

Supporting online material

Bioinformatics: NCBI BLASTP for NLRP1 and Orf63 was performed using the amino acid sequence of NLRP1 (NCBI accession # NP_127499) and the amino acid sequence of KSHV Orf63 (NCBI accession# YP_001129420.1). The ClustalW2 program was used to align the full-length sequence of NLRP1 isoform 1 (NCBI accession # NP_127497), NLRP1 isoform 3 (NCBI accession # NP_127499.1), NOD2 (NCBI accession # Q9HC29), KSHV Orf63 (NCBI accession # YP_001129420.1), and RRV Orf63 (NCBI accession # AAF60049).

Reagents: Total RNA was harvested from THP-1 cells with TriZol and, cDNA synthesis was performed using Superscript III reverse transcriptase. PCR reactions were performed using Pfx DNA polymerase with primers designed to the open reading frames of procaspase-1, pro-IL-1beta, NOD1, NOD2, and NLRP3. The amplicons were cloned into pCDNA3.1D to make V5 tagged pcDNA3-procaspase1-V5, pcDNA3-pro-IL-1 β -V5, and pcDNA3-NOD2-V5 plasmids. Epitope tags were interchanged using annealed oligonucleotides for FLAG (pcDNA3-ASC-FLAG, pcDNA3-NOD1-FLAG) or Myc (pcDNA3-NLRP3-myc) using standard procedures. Sequence fidelity was verified by DNA sequencing. Expression was verified by immunoblot analysis to epitope tags of transfected plasmids. pUNO-NLRP1 was purchased from Invivogen. pcDNA3-NLRP1-V5 was generated by amplifying isoform 1 of NLRP1 by PCR and subcloning into pcDNA3. KSHV Orf63 was amplified by PCR using primers adding BamH1 and EcoRV restriction sites with a FLAG epitope tag at the c-terminus. Amplified Orf63 was cloned into pcDNA3 vector to generate pcDNA3-Orf63-FLAG. The same procedure was used to

generate pcDNA3-Orf63-V5. Orf63-N-FLAG and Orf63 Δ N-FLAG were generated by cloning fragments encoding amino acids 1-200 (Orf63-N) and 201-928 (Orf63 Δ N). To generate GST-tagged Orf63, Orf63 was subcloned from pcDNA3-Orf63-FLAG using BamH1 and Not1 restriction sites and cloned into pGex5p-1 vector. pcDNA-NLRP1-myc, accompanying myc-tagged mutants and GST-tagged NLRP1 were previously described (15).

Cell lines: BCBL-1, THP-1-Orf63 and THP-1-Control cells were maintained in RPMI medium 1640 (Cellgro) containing 10% FBS and 1% penicillin-streptomycin (PS). 293T and lentivirus producer 293FT cells were maintained in DMEM medium (Cellgro) with 10% FBS and PS. KSHV-293T were maintained as 293T with 1mg/ml Puromycin. Cells were maintained at 37 °C in 5% carbon dioxide. BCBL-1-shRNA-NLRP1 and BCBL-1-shRNA-non-targeting cell lines were generated by nucleofecting BCBL-1 cells with shRNA-NLRP1 or shRNA-non-targeting (Santa Cruz) control plasmids. Nucleofected cells were selected for expression of shRNA plasmids by the addition of 1.0ug/ml puromycin. Knock-down of NLRP1 was confirmed by immunoblotting for NLRP1 (Cell Signaling).

NLRP1 Inflammasome Reconstitution Assays: 0.2×10^6 HEK293T cells were seeded in 24-well plates in cell culture media lacking antibiotics. 24 hours later, cells were transfected with 200ng pUNO-NLRP1 (Invitrogen), 5ng ASC-FLAG, 5ng pro-caspase-1-V5 and either 5ng human pro-IL-1 β -V5 or 20ng mouse pro-IL-1 β (14) using lipofectamine2000 (Invitrogen). 24 hours later, cell lysates and supernatants were

harvested and analyzed for IL-1 β secretion and expression by ELISA (BD) or immunoblot, respectively. Secreted IL-1 β levels were generally normalized by using a LDH release assay (see below) to normalize for cell death. For immunoblotting, cleaved IL-1 β (Cell signaling) or murine IL-1 β (R&D Systems) antibodies were used to detect human cleaved- or mouse pro- and cleaved-IL-1 β expression, respectively. Immunoblots of extracellular IL-1 β were performed after precipitation with trichloroacetic acid.

Orf63-Lentivirus Production and Infection: Orf63-FLAG was subcloned into pLenti7.3 lentivirus expression system (Invitrogen) according to manufacturer's instructions. Briefly, Orf63-FLAG was PCR amplified to include a 5'-terminus KOZAK sequence and subcloned into pENTR Gateway vector to yield pENTR-Orf63-FLAG (Note: stop codon not mutated to enable FLAG epitope detection). Next, pLenti7.3-Orf63-FLAG was generated by performing LR recombination reaction between pENTR-Orf63-FLAG and pLenti7.3 expression vector. A clone containing pLenti7.3 that did not recombine was isolated as a negative control. Orf63 expression was confirmed by transfecting into 293T cells and performing anti-FLAG immunoblots. To generate lentivirus, pLenti7.3-Orf63-FLAG or control pLenti7.3 were transfected into 293FT cells (Invitrogen) with ViraPower Packaging Mix. Virus was harvested 72 hours later. Transduction of THP-1 cells was performed by centrifugation of 5×10^6 cells with 1 ml virus, 8 μ g/ml polybrene for 3 hours at 3000 rpm at room temperature (RT). Twenty-four hours later, the transduction was repeated to enhance infection. The transduced cells were sorted for GFP expression by flow cytometry and Orf63 expressing clones isolated by

limiting dilution. Expression of FLAG-tagged Orf63 in THP-1-Orf63 cells was confirmed by immunoblot.

THP-1 Stimulations: 1×10^6 pLenti7.3 or pLenti7.3-Orf63 cells were plated in 12-well plates in 500ul RPMI containing 2% FBS and PS. Cells were either mock treated or primed with 5ng/ml lipopolysaccharide (Sigma) for 1 hour followed by treatment with 10 μ g/ml muramyl dipeptide (Sigma), 2.5mM adenosine triphosphate (ATP), or 130 μ g/ml Alum (Thermo Scientific) for 6 hours at 37 °C. Supernatants were harvested and clarified by centrifugation at 1500rpm at RT and cytokine analysis was performed as described below.

Immunoblot and Co-immunoprecipitation Assays: Cell lysates were prepared in 0.1% NP-40 lysis buffer containing protease inhibitor cocktail (Roche). Proteins were resolved on either 10% or 12.5% SDS-PAGE gels and transferred to PVDF membranes by wet-transfer. Membranes were blocked for 1 hour in 5% non-fat dry milk (NFDM) containing 0.1% TBS-Tween 20 and washed 3X in 0.1% TBS-Tween 20 at room temperature. Monoclonal, horse-radish peroxidase (HRP) conjugated antibodies (Sigma) to M2-FLAG, C-myc, and V5 epitopes were used to detect expression of epitope tagged proteins. IL-1 β and cleaved IL-1 β antibodies (Cell signaling) were used at 1:1000 in 5% bovine serum albumin overnight followed by anti-rabbit-HRP secondary antibody (Cell Signaling) to detect the 34kDa and 17kDa forms of IL-1 β . Detection of cleaved IL-1 β in supernatants was performed by concentrating supernatants using Millipore YP-10 centrifugal filter devices or trichloroacetic acid precipitation followed by immunoblot.

For co-immunoprecipitation studies, antibodies against the FLAG, V5 or c-myc epitopes were used to precipitate proteins in the presence of 20µl protein A/G beads (Santa Cruz) overnight at 4°C. Protein complexes were washed four times in either lysis or RIPA buffer, incubated at 95°C for 5 minutes and resolved by immunoblot. To detect Orf63's interaction with endogenous NLRP1, Orf63 was immunoprecipitated from THP-1 cells 24 hours following nucleofection (Amaxa) using anti-V5 monoclonal antibody (Sigma) as described above. Endogenous NLRP1 was detected by immunoblotting using human anti-NLRP1 antibody (Cell Signaling). For direct immunoprecipitation, Orf63 was expressed in BL21 Escherichia coli cells by induction with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 hours at 37°C. GST-Orf63 was purified with GST-sepharose (Amersham). GST-sepharose-Orf63 was incubated with precision protease overnight at 4°C. Cleaved Orf63 was further purified by incubation with anti-FLAG-M2 antibody for 1 hour at 4°C followed by incubation with protein A/G beads for 3 hours. Next, beads were washed 5 times with lysis buffer to remove unbound Orf63 and resuspended in 1ml lysis buffer and purified NLRP1. Mouse IgG (Santa Cruz) control reactions were also prepared. Orf63 and NLRP1 were incubated overnight at 4°C, washed 4 times with lysis buffer and NLRP1 interactions were detected by immunoblotting for NLRP1.

