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Supplemental Information

IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and reagents. Rabbit polyclonal antibodies detecting both the mature and unprocessed forms of human IL-1β (cat# 2022, cat# 2021) and caspase-1 (Cat# AHZ0082) were from Cell Signaling Technology, Beverly, MA and Invitrogen Corporation, Carlsbad, CA, respectively. Goat polyclonal antibodies against human ASC/TMS1 (cat# ER-03-0001) and Raybio Human IL-1β ELISA kits (ELH-IL-1beta-001) were from Ray Biotech, Norcross, GA. Mouse monoclonal antibodies against ASC (cat# D086-3) were from MBL International, Woburn, MA. Antibodies against human IFI16 (cat# sc-8023) and MNDA (cat# sc-6051) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Anti-AIM2 (cat# ab76423), anti-NALP3 (cat# ab17267), and anti-TATA binding protein antibodies (cat# ab818) were from Abcam Inc., Cambridge, MA. Anti-rabbit, anti-goat and anti-mouse antibodies linked to horseradish peroxidase, Alexa-488 and Alexa-594 were from KPL Inc., Gaithersburg, MD., or Molecular Probes, Eugene, OR. Protein G-Sepharose 4 beads were from GE Healthcare Bio-Sciences Corp., Piscataway, NJ.

Immunofluorescence microscopy (IFA). HMVEC-d cells infected with KSHV (20 -30 DNA copies/cell) were fixed, for 10 min with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min, washed and blocked with Image-iT™ FX signal enhancer (Invitrogen) for 20 min. Mock and KSHV infected cells were incubated with goat anti-ASC, rabbit anti-caspase-1 or mouse anti-IFI16 antibodies for 2h at 37^0C, followed by anti-goat Alexa Fluor 488, anti-rabbit Alexa Fluor 594 or anti-mouse-Alexa 594 labeled antibodies for 1h at 37^0C. The Olympus Fluoview 300 fluorescence confocal microscope or Nikon Eclipse 80i fluorescent microscope was used for imaging and analysis was performed using Fluoview software or Metamorph imaging software.

Immunofluorescence and fluorescence in situ hybridization. Uninfected and KSHV infected HMVEC-d cells were fixed as above and incubated with mouse anti-IFI16 antibodies followed by donkey anti-mouse Alexa Fluor 594 secondary antibodies. These cells were fixed with 2% paraformaldehyde for 10 min, permeabilized in 0.2% Triton X-100 for 5 min, and treated with 0.1 M Tris-HCl (pH 7.0) for 2 min and 2x SSC twice for 2 min. The cells were treated with denaturing solution (70% formamide in 2x SSC) at 70°C for 2 min, washed, dried, and subjected to in situ hybridization. KSHV FISH probe was developed and validated by Anatomic Pathology FISH Laboratory, Medical College of Virginia, Richmond, VA**.** Spectrum green labeled BAC36 KSHV entire genome was

used as a probe. The KSHV probe was diluted (1:10) in DenHyb hybridization solution (Insitus Biotechnologies, Albuquerque, NM) and incubated with cells for hybridization in a humidified chamber at 37^{o}C overnight. The slides were sequentially washed in 2x SSC (10min) and deionized water (1min), nuclei counter stained with DAPI and examined.

shRNA lentivirus system. IFI16, ASC and AIM2 expression were knocked down using shRNA lentivirus particles from Santa Cruz Biotechnology Inc. HMVEC-d cells in early passage were transduced with lentivirus particles following the manufacturer's instructions. These products are a pool of concentrated, transduction-ready viral particles containing 3 target-specific constructs that encode 19-25 nt (plus hairpin) shRNA designed to knockdown gene expression. For IFI16, three different individual target specific shRNA lentivirus particles were also used. The sequence information is given in supplementary Table 2. HMVEC-d cells at 50% confluency were incubated with 40 μl of ASC, IFI16 or control shRNA lentivirus particles in 1 ml of serum free media containing 5 μg/ml of polybrene. The transduced cells were selected with 50 μg/ml of puromycin for 4 days and selected cells were expanded. Protein knockdown in the selected cells was confirmed by Western blotting.

