Kaposi’s Sarcoma-Associated Herpesvirus Reduces Cellular Myeloid Differentiation Primary-Response Gene 88 (MyD88) Expression via Modulation of Its RNA

Amy Lingel
*University of Nebraska-Lincoln, alingel2@unl.edu*

Erica Ehlers
*University of Nebraska-Lincoln*

Qianli Wang
*University of Nebraska-Lincoln*

Mingxia Cao
*University of Nebraska-Lincoln*

Charles Wood
*University of Nebraska-Lincoln, cwood1@unl.edu*

Follow this and additional works at: [http://digitalcommons.unl.edu/virologypub](http://digitalcommons.unl.edu/virologypub)

Part of the Biological Phenomena, Cell Phenomena, and Immunity Commons, Cell and Developmental Biology Commons, Genetics and Genomics Commons, Infectious Disease Commons, Medical Immunology Commons, Medical Pathology Commons, and the Virology Commons


[http://digitalcommons.unl.edu/virologypub/340](http://digitalcommons.unl.edu/virologypub/340)

This Article is brought to you for free and open access by the Virology, Nebraska Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Virology Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Kaposi’s Sarcoma-Associated Herpesvirus Reduces Cellular Myeloid Differentiation Primary-Response Gene 88 (MyD88) Expression via Modulation of Its RNA

Amy Lingel, Erica Ehlers, Qianli Wang, Mingxia Cao, Charles Wood, Rongtuan Lin, Luwen Zhang

School of Biological Sciences and Nebraska Center for Virology, University of Nebraska, Lincoln, Nebraska, USA; Lady Davis Institute for Medical Research, Department of Medicine, McGill University, Montreal, Quebec, Canada

ABSTRACT
Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV) is a human gammaherpesvirus associated with several human malignancies. The replication and transcription activator (RTA) is necessary and sufficient for the switch from KSHV latency to lytic replication. Interleukin 1 (IL-1) is a major mediator for inflammation and plays an important role in both innate and adaptive immunity. Myeloid differentiation primary response gene 88 (MyD88) is an essential adaptor molecule for IL-1 as well as most Toll-like receptor signaling. In this study, we identified a novel mechanism by which KSHV interferes with host inflammation and immunity. KSHV RTA specifically reduces the steady-state protein levels of MyD88, and physiological levels of MyD88 are downregulated during KSHV lytic replication when RTA is expressed. The N-terminal region of RTA is required for the reduction of MyD88. Additional studies demonstrated that RTA targets MyD88 expression at the RNA level, inhibits RNA synthesis of MyD88, and may bind MyD88 RNA. Finally, RTA inhibits IL-1-mediated activation of NF-κB. Because IL-1 is abundant in the KS microenvironment and inhibits KSHV replication, this work may expand our understanding of how KSHV evades host inflammation and immunity for its survival in vivo.

IMPORTANCE
MyD88 is an important molecule for IL-1-mediated inflammation and Toll-like receptor (TLR) signaling. This work shows that KSHV inhibits MyD88 expression through a novel mechanism. KSHV RTA may bind to MyD88 RNA, suppresses RNA synthesis of MyD88, and inhibits IL-1-mediated signaling. This work may expand our understanding of how KSHV evades host inflammation and immunity.

Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus. It is believed to be the etiological agent of KS (1–3). In addition, KSHV is implicated in the pathogenesis of AIDS-associated primary effusion lymphoma (PEL, also called body cavity-based lymphoma [BCBL]) and a lymphoproliferative disorder known as multicentric Castleman’s disease (3–5).

Like other herpesviruses, KSHV goes through both latency and lytic replication cycles. The expression of the KSHV replication and transcription activator (RTA) is necessary and sufficient for the switch from latency to lytic replication (4, 5). RTA is an immediate early gene (6–8) and a sequence-specific DNA-binding protein (7–13). RTA also interacts with other factors, such as cellular recombination signal sequence-binding protein Jκ (RBP-Jκ) (also known as CBF-1 and CSL), to modulate its transcription potential (14–16). Other than transcriptional regulation, RTA modulates protein degradation. RTA was first documented as a ubiquitin E3 ligase for degradation of cellular interferon regulatory factor 7 (IRF-7) (17). Many more proteins, including TIR domain-containing adapter-inducing interferon beta (TRIF) and KSHV-RTA binding protein (K-RBP), have been identified as targets by RTA for degradation with the same pathway (18–21).

The host inflammation and immune responses are essential for the initial detection of invading viruses and subsequent antagonizing invaders. The interleukin 1 (IL-1) family is a group of 11 cytokines which play a central role in the regulation of immune and inflammatory responses to infections. They act on innate immune cells to influence their survival and function. In addition, they act directly on lymphocytes to reinforce certain adaptive immune responses (for a review, see reference 22). Myeloid differentiation primary response gene 88 (MyD88) is an adaptor molecule that plays an essential role in mediation of IL-1 signaling. In addition, MyD88 is a critical adaptor for all Toll-like receptor (TLR) signaling pathways, except that of TLR3, to activate NF-κB (23–25). TLRs are able to recognize molecular patterns unique to pathogens and activate host innate immunity against the pathogens (26, 27).