In-vitro transcription/translation: [35S]-methionine (Perkin-Elmer) labeled NLRP1, NLRP3 and control luciferase proteins were prepared using TnT T7 Quick Coupled Transcription/Translation System (Promega) according to manufacturer's instructions.

pcDNA3-NLRP1 and pcDNA3-NLRP3 plasmids were used for the transcription/translation reaction.

GST binding Assay: Expression and purification of GST fusion proteins were performed as previously described (29) and equal amounts of GST and GST-Orf63 were bound to glutathione beads (29). Equivalent amounts (1×10^5 cpm) of ^{35}S -methionine labeled NLRP1, NLRP3 and luciferase proteins were incubated with the GST and GST-Orf63 bound protein in NETN+ buffer plus protease inhibitors for an hour at 4°C. NETN+ buffer comprised of 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT. The bound complexes were washed five times (5 minute washes) in NETN+ buffer. The samples were subjected to SDS-PAGE. The gel was fixed, dried and exposed to a phosphoimager.

Gel Filtration Assay: HEK293T cells were plated in 15cm dishes in antibiotic-free media. 24 hours later, cells were transfected with NLRP1-myc or NLRP1-myc and Orf63-flag expression plasmids with lipofectamine2000 as described. 24 hours post-transfection, cells were washed with ice-cold 1X PBS followed by lysis in hypotonic buffer (20mM HEPES-KOH pH7.5, 10mM KCL, 1.5mM MgCl_2 , 1mM EDTA, 1mM EGTA and Roche Protease Inhibitor Cocktail) and shearing with 27-gauge needle. Soluble lysates were run over a Superose 6 gel filtration column (GE Lifesciences) using a Bio-Rad Duoflow chromatography system. Column elution fractions were analyzed for comigration of NLRP1 and Orf63 by immunoblot for myc and FLAG, respectively.

Caspase-1 Fluorescence Assay: Caspase-1 fluorescence assay (R&D systems) was performed according to manufacturer's instructions: Briefly, 293T cells were transfected as described above. Twenty-four hours later, cells were washed in ice-cold 1X PBS, and whole cell extracts prepared. 50 μ L of extract was added to 50 μ L of 2X reaction buffer containing DTT, followed by 5 μ L of WEHD- 7-amino-4-trifluoromethyl coumarin (AFC) substrate. Caspase-1 activity was measured every 15 minutes over 2 hours using a fluorescence plate reader at 505nm.

ELISA: Supernatants were analyzed for cytokine expression using IL-1 β , TNF- α (BD Biosciences) and IL-18 (R&D Systems) ELISAs according to the manufacturer's instructions. IL-1 β secretion was normalized by the LDH release assay.

Lactate-Dehydrogenase Release Assay: LDH release assays (Promega) were performed according to the manufacturer's instructions. LDH release data was used to normalize IL-1 β secretion in 293T inflammasome reconstitution and THP-1 stimulation assays to account for cell death.

Reactivation Assays: KSHV-293T cells were seeded at 0.5×10^6 cells per well in a 6-well plate. Cells were transfected 24 hours later with plasmids encoding procaspase-1, ASC, pro-IL-1 β , RTA or vector control, along with either 50nM Orf63 ON-TARGET (Dharmacon) siRNA (Sense: AGACAAAGCUGUUGAUGGAUU, Antisense: UCCAUCAACAGCUUUGUCUUU) or control non-targeting siRNA (Dharmacon). Cells and supernatants were harvested forty eight hours later. Supernatants were

subjected to IL-1 β ELISA while total RNA was isolated from the cell pellets. In order to confirm knockdown of Orf63, cDNA was generated and used as a template for PCR with Orf63 primers (Sense: CCCACTACGCGGATCAGATA, Antisense: GCTCTTGCATAATGCCTCTA). GAPDH primers were used for loading control. PCR products were resolved on a 1% agarose gel. NLRP1 inhibition of reactivation was performed by coexpressing NLRP1 inflammasome components as described above with RTA or vector control. vIL-6 expression was analyzed by immunoblot 72 hours post-reactivation.

Inhibition of KSHV reactivation in BCBL-1. 1×10^6 BCBL-1-shRNA-NLRP1 or BCBL-1-shRNA-control cells were either mock treated or treated with 25ng/ml TPA (Sigma). Viral genomes were analyzed by qPCR using primers specific for Orf49 as previously described 96 hours post-reactivation (6).

Analysis of KSHV transcription: 2.5×10^6 BCBL-1 cells were nucleofected with siRNAs against Orf63 or non-targeting control. 24 hours later, cells were either mock stimulated or treated with 25ng/ml TPA for 96 hours. Orf49 and Orf57 viral gene transcription was analyzed as previously described (6).

Human Primary Monocyte Infections: KSHV was grown and purified as previously described (7). Primary human monocytes were isolated from peripheral blood mononuclear cells by negative selection using the monocyte isolation kit II from Milltenyi Biotec. Purification of monocytes was verified by flow cytometry for CD14

(Miltenyi Biotec) and average purities were greater than 98%. Immediately following purification, 1.5×10^7 monocytes were infected with KSHV by centrifugation at 2000 rpm at 30°C for 1 hour. KSHV-infected monocytes were cultured in 20% FBS. 48 hours post-infection, media was changed to antibiotic free media. 72 hours post-infection, productive infection was confirmed by fluorescent microscopy. Next, siRNAs against Orf63 or non-targeting control were transfected by Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instruction. 120 hours post-infection, supernatants were analyzed for IL-1 β expression by ELISA and knockdown of Orf63 tested by PCR. qPCR for Orf49, Orf50 and Orf57 transcription was assessed as previously described (6).

Statistical Analysis: Data were analyzed for statistical significance by two-tailed student's t-test in Excel (Microsoft). Differences in *P* values ≤ 0.05 were considered statistically significant.

BLASTP alignment of the LRR domain of NLRP1 with Orf63

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Orf63      230  SRSNSTETAVTMPVSKERALAIYRVFAKGEVVAENTPILAF---TDVELSTLKPHYLFIY 286
             S S+S  ++  + + R L      A  + AE+  LAF  +  L+ L  +  +
NLRP1     819  SLSHSAVKSLCKTLRRRPRCLETLRLAGCGLTAEDCKDLAFGLRANQTLTELDLSFNVL 878
             * * * *      * *      * *      * *      * *      * *
Orf63      287  DFIIIEALCKSYTY-SCTQARLESFLSRGI--DFMTDLGQYLDTATSQKQ-QLTHSQIKEI 342
             D  + LC+      SC  RL+  +S G+  D  DL  L  +  S K+  L  +  +  ++
NLRP1     879  DAGAKHLQQLRQPSCKLQRLQ-LVSCGLTSDCCQDLASVLSASPSLKELDLQNNLDDV 937
             *      * *      * *      * *      * *      * *      * *      * *
Orf63      343  KYRLLSGGLSASACDVFR 360
             RLL  GL  AC  + R
NLRP1     938  GVRLLEGLRHPACKLIR 955
             * * * *      * *      * *

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ClustalW2 alignment of a section of the NBD domain of NLRP1 isoforms 1 and 3 with Orf63

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NLRP1 isoform 1  301 PDYVEENRGHLIEIRD LFGPGLDTQEPRIVILQGAAGIGKSTLARQVKEAWGRGQLYGDR 360
NLRP1 isoform 3  301 PDYVEENRGHLIEIRD LFGPGLDTQEPRIVILQGAAGIGKSTLARQVKEAWGRGQLYGDR 360
Orf63           211 PSEEV LAKTLVTEHHELFVSRNSTETAVTMPVSKE-----FIIIE 291
             * .      :  : *  : : * * .  : : * . : : .
NLRP1 isoform 1  361 FQHVFYFSCRELAQSKVVSLAELIGKDGATAPAPIRQILSRPERLLFILDGVDEPGWVLQ 420
NLRP1 isoform 3  361 FQHVFYFSCRELAQSKVVSLAELIGKDGATAPAPIRQILSRPERLLFILDGVDEPGWVLQ 420
Orf63           247 -----RALAIYRVFAKGEVVAENTPILAF TDVELSTLKPHYLFIYD----- 291
             * * *      : * . : * : : : .  .  .  : : :      : * * * *      : : :
NLRP1 isoform 1  421 EPSSELCLHWSQPQPADALLGSLLGKTI LPEASFLITARTTALQNLIPSLEQARWVEVLG 480
NLRP1 isoform 3  421 EPSSELCLHWSQPQPADALLGSLLGKTI LPEASFLITARTTALQNLIPSLEQARWVEVLG 480
Orf63           292 ALCKSYTYSCTQARLESFLS--RGIDFMTDLGQYLDTATSQKQ----- 332
             . . .      : * . : . *      *      : : : .  .  : : * : . *

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Supplemental Figure 1A: Alignment of KSHV Orf63 with NLRP1