Transfection. HEK293T cells were transfected by the calcium phosphate method as described previously (Graham and van der Eb, 1973). HEK293T cells at 50% confluency in 6-well plates were transfected with a maximum of 4ug/well of plasmid DNA. The DNA was mixed with 100 μl of 0.25 M $CaCl_2$ and then with an equal volume of BES buffered solution (BBS: 50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95). The DNA mixture was added to the HEK293T cells in 2 ml of complete medium, incubated overnight at 3% CO_2 and fresh medium was added the next day.

Lentivirus vectors. The production and infection of lentivirus vectors were carried out as described by Vart et al. (Vart et al., 2007). Vesicular stomatitis virus-G envelope pseudotyped lentivirus was produced using a four-plasmid transfection system as previously described (Dull et al., 1998; Miyoshi et al., 1998). Lentivirus vector particles for overexpressing ORF71, ORF72, ORF73, ORF74, K8, K12 and were prepared as described previously (Sadagopan et al., 2009) This preparation was directly added to HMVEC-d cells to transduce the indicated KSHV genes. The cells were split at 24h after lentivirus transduction and subsequently used for examining inflammasome activation.

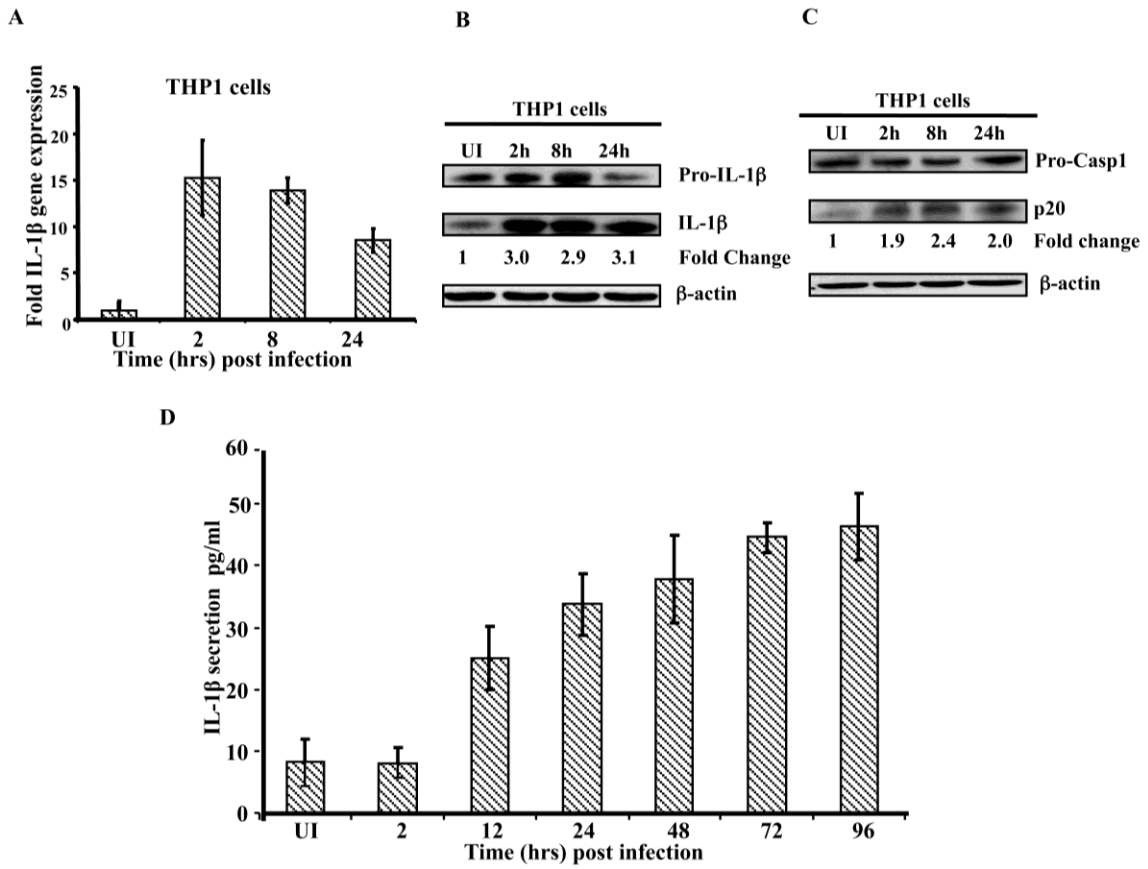

Figure S1. **KSHV Induces IL-1β Gene Expression and Secretion, Related to Figure 1**

