complex coordination or regulation that may occur at the level of RNA ligand-receptor interactions and/or in the downstream signaling pathways. Furthermore, RIG-I suppression may predispose cells to have an enhanced inflammatory or antiviral response to viral RNA ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ensoli B, Sturzl M. Kaposi's sarcoma: a result of the interplay among inflammatory cytokines, angiogenic factors and viral agents. *Cytokine Growth Factor Rev* 1998;9:63–83. [PubMed: 9720757]
2. Ensoli B, Sgadari C, Barillari G, Sirianni MC, Sturzl M, Monini P. Biology of Kaposi's sarcoma. *Eur J Cancer* 2001;37:1251–69. [PubMed: 11423257]
3. Ganju RK, Munshi N, Nair BC, Liu ZY, Gill P, Groopman JE. Human immunodeficiency virus tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases, and components of focal adhesion in Kaposi's sarcoma cells. *J Virol* 1998;72:6131–7. [PubMed: 9621077]
4. Cantaluppi V, Biancone L, Boccellino M, Doublier S, Benelli R, Carlone S, Albini A, Camussi G. HIV type 1 Tat protein is a survival factor for Kaposi's sarcoma and endothelial cells. *AIDS Res Hum Retroviruses* 2001;17:965–76. [PubMed: 11461682]
5. Miles SA, Rezai AR, Salazar-Gonzalez JF, Vander Meyden M, Stevens RH, Logan DM, Mitsuyasu RT, Taga T, Hirano T, Kishimoto T, et al. AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. *Proc Natl Acad Sci U S A* 1990;87:4068–72. [PubMed: 1693429]
6. Masood R, Cai J, Tulpule A, Zheng T, Hamilton A, Sharma S, Espina BM, Smith DL, Gill PS. Interleukin 8 is an autocrine growth factor and a surrogate marker for Kaposi's sarcoma. *Clin Cancer Res* 2001;7:2693–702. [PubMed: 11555581]
7. Ensoli B, Nakamura S, Salahuddin SZ, Biberfeld P, Larsson L, Beaver B, Wong-Staal F, Gallo RC. AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. *Science* 1989;243:223–6. [PubMed: 2643161]
8. Masood R, Cai J, Zheng T, Smith DL, Naidu Y, Gill PS. Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. *Proc Natl Acad Sci U S A* 1997;94:979–84. [PubMed: 9023368]
9. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* 2006;22:561–9. [PubMed: 16762830]
10. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature* 2006;442:39–44. [PubMed: 16823444]
11. Siegal B, Levinton-Kriss S, Schiffer A, Sayar J, Engelberg I, Vonsover A, Ramon Y, Rubinstein E. Kaposi's sarcoma in immunosuppression. Possibly the result of a dual viral infection. *Cancer* 1990;65:492–8. [PubMed: 2153430]
12. Herndier BG, Werner A, Arnstein P, Abbey NW, Demartis F, Cohen RL, Shuman MA, Levy JA. Characterization of a human Kaposi's sarcoma cell line that induces angiogenic tumors in animals. *AIDS* 1994;8:575–81. [PubMed: 7520247]
13. Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, Polentarutti N, Muzio M, Arditi M. Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J Biol Chem* 2000;275:11058–63. [PubMed: 10753909]
14. Maaser C, Heidemann J, von Eiff C, Luger A, Spahn TW, Binion DG, Domschke W, Luger N, Kucharzik T. Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. *J Immunol* 2004;172:5056–62. [PubMed: 15067088]
15. Morris GE, Parker LC, Ward JR, Jones EC, Whyte MK, Brightling CE, Bradding P, Dower SK, Sabroe I. Cooperative molecular and cellular networks regulate Toll-like receptor-dependent inflammatory responses. *FASEB J* 2006;20:2153–5. [PubMed: 16935934]
16. Berghall H, Siren J, Sarkar D, Julkunen I, Fisher PB, Vainionpaa R, Matikainen S. The interferon-inducible RNA helicase, mda-5, is involved in measles virus-induced expression of antiviral cytokines. *Microbes Infect* 2006;8:2138–44. [PubMed: 16782388]
17. Yang J, Hagan MK, Offermann MK. Induction of IL-6 gene expression in Kaposi's sarcoma cells. *J Immunol* 1994;152:943–55. [PubMed: 8283061]
18. Yang J, Xu Y, Zhu C, Hagan MK, Lawley T, Offermann MK. Regulation of adhesion molecule expression in Kaposi's sarcoma cells. *J Immunol* 1994;152:361–73. [PubMed: 7504714]
19. Ciufu DM, Cannon JS, Poole LJ, Wu FY, Murray P, Ambinder RF, Hayward GS. Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with

- mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. *J Virol* 2001;75:5614–26. [PubMed: 11356969]
20. Grundhoff A, Ganem D. Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. *J Clin Invest* 2004;113:124–36. [PubMed: 14702116]
 21. Grossmann C, Podgrabinska S, Skobe M, Ganem D. Activation of NF-kappaB by the latent vFLIP gene of Kaposi's sarcoma-associated herpesvirus is required for the spindle shape of virus-infected endothelial cells and contributes to their proinflammatory phenotype. *J Virol* 2006;80:7179–85. [PubMed: 16809323]
 22. Kaiser WJ, Kaufman JL, Offermann MK. IFN-alpha sensitizes human umbilical vein endothelial cells to apoptosis induced by double-stranded RNA. *J Immunol* 2004;172:1699–710. [PubMed: 14734752]
 23. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. *J Immunol* 2006;176:4894–901. [PubMed: 16585585]
 24. Elco CP, Sen GC. Stat1 required for interferon-inducible but not constitutive responsiveness to extracellular dsRNA. *J Interferon Cytokine Res* 2007;27:411–24. [PubMed: 17523873]
 25. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;413:732–8. [PubMed: 11607032]
 26. Marshall-Clarke S, Downes JE, Haga IR, Bowie AG, Borrow P, Pennock JL, Grencis RK, Rothwell P. Polyinosinic Acid Is a Ligand for Toll-like Receptor 3. *J Biol Chem* 2007;282:24759–24766. [PubMed: 17573354]
 27. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 2006;314:994–7. [PubMed: 17038590]
 28. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 2006;314:997–1001. [PubMed: 17038589]
 29. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006;441:101–5. [PubMed: 16625202]
 30. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 2005;175:2851–8. [PubMed: 16116171]
 31. Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol* 2007;81:1401–11. [PubMed: 17108032]

Fig. 1.

Constitutive expression of Toll-like receptors 1–10 mRNA in SLK cells and human umbilical vein endothelial cells. (A) Human umbilical vein endothelial cells (HUVEC), a human Kaposi's sarcoma cell line (SLK), and human peripheral blood mononuclear cells (PBMC) were analyzed for the expression of mRNA for the genes listed by endpoint PCR as described in Materials and Methods. (B) HUVEC and SLK cDNAs were analyzed by QPCR using the standard curve method as described in Materials & Methods. Toll-like receptor mRNA levels were normalized to 18S rRNA.

Fig. 2.

SLK cells release IL-8 in response to the synthetic double-stranded RNA analog poly(I:C) and constitutively express mRNA for RNA receptors RIG-I and MDA5. (A) SLK cells or HUVECs were treated with 25 μ g/ml poly(I:C) or control buffer for 24 hrs. Supernatants were collected and human IL-8 levels were determined by ELISA. Means and standard deviations of representative results from three independent experiments are shown. (B) Levels of RIG-I and MDA5 were determined by endpoint PCR. GAPDH levels were determined for normalization. (C) Levels of RIG-I and MDA5 were determined by QPCR using the standard curve method as described in Materials & Methods. Levels of each gene were normalized to 18S rRNA.

Fig. 3.

Poly(I:C) treatment results in increased IκB-α degradation, and increased inflammatory cytokine and chemokine mRNA and protein in SLK cells. (A) SLK cells were treated with 25 μg/ml poly(I:C) for the times indicated, whole cell lysates were collected, and IκB-α levels were determined by western blot analysis. Results shown are representative of two separate experiments. (B) SLK cells were treated with 25 μg/ml poly(I:C) for the times indicated, and mRNA levels of IL-6 and IL-8 were determined by RT-PCR. Results shown are representative of two separate experiments. (C–E) SLK cells produce cytokines and chemokines in response to poly(I:C). SLK cells were stimulated for 24 hours with 25 μg/ml poly(I:C), and culture supernatants were collected and levels of secreted (C) IP-10 (CXCL10), (D) RANTES (CCL5), and (E) IL-6 proteins were determined by Luminex multiplex cytokine detection. Means and standard deviations of two independent experiments are shown.

Fig. 4.

The cytokine and chemokine release in response to poly(I:C) in SLK cells is reduced by TLR3 silencing and enhanced by RIG-I silencing. (A) SLK cells were transfected with 40 nM of the indicated siRNAs using optimized conditions. At 48 hours post-transfection cells were stimulated with 25 μ g/ml poly(I:C) or control buffer, then 24 hours later RNA was isolated for RT-PCR analysis of the genes listed. (B–E) SLK cells were transfected and treated with or without poly(I:C) as described before, and 24 hours later supernatants were collected and assayed for (B) human IL-8 secreted protein by ELISA or (C) IP-10, (D) RANTES, and (E) IL-6 by Luminex multiplex cytokine analysis. Means and standard deviations from 2–3 independent experiments are shown; (**) $p < 0.01$ relative to non-targeting (NT) siRNA control + poly(I:C); (*) $p < 0.05$ relative to non-targeting (NT) siRNA control + poly(I:C); (–) transfection only (no siRNA).

Fig. 5. Enhanced cytokine and chemokine release in RIG-I knockdown cells is lost by concomitant silencing of TLR3. SLK cells were transfected with 20 nM of each siRNA listed, except non-targeting siRNA (NT) at 40 nM. At 48 hours post-transfection cells were stimulated with 25 μ g/ml poly(I:C) or control buffer, then 24 hours later (A) RNA was collected for RT-PCR analysis to confirm knockdown of the indicated genes and (B) supernatants were collected to determine levels of secreted human IL-8 by ELISA (means and standard deviations are shown).