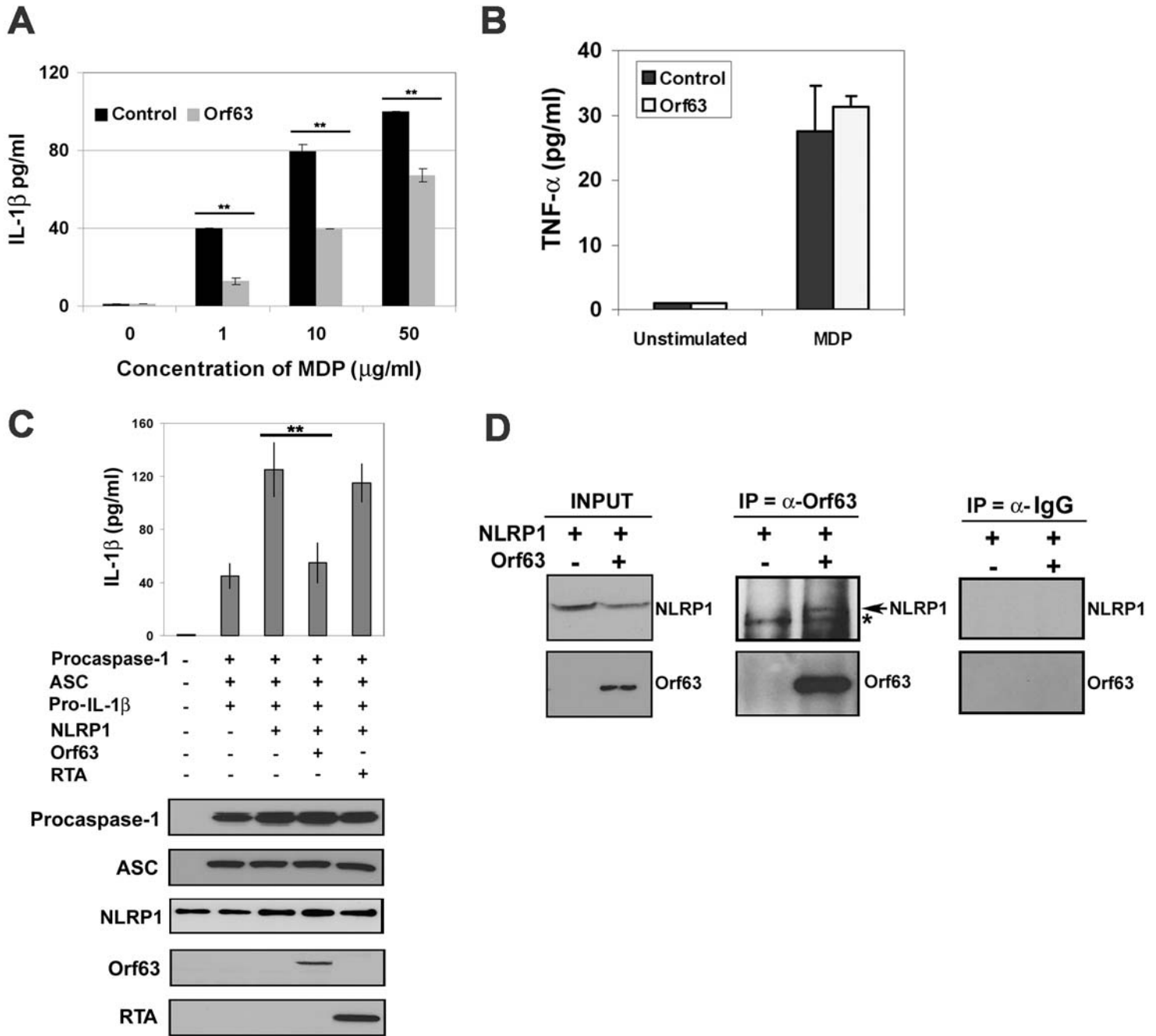
Amino acid alignments were performed using BLASTP and ClustalW2 bioinformatics programs with Orf63 (NCBI accession# YP_001129420.1) and human NLRP1 (NCBI accession # NP_127499). The LRR and NBD homology regions are shown in detail.

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Orf63 MDG-----TDALKLTKGLSGGGSLHQTKLLMEFQLRGLPVPA 39
NLRP1 MAGGAWGRLACYLEFLKKEKELKEFQLLANKAHSRSSGETPAQPEKTSGMVEVASYLVAQ 60
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Orf63 LLNSTTEQFLNTVAQLPT---DLSKFIIRDYRVFALVRAAYFLEPPSS----- 84
NLRP1 YGEQRADLALHTWEQMGRLSLCAQAQEGAGHSPPFPYSPSEPHLGSPTSQTSTAVLMPW 120
      : : : *:* * : : : : : * : : * . : . * . * . * . * . * . * . * . * .
Orf63 IDPLEAARALGRLVDILSSQPPQN-----TAPAQPPTSDDTLNNCTLL 127
NLRP1 IHELPACTQGSERRVLRQLPDTSGRRWREISASLLYQALPSSPDHESPSQESPNAPTST 180
      * . * . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Orf63 KLLAHYADQIAGFKTFALPPVP-----PGI 152
NLRP1 AVLGSWGSFPQSLAPREQEAPGTQWPLDDETSGIYYTEIREREREKSEKGRPPWAAVVTG 240
      : * . : . . * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Orf63 IGLFTCVEQMYHACFOKYWAALPPMNIITYDPPTSPLDQNLVAVGN--KEGILLPSGI 210
NLRP1 FPAQHTSLQPHHHPWEPVRESLCSTWPKNEDPNQKFTQLLLQRPHPRSQDPLVKRSW 300
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Orf63 PSEEVLAKTLVTEHHELFVSRSNSTETAVTMPVSKE----- 246
NLRP1 PDYVENRGLHIEIRDLFQGLDTQEPRIIVLQGAAGIGKSTLARQVKEAWGRGQLYGD 360
      * . : : : * * : * * * * . : : * . : : .
Orf63 -----RALAIYRVFAKGEVVAENTPILAFDVELSTLKPHTLFIYD-----FII 291
NLRP1 FQHVYFSCRELAQSKVSLAELIGKDGATAPAPIRQILSRPERLLFILDGVDPEGVVW 420
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Orf63 ALCKSYTYSCTOARLESFLS--RGIDFMTDLGQYLDTATSGKQ----- 332
NLRP1 EPSSELCHWSQPQADALLGSLGKTLPEASPLITARTALQNLIPLSLEQARWVEVLG 480
      . . . * : * . * * * : : : . : : * : * *
Orf63 -----QLTHSQIKEIKY 344
NLRP1 FSESSRKEYFYRYFTDERQAIRAFRLVKSNEKELWALCLVPVWVSWLACTCLMQQMKRKEKL 540
      * : . : : *
Orf63 RLLSCGLSASACDVFRPTVIMTLPYRPTNLANLSTPMGMVHQLTMFGHYFYRCLG---- 399
NLRP1 TLTSKTTTLCHHYLAQALQAFQPLGQDLRDLCSLAEBGWKKTLPSPDDLRKHGLDGA 600
      * * : : . : : : : * * . : * : : * : * . * *
Orf63 -----SYSPTGLAFTLQKILTRASAEQTERNPWR----- 429
NLRP1 ISTFLKMGILQEHPIPLSYSFHLCFQEFFAAMSIVLEDEKGRGKHSNCIIDLEKTL 660
      * * * * * * * * * * : : . : : * .
Orf63 -----HPGISDIPLRWKISRALAFVFPVAPINTLQ 459
NLRP1 GIHGLFGASTTRFLGLLSDBGEREMENIFHCRLSQGRNLMQWVPSLQLLQPHSLES 720
      * * : * : : : * * : * : * : * * : * * : * * : * * : * * : * * : * *
Orf63 RVYAALPSQLMRAIFEISVKT-----TWG 483
NLRP1 CLYETRNKTFLTQVMAHFEEMGMCVETDMELLVCTFCIKFSRHVKKLQLEGRQHRST 780
      : * : . : : : : * : * * * *
Orf63 GAVPANLARDIDTGPNTQHISSTPPPT--LKDVEYTCQGLRVGDEYDEDIVRSP---LF 538
NLRP1 PTMVVFLRWVPTDAYWQILFVSVLKVTRNLKELDLSGNSLSHSAVSKLCTLRPRCLLE 840
      : : . : * . * * * * * * * * * * : * * . . : : * * * * *
Orf63 ADAFTKSHLLPILREVLENRLQKNRALFQIRNLIIPAAEAATGLIPARRPLARAYFHIM 598
NLRP1 TRLRAGCGLTAEDCKDLAFGLRANQTLTELDLSPNVLTDAKAKLQRLRQPSCKLQRLQ 900
      : : : * . : * * * * * : * : * * : * * . : : :
Orf63 ILEERHSQDALYNLLDCIQEL-----FTHIRQAVPD 629
NLRP1 LVSCGLTSDCCDLASVLSASPSLKELDLQNNLDDVGVRLCEGLRHPACKLIRLGLDQ 960
      : : . : * . * . : . : * * : :
Orf63 AQCPhaFLQSLFVFQFRP---FVLKHQQVTLFLDGLQ----- 665
NLRP1 TTSDEMQRQELRALQEKQQLLIFSRKPSVMTPTTEGLDGTGEMSNSTSLKRQRLG 1020
      : . . : * * : : . : : : * * * * *
Orf63 -----SLPPVISLANLGDKLCR-----LEFYDSEGDFVR 695
NLRP1 ASHVAQANLKLDDVSKIFFIAEIAEESPEVVPVELLCVPSPASQGDHLTKPLGTDDDF 1080
      . : . : * : * : : * . : : * * *
Orf63 VFPVAPPEQPPHVLHSHFKKTIQIEQATREATVAMTTIAKPIYPAYIRLLQRLVYLNRLN 755
NLRP1 GFTGPVATEVVDKKNLYRVHFPVAGSYRWNTGLCFVMREAVTVEIFEVCVWDQFLGEIN 1140
      * . * * * : : : : . : : * . . . : : . . * . : : * * : * *
Orf63 --HHILRIFFPQDALSELQETYLAAFARLTKLAADAANTCSYSLTKYFG----- 802
NLRP1 PQHSWMVAGPLLDIKAEPGAVEAVLPHFVALQGGHVDTSLFQMAHFKEBGMLEKPARV 1200
      * : * * * . : : : * . . : * . : : :
Orf63 ----VLFQHQLVPTAIVKLLH----- 820
NLRP1 ELHHIVLENPSFPLGVLLKMIHNLRFIPVTSVVLLYHRVHPEEVTPHLYLIPSDCSIR 1260
      * * : : * : * : * *
Orf63 ----FDEAKDTTEAFLQSLAQPVVQGRQGAAGSG--VLTQKELELLNK----- 864
NLRP1 KAIDDLKMFQFVRIHKPPPLTPLYMGCRTVTSGSGSMLLEILPKELELCYRSPGEDQLF 1320
      : : . : . . * : * * . : . : * * * * *
Orf63 -----INPQFTDAQANIPPSIKRSYSNKYDVPEVSV 895
NLRP1 SEFYVGHLSGIRLQVKDKKDETLVWEALVKPGLMFATTLPARIAVPSPLDAPQLH 1380
      : * : * : : * . : : . * * : *
Orf63 DWETYSR----SAFEAPDDELRFVPLTLAGLRKLFVE----- 928
NLRP1 FVDQYREQLIARVTSVEVLDKLHGQVLSQEQYERVLAENTRPSQMRKLFSLSQSWDRKC 1440
      : * . : : * . * * * * : : : *
Orf63 -----
NLRP1 KDGLYQALKETHPHLIMELWEKSGKGLLPLSS 1473