(A) THP1 cells were infected with KSHV (30 DNA copies per cell) for the indicated time points. At different times p.i., IL-1β gene expression was examined by real-time PCR. Each bar represents the fold increase in gene expression ± SD of three independent experiments. These fold changes were calculated after normalizing with expression of the 18s rRNA gene. (B and C) THP1 cells were uninfected (UI) or infected with live KSHV for 2h. The uninfected and infected cells were washed to remove uninternalized cell-free virus and cells were either harvested immediately (2h) or incubated for different time points before harvesting. Proteins were analyzed by immunoblotting for pro- and mature-IL-1β (B) and pro-caspase-1 and activated caspase-1 (p20) (C). THP1 cells were infected with KSHV (30 DNA copies per cell) for the indicated time points. At different times p.i., culture supernatant from infected and uninfected (UI) THP1 cells were collected and secreted IL-1β was quantified by ELISA (D). Each bar represents the IL-1β secretion (pg/ml) + SD of three independent experiments.

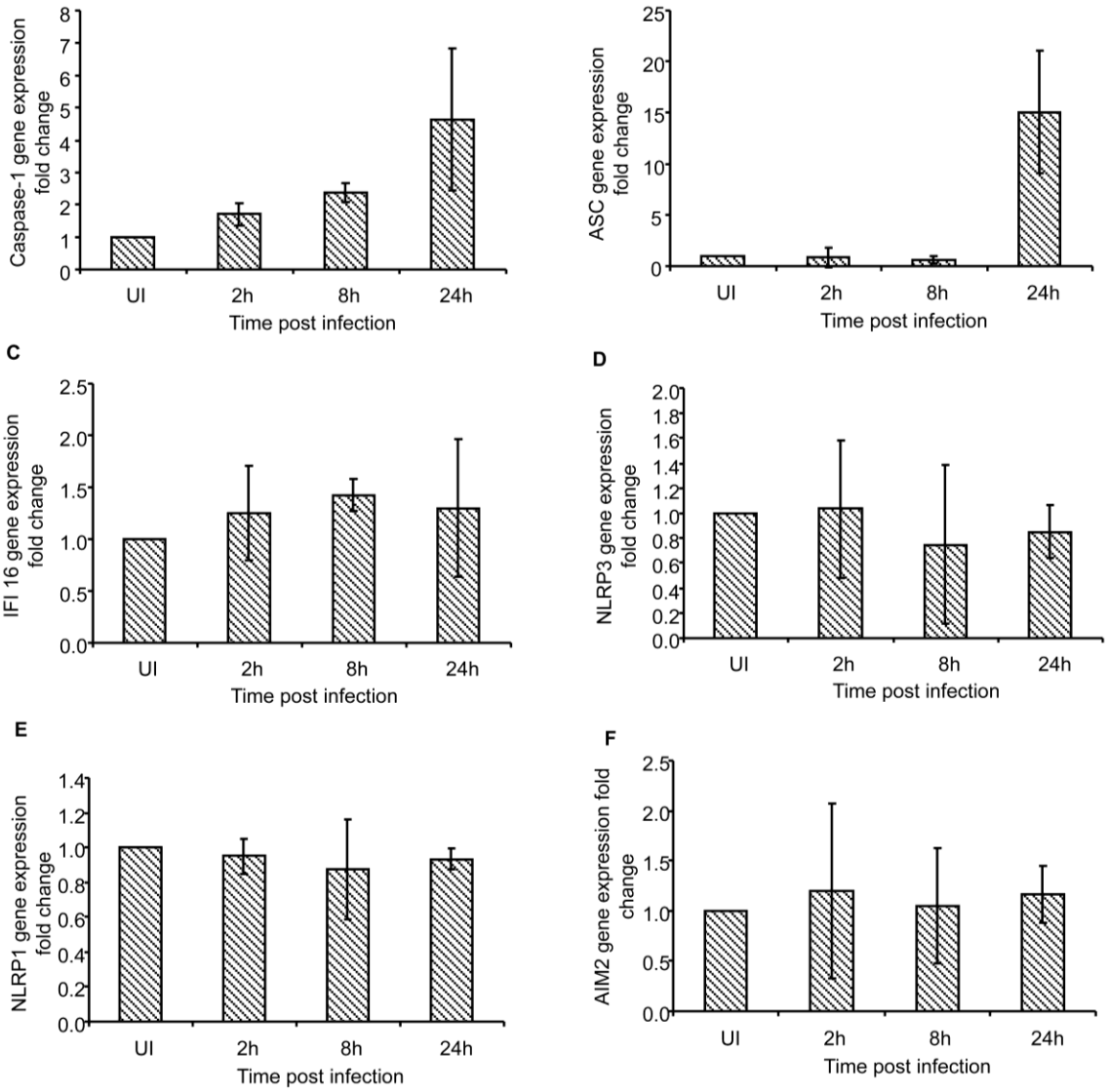

Figure S2. **Expression of Inflammasome Proteins in KSHV Infected HMVEC-d Cells, Related to Figure 3**

(A-F) HMVEC-d cells were infected with KSHV (30 DNA copies per cell) for 2h, uninternalized viruses removed by washing and infected and uninfected cells further incubated at 37^{o}C for the indicated time. RNA was isolated from infected and uninfected cells and gene expression was examined by quantitative real-time PCR using specific primers for caspase-1, *Asc*, *Ifi16*, *Nlrp3*, *Nlrp1*, and *Aim2*. Data for *Ifix* and *Mnda* are not shown as no expression was detected in these cells. Fold changes shown were calculated after normalizing with expression of the GAPDH gene. Each bar represents fold change in the gene expression + SD of three independent experiments.