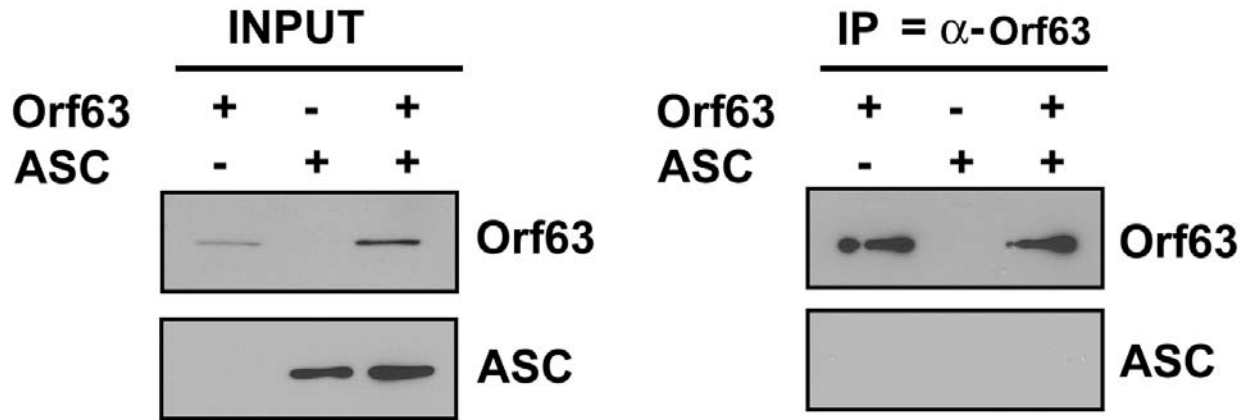
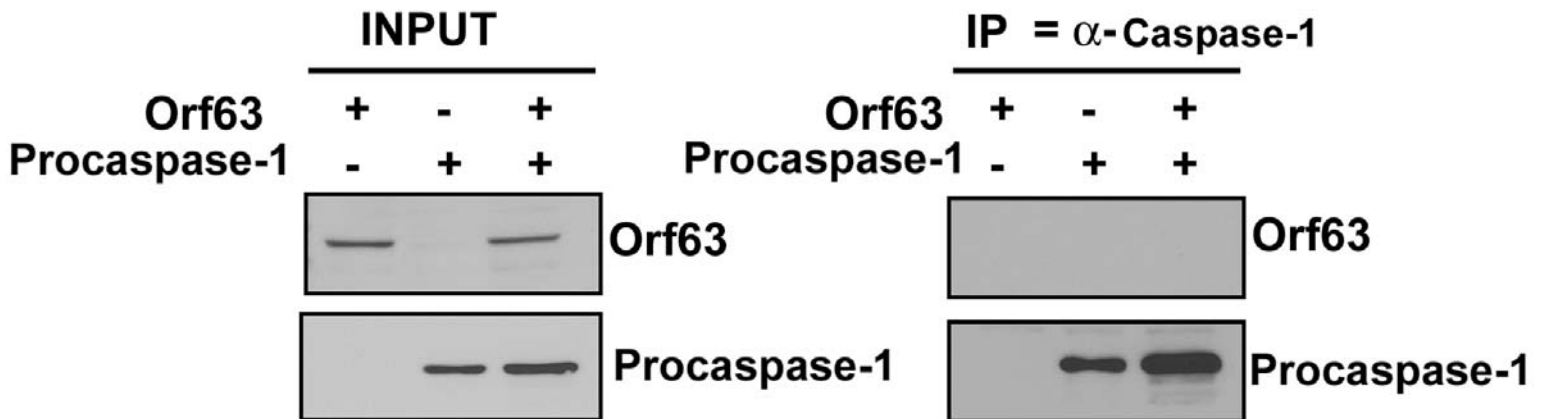
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Supplemental Figure 1B: Alignment of KSHV Orf63 with NLRP1
 Amino acid alignment of the full-length KSHV Orf63 and human NLRP1 proteins performed using the ClustalW2 program are shown.

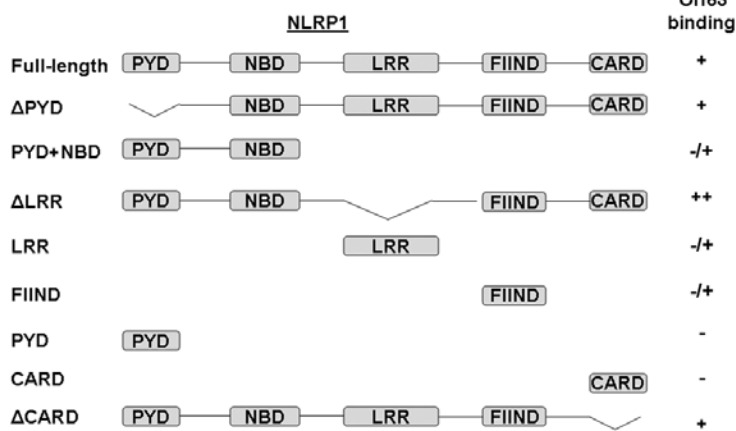
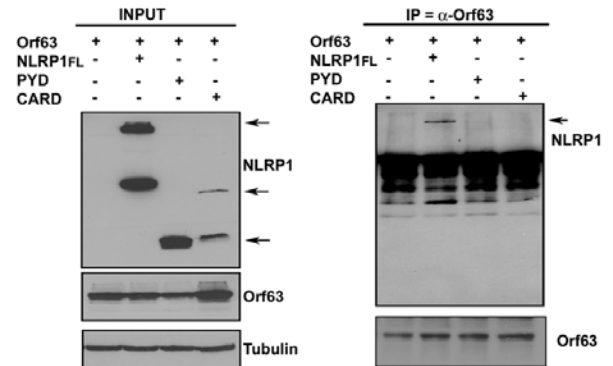
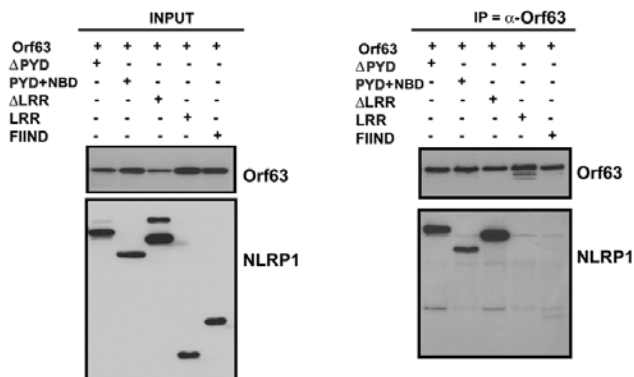
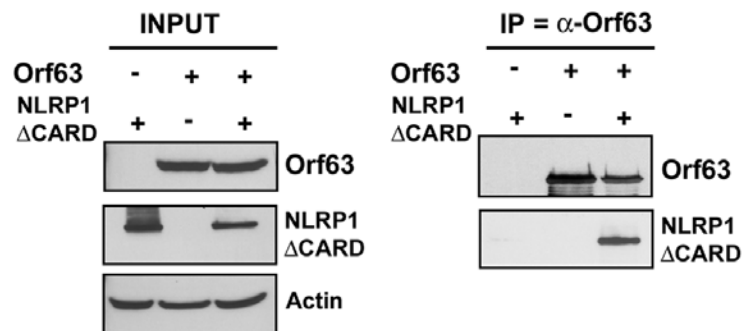


Supplemental Figure 2: KSHV Orf63 but not KSHV RTA inhibits NLRP1-mediated IL-1β secretion.

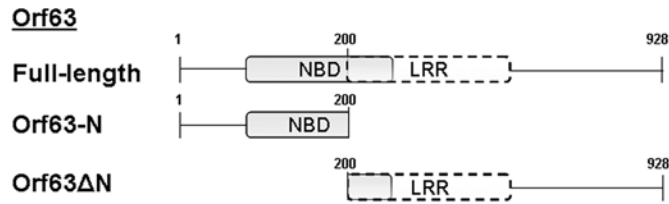
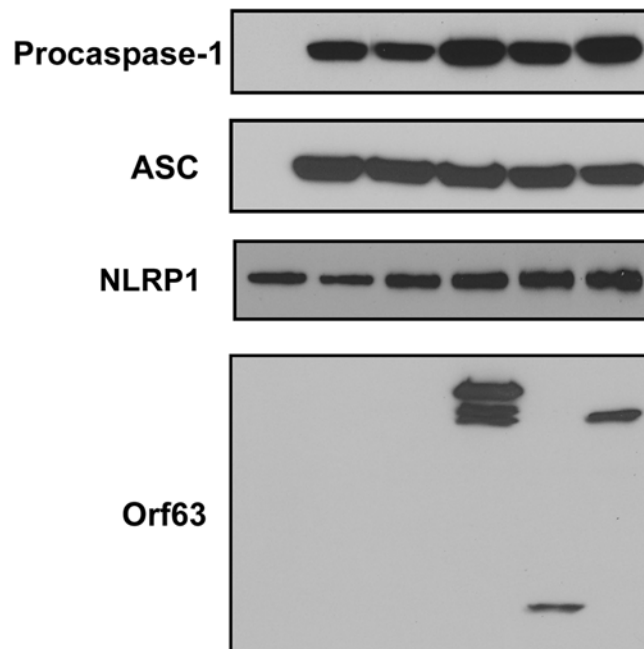
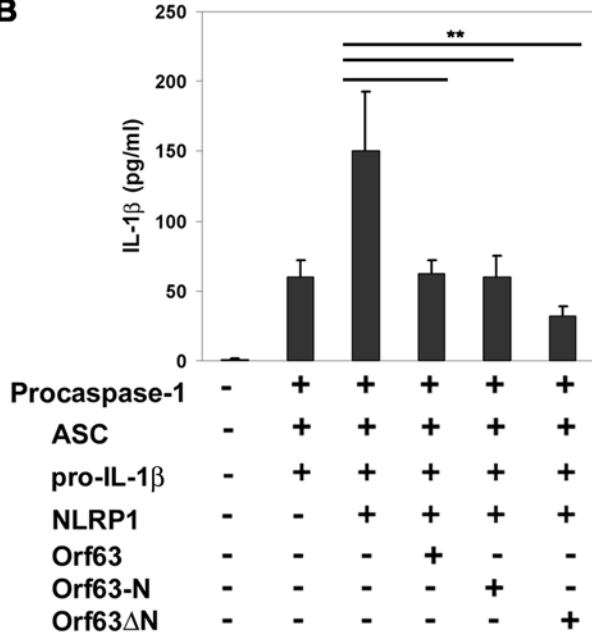
(A) 0.5×10^6 THP-1 vector control cells or Orf63-expressing cells were primed with 5ng/ml LPS for 1 hour followed by stimulation with 1μg/ml, 10μg/ml and 50μg/ml MDP. Supernatants were harvested 6 hours later followed by an ELISA for extracellular IL-1β secretion. The IL-1β values were normalized to LDH (B) THP1 cells were treated as described above with 10 μg/ml MDP and TNF-α expression was analyzed by ELISA. (C) NLRP1, procaspase-1, ASC and pro-IL-1β expression plasmids were transfected into 293T cells with Orf63, RTA or vector control. Supernatants were harvested 24h later and subjected to ELISA for extracellular IL-1β expression and expression of each component was depicted by immunoblot. (D) Immunoprecipitation of Orf63 from THP-1 cells followed by detection of endogenous NLRP1 by immunoblot using anti-NLRP1 antibody. Asterisk indicates non-specific band. ** indicates statistical significance $P \leq 0.05$ by two-tailed student's t-test. Data are representative of a minimum of three experimental replicates.

A**B**

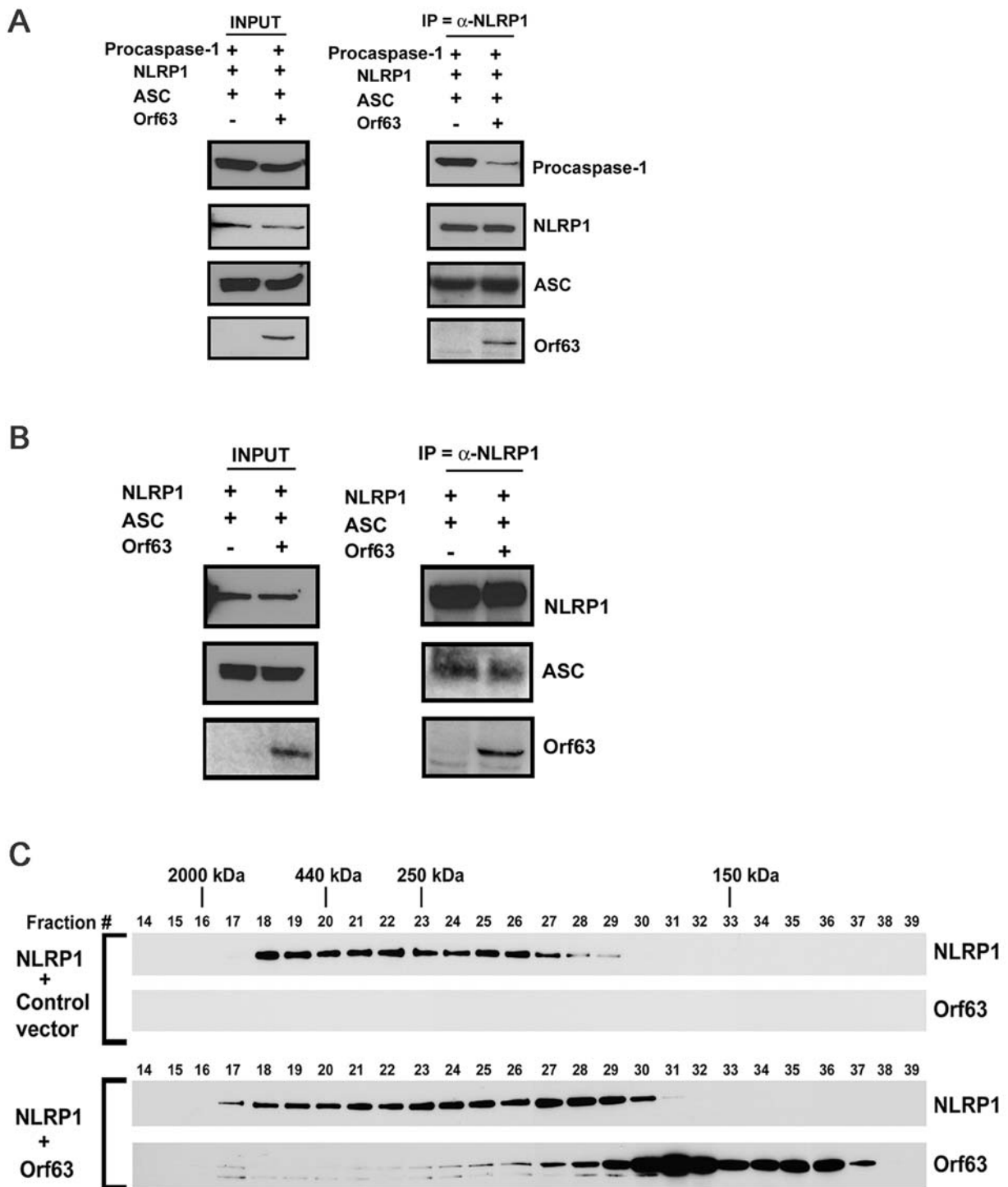
Supplemental Figure 3: Orf63 does not interact with NLRP1 inflammasome components ASC and caspase-1 and blocks the interaction of caspase-1 with NLRP1. (A-B) 293T cells were transfected with expression plasmids for Orf63 and either ASC (A) or caspase-1 (B) and 48h later, immunoprecipitation of Orf63 (A) or caspase-1 (B) was performed followed by immunoblot for ASC or Orf63, respectively.