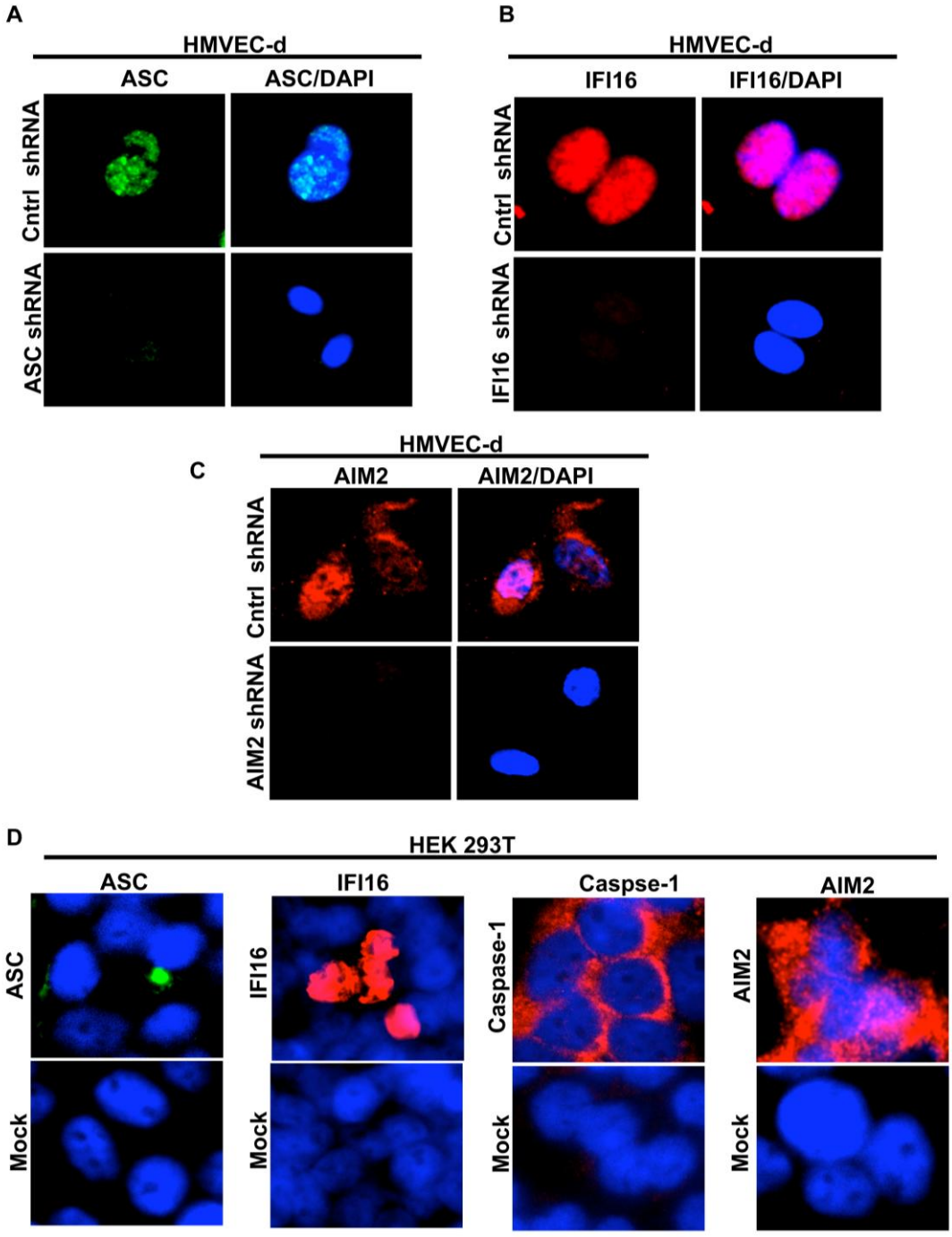

Figure S3. Specificity of Caspase-1, ASC, IFI16 and AIM2 Antibodies, Related to Figure 5

HMVEC-d cells transduced with control (Cntrl), ASC, IFI16 or AIM2 knockdown shRNA lentivirus particles were stained with (A) ASC (B) IFI16 and (C) AIM2 antibodies followed by donkey anti-goat Alexa-488, donkey anti-mouse Alexa-594 or donkey anti-rabbit Alexa-594 secondary antibodies, respectively. (D) HEK293T cells transfected with empty vector (mock) or with ASC or IFI16 or pro-caspase-1 or AIM2 expression plasmids. 24 h post-transfection, the cells were reacted with anti-ASC, IFI16, pro-caspase-1 or AIM2 antibodies followed by donkey anti-goat Alexa-488, donkey anti-mouse Alexa-594, donkey anti-rabbit Alexa-594 or donkey anti-rabbit Alexa-594 secondary antibodies, respectively.

Table S1. Related to Figure 1

A. Gene expression of inflammatory cytokines and chemokines upon KSHV infection of THP1 cells

Cytokines					
		UI	2h	12h	24h
1.	IL8	1	56.53	25.31	5.70