A**B****C****D**

Supplemental Figure 4: The NBD of NLRP1 is required, but not sufficient for interactions with Orf63. (A) Summary of NLRP1 mutants and Orf63's interactions with NLRP1. (B-D) Expression plasmids encoding PYD and CARD (B) or ΔPYD, PYD+NBD, ΔLRR, LRR and FIIND (C) or ΔCARD (D) mutants of NLRP1 were cotransfected into 293T cells with Orf63. Forty-eight hours post-transfection, Orf63 was immunoprecipitated and western blots for NLRP1 mutants were performed.

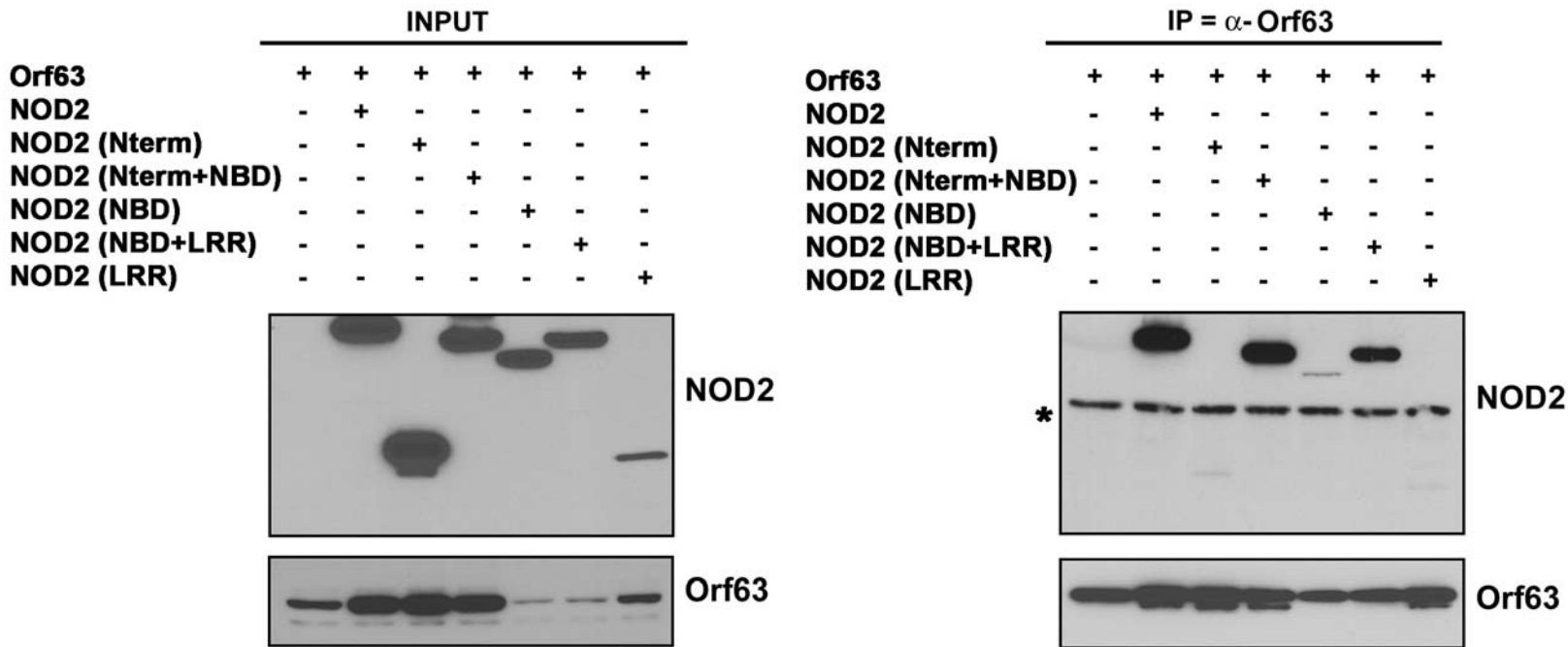
A**B**

Supplemental Figure 5: Orf63-N and Orf63ΔN mutants are capable of inhibiting NLRP1 activity. (A) Panel depicting Orf63 mutants. Orf63-N, which contains amino acids 1-200, and Orf63ΔN, which contains amino acids 201-928. **(B)** NLRP1 inflammasome was reconstituted by transfecting 293T cells with NLRP1, ASC, procaspase-1 and pro-IL-1β expression plasmids and wild-type Orf63, Orf63-N or Orf63ΔN mutants. Twenty four hours post-transfection, supernatants were analyzed for IL-1β by ELISA. ** indicates statistical significance $P \leq 0.05$ by two-tailed student's t-test. Data are representative of a minimum of three experimental replicates.

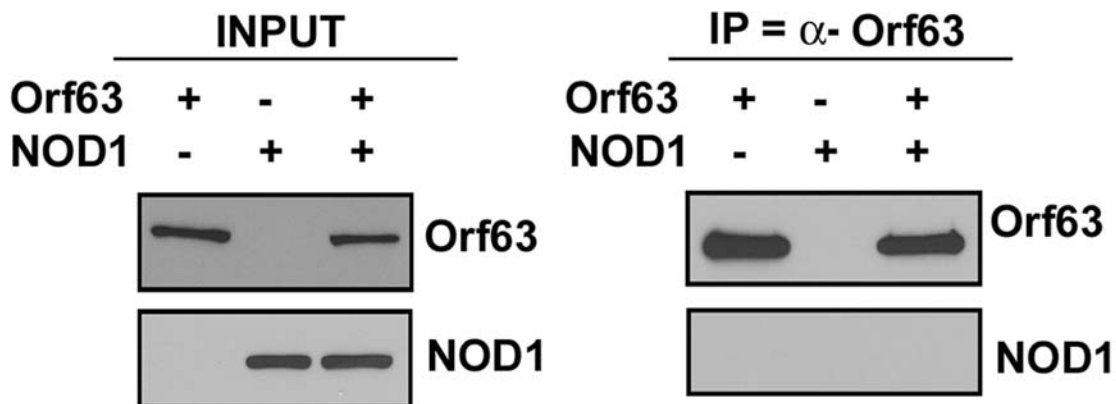


Supplemental Figure 6: Orf63 inhibits the interaction of procaspase-1 with NLRP1 and NLRP1 oligomerization. (A-B) Expression plasmids encoding NLRP1, ASC (A) and procaspase-1 (B) with either vector control or Orf63 were transfected into 293T cells. 48h later, NLRP1 was immunoprecipitated and immunoblots for each protein were performed. **(C)** 293T cells expressing NLRP1 or co-expressing NLRP1 and Orf63 were lysed under native conditions and subject to gel filtration analysis. Fractions were isolated and subjected to immunoblotting to detect the presence of NLRP1 and Orf63.

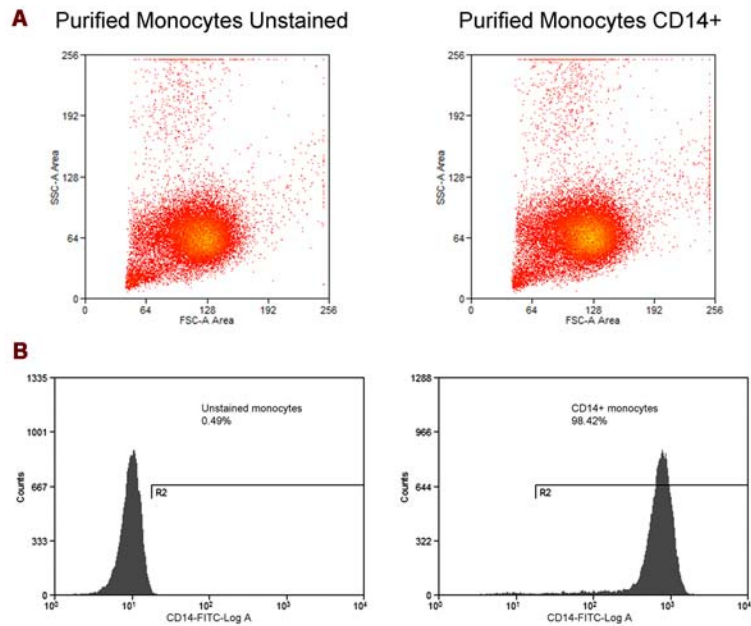
A



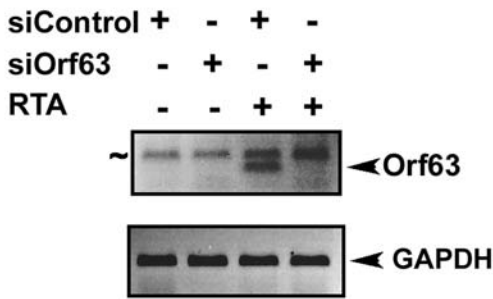
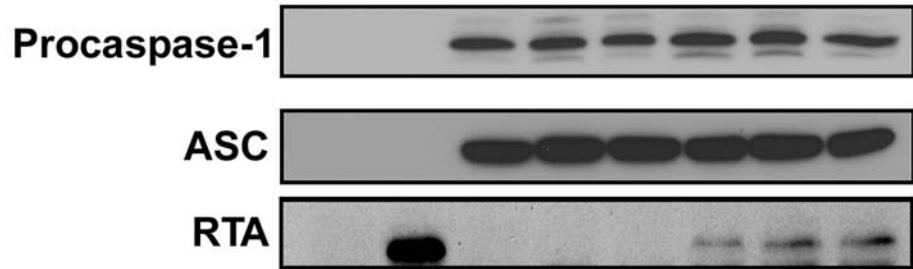
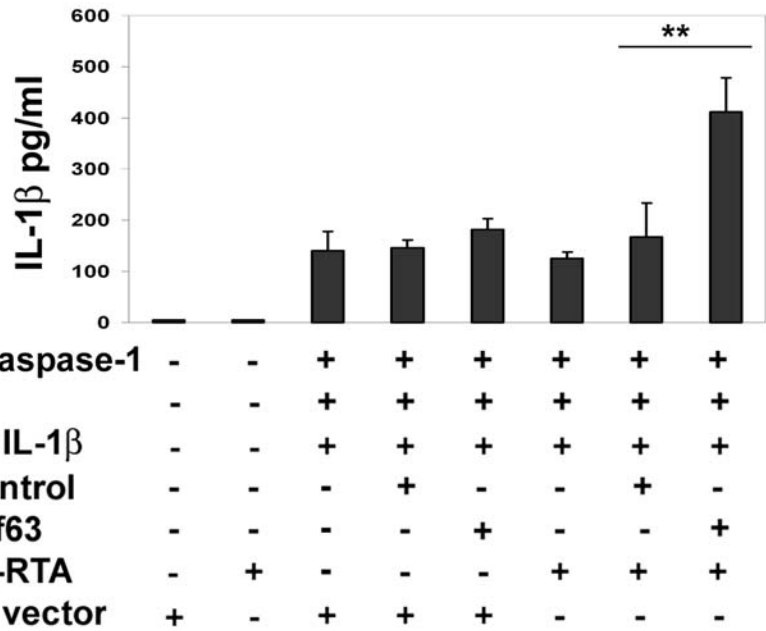
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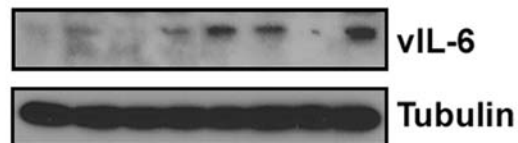
Supplemental Figure 7: NOD2 NBD is required for interactions with Orf63, and Orf63 does not interact with NOD1. (A) Full-length NOD2 or NOD2 mutants were transfected into 293T cells. 48h later, Orf63 was immunoprecipitated and NOD2 interactions detected by immunoblot. * indicates non-specific band. (B) 293T cells were transfected with Orf63 and NOD1 expression plasmids for 48h. Orf63 was immunoprecipitated and the immunoprecipitates were subjected to western blot analysis for NOD1.



Supplemental Figure 8: Purity of primary human monocytes purified from healthy donors. (A-B) Human monocytes were isolated from peripheral blood mononuclear cells (PBMC) and analyzed by flow cytometry. Forward and side scatter (A) and CD14 expression (B) plots are shown to demonstrate purity. Percentage of cells that are CD14+ is determined by calculating the number of cells within the R2 marker.

A**B****C**

Procaspase-1	-	+	+	+	-	+	+	+
Pro-IL-1β	-	+	+	+	-	+	+	+
ASC	-	+	+	+	-	+	+	+
NLRP1	-	-	+	+	-	-	+	+
LRR	-	-	-	+	-	-	-	+
RTA	-	-	-	-	+	+	+	+

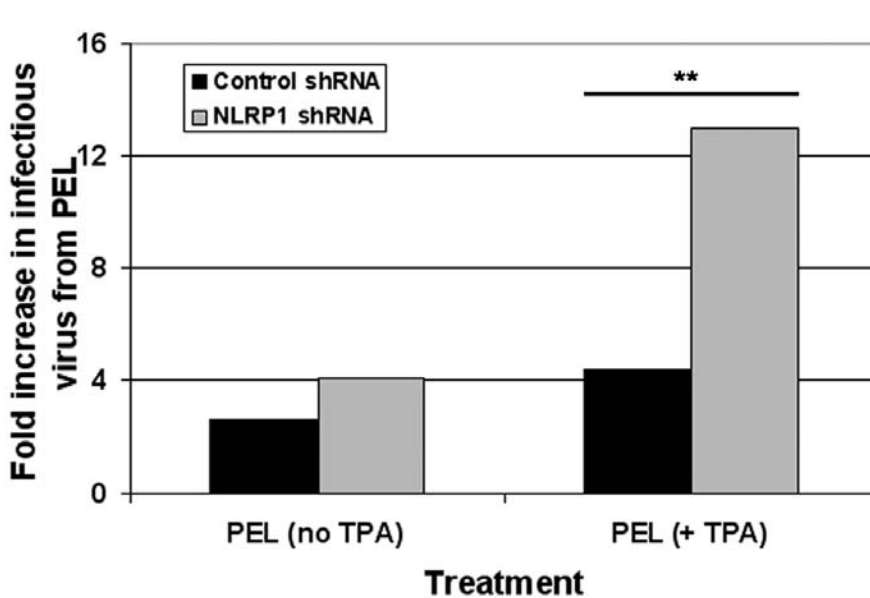
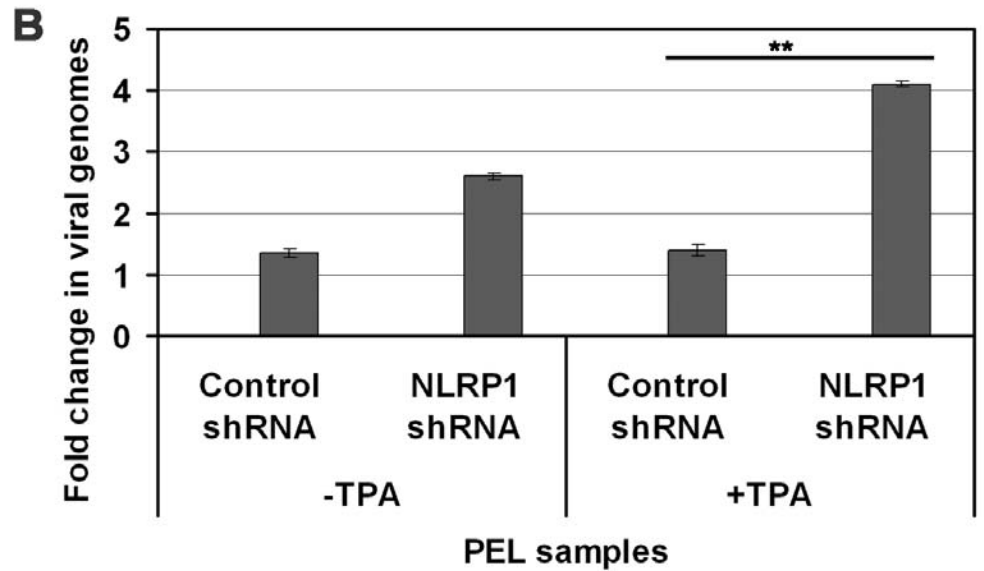
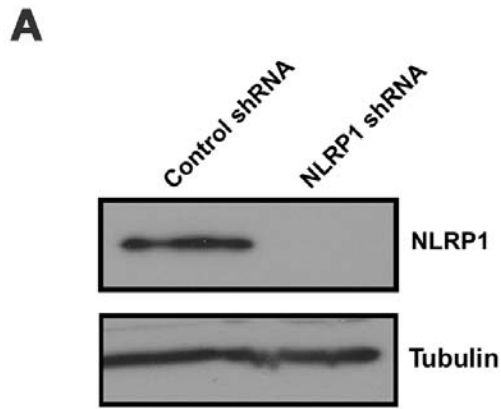


Supplemental Figure 9: Orf63 inhibits IL-1β expression during KSHV reactivation.

(A) KSHV-293T cells were transfected with RTA or vector control expression plasmids and either control siRNA (siControl) or Orf63-targeting siRNA (siOrf63) as well as plasmids encoding procaspase-1, ASC and pro-IL-1β. RNA was harvested 24 hours later and cDNA was synthesized. Orf63 and GAPDH transcription levels were determined by PCR amplification and agarose gel electrophoresis. ~ indicates non-specific band.