2.	IL10	1	27.75	5.34	3.37
3.	IL1β	1	26.28	11.58	6.70
4.	TNF	1	13.61	2.09	1.85
5.	SPP1	1	7.77	3.64	1.87
6.	IL1A	1	6.83	2.55	1.35
7.	LTA	1	5.85	4.59	1.44
8.	IL1RN	1	3.97	1.20	2.11
9.	IL8RA	1	2.66	1.26	1.94
10.	MIF	1	2.00	1.15	1.35
11.	IL5RA	1	1.35	1.53	1.70
12.	IL1F8	1	1.29	4.16	2.84
13.	IL13	1	1.26	1.55	2.06
14.	IL1F9	1	1.15	1.58	2.07
15.	IL22	1	1.08	1.20	1.75
16.	IL1F7	1	1.07	1.20	2.19
17.	IL9R	1	1.03	1.21	1.64
18.	IL1F5	1	1.02	1.20	1.99
19.	IL1F6	1	1.00	1.20	1.83
20.	IL9	1	1.00	1.20	1.68
21.	IL1F10		1.00	1.20	1.55

Chemokines					
		UI	2h	12h	24h
1.	CCL4	1	804.62	47.77	6.78
2.	CCL20	1	547.95	14.01	2.67
3.	CXCL10	1	89.77	72.42	19.55
4.	CCL3	1	89.60	8.30	3.31
5.	CXCL9	1	33.50	13.30	3.34
6.	CCL8	1	17.46	20.09	17.36
7.	CXCL3	1	9.26	4.20	3.23
8.	CCL2	1	8.05	7.12	9.20
9.	CXCL11	1	6.69	26.95	7.11
10.	CXCL2	1	6.69	3.27	1.62
11.	CCL18	1	3.66	3.07	2.35
12.	CCL7	1	3.55	5.03	3.83
13.	CCR7	1	3.19	1.14	1.73
14.	CXCL1	1	2.84	2.12	1.81
15.	XCR1	1	2.56	1.33	1.91
16.	XCR1	1	2.56	1.33	1.91