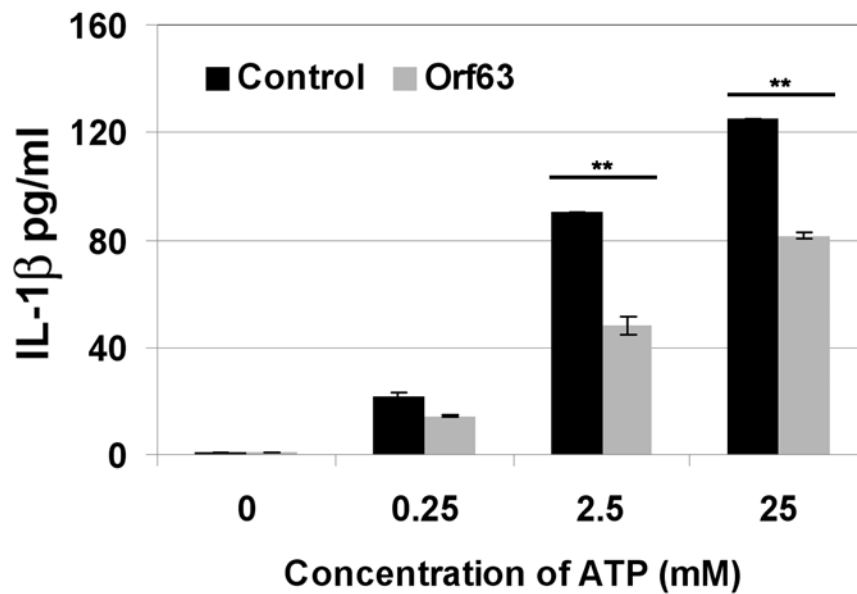
(B) KSHV-293Ts were transfected as described above and supernatants harvested 48 hours later.

Extracellular IL-1β was determined by ELISA. (C) KSHV-293T cells were transfected with procaspase-1, pro-IL-1β, ASC, NLRP1 and either vector control or RTA expression plasmids. 72 hours post-transfection, reactivation and vIL-6 expression (indicator of lytic protein expression) was analyzed by immunoblot. ** indicates statistical significance $P \leq 0.05$ by two-tailed student's t-test. Data are representative of a minimum of three experimental replicates.



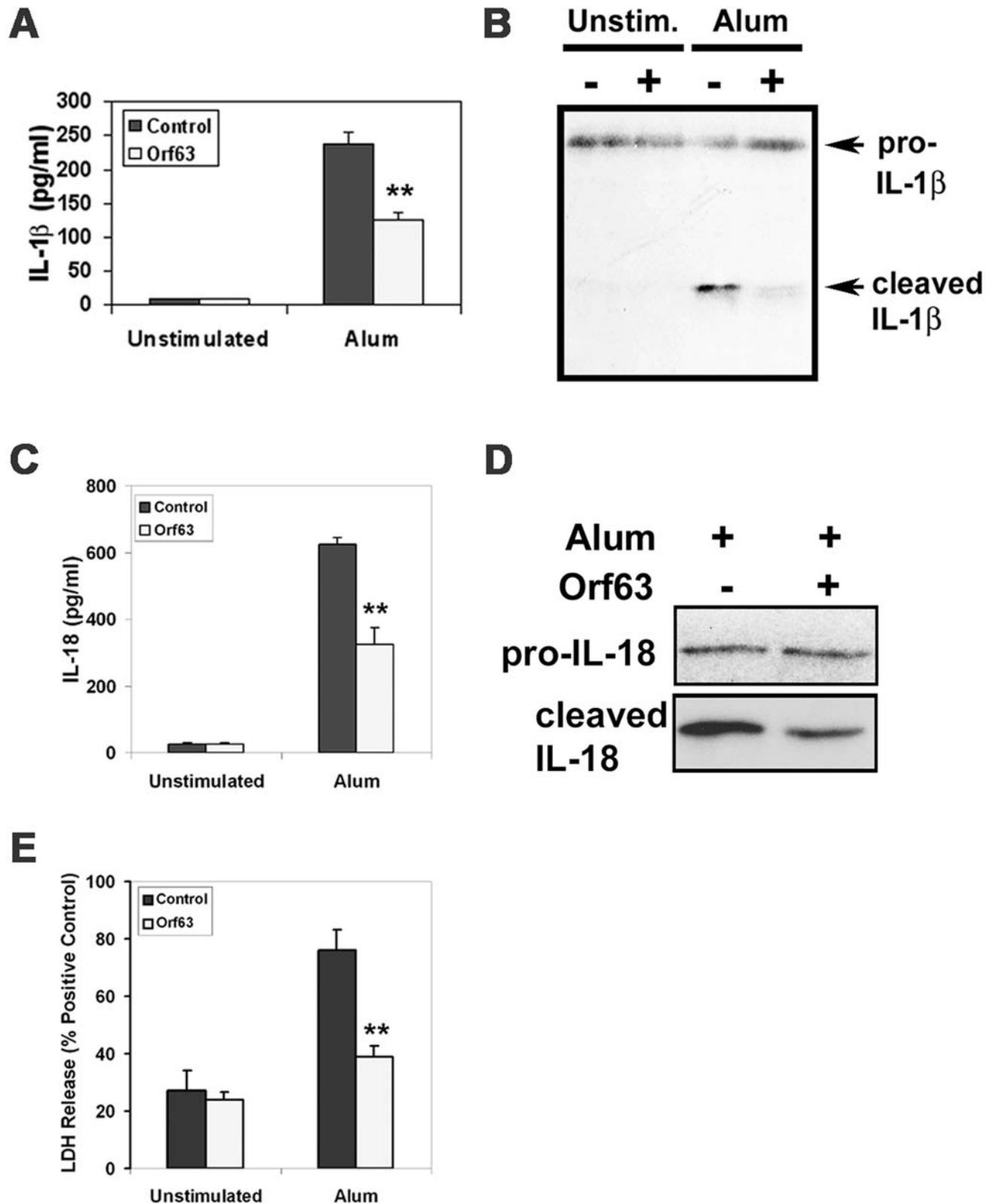
Supplemental Figure 10: NLRP1 inhibits KSHV reactivation from latency and production of infectious progeny virus.

(A) BCBL-1-shRNA-control and BCBL-1-shRNA-NLRP1 stable knockdown cell lines were analyzed for NLRP1 expression by immunoblot. (B) BCBL-1 or BCBL-1-shRNA-NLRP1 stable knockdown PEL cells were mock treated or treated with 25ng/ml TPA followed by analysis of viral genomes 96 hours post-treatment by qPCR. (C) BCBL-1-shRNA-control or BCBL-1-shRNA-NLRP1 PEL cells were treated as in panel B, and 96 hours later, supernatants were used to infect naïve Vero cells. 72 hours later, KSHV viral load in the infected Vero cells was determined by qPCR.



Supplemental Figure 11: Orf63 inhibits NLRP3 in a dose-dependent manner.

A dose-response graph for ATP treatment in control versus Orf63-expressing THP-1 cells. THP-1 control cells or Orf63-expressing cells were stimulated with LPS as described followed by stimulation with 0.25mM, 0.5mM or 25mM ATP. Supernatants were harvest 6 hours later and IL-1 β secretion was analyzed by ELISA. The IL-1 β values were normalized to LDH. ** indicates statistical significance $P \leq 0.05$ by two-tailed student's t-test. Data are representative of a minimum of three experimental replicates



Supplemental Figure 12: Orf63 inhibits the NLRP3 inflammasome. (A-D) THP-1-control or THP-1-Orf63 cells were mock treated or primed with 5ng/ml LPS for 1 hour followed by stimulation with 130µg/ml Alum for 6 hours. Supernatants were harvested and analyzed by ELISA for IL-1β (A) or for pro-IL-1β and cleaved IL-1β by immunoblot (B). Alum-stimulated cells were also examined for IL-18 expression by ELISA (C) and pro-IL-18 and cleaved IL-18 by immunoblot (D). For both IL-1β and IL-18 ELISAs, values were normalized to LDH. **(E)** THP-1-control or THP-1-Orf63 cells were treated with ATP as described above and subjected to an LDH release assay. ** indicates statistical significance $P \leq 0.05$ by two-tailed student's t-test. Data are representative of a minimum of three experimental replicates.

BLASTP alignment of the LRR domain of NLRP1 with RRV Orf63

NLRP1	308	CKNQAYGCSRNAIEHFIKKGIDFMAELGAFIEKTCGYRSTVLSNSVRAVKARLASCGLSK	367
		CK+ A+G N + + + GA + C + + + +L SCGL+	
RRV Orf63	854	CKDLAFGLRANQTLTELDLSFNVLTDAGA--KHLC--QRLRQPCKLQRLQLVSCGLTS	908
NLRP1	368	EACEDFRAMILMTP	381
		+ C+D +++ +P	
RRV Orf63	909	DCCQDLASVLSASP	922

Supplemental Figure 13: Rhesus monkey rhadinovirus (RRV) Orf63 demonstrates homology to NLRP1. Amino acid alignments were performed using BLASTP and ClustalW2 (depicted) bioinformatics programs with RRV Orf63 (NCBI accession# YP_001129420.1) and human NLRP1 (NCBI accession # NP_127499). The E value of the BlastP alignment of RRV Orf63 and NLRP1 was 0.002.