B. Secretion of inflammatory cytokines and growth factors upon KSHV infection of THP1 cells

Cytokines					
		UI	2h	12h	24h
1.	IL-6	1.0	2.0	8.4	0.3
2.	IL-5	1.0	2.2	5.5	1.8
3.	I-309	1.0	3.7	0.0	3.0
4.	IL-3	1.0	4.2	3.2	2.3
5.	IL12-p40	1.0	5.6	0.0	4.8
6.	IL-2	1.0	6.6	1.0	2.6
7.	TNF-β	1.0	1.1	2.9	0.6
8.	LIGHT	1.0	1.8	2.7	1.7
9.	IL-4	1.0	3.4	4.7	0.5
10.	IL-10	1.0	1.6	5.9	1.1
11.	IL-1β	1.0	9.0	7.3	5.4
12.	IL-1α	1.0	6.2	0.0	2.9
13.	IFN- γ	1.0	5.4	1.5	1.6
14.	TNF-α	1.0	10.4	6.9	1.1
15.	GRO	1.0	1.2	2.9	0.8
16.	IL-7	1.0	0.7	1.4	0.4
17.	IL-8	1.0	0.8	1.4	0.6
18.	IL-5	1.0	2.2	5.5	1.8
19.	IL-10	1.0	1.6	5.9	1.1
20.	IL-13	1.0	1.7	1.0	2.3
21.	IL-15	1.0	7.3	0.2	3.2
22.	LIF	1.0	2.6	1.2	5.9
Growth Factors					
1.		UI	2h	12h	24h
2.	BDNF	1.0	1.5	1.5	0.8
3.	EGF	1.0	0.8	1.6	0.1
4.	FGF-4	1.0	3.0	3.4	12.0
5.	FGF-6	1.0	2.0	0.5	10.1
6.	FGF-7	1.0	2.2	3.2	8.9
7.	FGF-9	1.0	2.3	7.3	4.9
8.	Flt-3 Ligand	1.0	3.2	2.4	5.4
9.	G-CSF	1.0	1.1	2.1	0.0
10.	GDNF	1.0	1.2	2.3	1.4
11.	GM-CSF	1.0	5.8	2.5	0.0
12.	GRO	1.0	1.2	2.5	0.8
13.	HGF	1.0	0.9	4.4	0.7
14.	IGF-1	1.0	0.3	2.5	0.0
15.	Leptin	1.0	3.6	2.9	3.5
16.	M-CSF	1.0	2.7	1.3	2.2
17.	PDGF-BB	1.0	4.7	3.9	7.6
18.	PlGF	1.0	3.9	5.9	7.5
19.	TGF-beta 1	1.0	3.1	2.1	1.6
20.	TGF-beta 2	1.0	1.5	1.3	1.8
21.	TGF-beta 3	1.0	1.1	0.6	1.4

C. Secretion of inflammatory cytokines and growth factors upon KSHV infection of HMVEC-d cells

		Cytokines					
		UI	4h	8h	24h	4days	5 days
1.	GRO	1.0	1.8	3.4	2.7	2.7	3.7
2.	GRO-α	1.0	1.9	2.3	1.7	1.8	3.3
3.	IL-1α	1.0	1.8	2.3	1.9	1.8	3.4
4.	IL-1β	1.0	1.9	2.6	2.0	2.4	3.1
5.	IL-2	1.0	1.9	2.5	2.0	2.0	3.9
6.	IL-3	1.0	1.8	2.0	2.0	1.5	3.1
7.	IL-6	1.0	1.9	2.8	2.0	2.0	4.0
8.	IL-7	1.0	1.7	3.2	2.1	2.1	3.4
9.	IL-8	1.0	1.5	1.9	1.5	1.4	1.4
10.	IL12-p40	1.0	1.7	3.0	2.5	2.4	3.7
11.	TNF-beta	1.0	2.0	2.9	1.9	2.7	3.7
12.	TNF-alpha	1.0	1.6	2.2	2.2	1.7	3.9
13.	IFN-gamma	1.0	1.9	2.7	2.0	2.0	4.0
14.	IL-4	1.0	1.5	2.0	2.0	1.7	4.0
15.	IL-5	1.0	1.5	2.5	2.0	1.9	3.9
16.	IL-10	1.0	0.8	4.0	4.6	4.2	3.5
17.	IL-13	1.0	1.1	2.7	2.0	2.0	3.8
18.	IL-15	1.0	1.2	2.7	2.0	2.0	3.9

		Growth and angiogenic factors					
		UI	4h	8h	24h	4days	5 days
1.	IGF-1	1.0	3.2	2.9	2.2	2.2	4.3
2.	PDGF-BB	1.0	1.5	2.9	2.1	2.1	3.8
3.	EGF	1.0	1.3	2.8	2.3	2.3	4.4
4.	M-CSF	1.0	1.2	2.6	2.0	1.9	3.9
5.	G-CSF	1.0	1.1	3.0	2.1	2.1	3.8
6.	GM-CSF	1.0	1.2	3.5	2.7	2.7	3.8
7.	Angiogenin	1.0	1.5	2.5	2.0	2.9	4.0
8.	Oncostatin M	1.0	2.2	3.0	2.2	2.1	4.3
9.	TPO	1.0	2.7	2.9	2.2	2.3	3.9
10.	VEGF	1.0	3.7	2.5	2.0	2.6	2.9
11.	SDF-1	1.0	1.3	3.0	2.0	2.0	4.0
12.	SCF	1.0	1.2	3.0	2.0	2.0	3.4
13.	TGF-beta1	1.0	1.2	2.9	1.9	2.2	3.4
14.	Leptin	1.0	1.4	3.0	2.2	2.2	3.9

(A) Gene expression profile of inflammatory cytokines and chemokines in THP1 cells. $1X10^6$ THP1 cells were serum starved for 8h and infected with 30 KSHV genome copies/cell for 2, 12 and 24h. At different times p.i., total RNA from infected and uninfected THP1 cells was reverse-transcribed to cDNA and then qPCR was performed using an array of 84 genes consisting of several cytokines, chemokines and cytokine and chemokine receptors. KSHV induced cytokine gene expression was represented as fold

change compared to uninfected cells. IL-1β gene expression is highlighted in red. The values from one representative of the three experiments have been shown here.
(B and C) Secretion of inflammatory cytokines and growth factors. Profiling of secreted cytokines and growth factors in THP1 (B) and HMVEC-d (C) cells was done using Ray Biotech human cytokine antibody 3.1 according to manufacturer's instructions. Fold induction in cytokine levels was calculated using levels in uninfected supernatant as 1 - fold. IL-1β and related cytokines are highlighted in red. The values from one representative of the three experiments have been shown here.

Table S2. Sequences of shRNA Lentiviruses Used (Santa Cruz Biotechnology Inc.), Related to Figure 5

IFI16	shRNA#1	Sense	CCACAAUCUACGAAAUUCAtt
		Anti-sense	UGAAUUUCGUAGAUUGUGGtt
	shRNA#2	Sense	CCAUCCAGCAGUUUCUUCAtt
		Anti-sense	UGAAGAAACUGCUGGAUGGtt
	shRNA#3	Sense	GGAAGGAGAUAAACUGAAAtt
		Anti-sense	UUUCAGUUUAUCUCCUUCCtt
ASC	shRNA#1	Sense	CGAGGGUCACAAACGUUGAtt
		Anti-sense	UCAACGUUUGUGACCCUCGtt
	shRNA#2	Sense	CGGAAGCUCUUCAGUUUCAtt
		Anti-sense	UGAAACUGAAGAGCUUCCGtt
	shRNA#3	Sense	CCCACCAAAUCAUCCUGAAtt
		Anti-sense	UUCAGGAUGAUUUGGUGGGtt
AIM2	shRNA#1	Sense	CUGGAUAGGUUUAAGUUCUtt
		Anti-sense	AGAACUUAAACCUAUCCAGtt
	shRNA#2	Sense	GCAAGCAGGAGAUGUUUCAtt
		Anti-sense	UGAAACAUCUCCUGCUUGCtt
	shRNA#3	Sense	GUUCGACUUACAUUCUUCAtt
		Anti-sense	UGAAGAAUGUAAGUCGAACtt

Supplemental References

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