KSHV needs to counteract host inflammation and innate-immunity-mediated antiviral responses. KSHV has abundant IL-1 in its microenvironment, and IL-1 inhibits its replication (28–31). KSHV uses microRNAs (miR-K9 and miR-K5) to target the IL-1 downstream components IRAK1 and MyD88 (32). TLR4 has been identified as an important molecule against KSHV infection, and KSHV has developed a mechanism for rapid suppression of TLR-4

Editor: R. M. Longnecker
Address correspondence to Luwen Zhang, l_zhang2@unl.edu
Copyright © 2015, American Society for Microbiology. All Rights Reserved.
expression (33). Also, murine gammaherpesvirus 68 (MHV68) is another herpesvirus with significant similarities to KSHV. Activation of the TLR3/TLR4 pathway potently inhibits the replication of MHV68 in vivo (34). Thus, a successful counteraction of host inflammation and immunity may be a necessity for the survival of KSHV in vivo.

Previously, we have found that RTA degrades TRIF and blocks TLR3-mediated antiviral responses (18). In this study, we found that RTA also downregulates expression of MyD88 to block the host inflammation and innate immunity. Interestingly, the reduction of MyD88 was at the RNA level rather than by protein degradation. RTA may be a RNA-binding protein that targets MyD88 RNA and consequently inhibits its synthesis. This report describes another mechanism by which KSHV counteracts host defense systems following infection.

**MATERIALS AND METHODS**

**Plasmids, antibodies, and drugs.** Expression plasmids of KSHV RTA and its mutants (RTA-K152E, RTA-ΔC, RTA-ΔN, RTA-H145L, and RTA-C141S), Epstein-Barr virus (EBV) RTA, MyD88, and TRAM were described previously (35–39). Mutagenesis to generate MyD88 mutants (MyD88-N and MyD88-C) was done by the use of PCR and cloned into a 3-FLAG vector (Sigma). The expression clones were all sequenced to verify their identity. MyD88 antibody was described previously (40). Tubulin (T6557) and FLAG (F1804) antibodies were obtained from Sigma. The antibodies for GAPDH (sc-47724), MyD88 (sc-11356), MDM2 (sc-965), and IRF-1 (sc-497) were from Santa Cruz. TRIF antibody was from Cell Signaling (catalog number 4596). The proteasome inhibitor lactacystin was purchased from Assay Designs and used at 1 to 10 μg/ml. For lactacystin treatment, cells were transfected, and medium was removed 4 to 6 h after transfection. Fresh medium plus the appropriate concentrations of drugs were added. Cells were collected 12 to 24 h later.

**Cell culture and transient transfection.** 293T is a human fibroblast line, and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% penicillin-streptomycin (PS) at 37°C with 5% CO2 incubation. P3HR1 is a KSHV-negative but EBV-positive Burkitt’s lymphoma cell line. BCBL1 is a KSHV-positive, EBV-negative primary effusion lymphoma line. These cells were maintained in RPMI 1640 plus 10% FBS.

**Transient transfection, cytokines, and reporter assays.** The transfection reagent Attractene (Qiagen catalog no. 300107) was used for the transfection of 293T cells following the manufacturer’s recommendations. Interleukin 1β (IL-1β; catalog no. 201-LB) and tumor necrosis factor alpha (TNF-α; catalog no. 210-TA) were purchased from R&D Systems. Luciferase assays were performed using the luciferase assay kit (catalog no. E1501) from Promega according to the manufacturer’s recommendations. β-Galactosidase assays were also performed for transfection efficiency. Data were averaged from triplicate experiments.

**Protein and RNA stability assays.** The protein biosynthesis inhibitor cycloheximide (Sigma catalog no. C4859) was used at 50 to 100 μg/ml. Cells were transfected in 10-cm dishes, and transfected cells were split 4 to 6 h after transfection into 6-well plates. Next day, the cells were treated with cycloheximide for various periods, and cell lysates were used for Western blot analysis. For RNA stability, the RNA synthesis inhibitor actinomycin D (Sigma catalog no. A1410) was used at 10 μg/ml. Transfected cells were split 4 to 6 h after transfection into 6-cm dishes. The next day, the cells were treated with actinomycin D for various periods and collected for total-RNA isolation.

**Western blot analysis, RNA extraction, and reverse transcription-PCR (RT-PCR) analysis.** Standard Western blot analysis was performed as described previously (41–43). Total RNA was isolated from cells using TRIzol extraction. cDNA was synthesized with SuperScript reverse transcriptase (Invitrogen catalog no. 18064-014) and random hexamers (Invitrogen catalog no. 48190-011). The primers used for PCR analysis are listed in Table 1. The signal strengths were enumerated by the use of the Bio-Rad software Quantity One (version 4.6.7).

**Isolation of newly transcribed RNA.** Newly transcribed RNA was isolated using the Click-IT nascent-RNA capture kit from Life Technology (catalog no. C10365). This method enables the metabolic incorporation of ethynyl uridine (EU)—a “clickable” ribonucleotide—onto RNA during nascent-RNA synthesis. Biotin is then “clicked” onto the nascent chain, and streptavidin magnetic beads are used to capture the nascent RNA. The cells were labeled with EU (200 mM) for 30 to 60 min. The newly synthesized RNA (with EU incorporation) was isolated from total RNA by following the manufacturer’s recommendations. The cDNA was synthesized with a SuperScript VILO cDNA synthesis kit (Life Technology catalog no. 11754-050) as recommended by the manufacturer.

**RNA-ChIP assay.** RNA-chromatin immunoprecipitation (RNA-ChIP) essentially followed the published protocol (44) and the corresponding online protocol ["RNA-chromatin immunoprecipitations (RNA-ChIP) in mammalian cells (PROT28)""] (http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=3241). Briefly, cells were grown in a 10-cm culture dish and transfected with plasmids. One day later, cells were cross-linked with formaldehyde for 30 min at room temperature. Glycine was added, and the cells were washed twice with ice-cold PBS containing 1% protease inhibitor cocktail (Roche catalog no. 05892). Cell lysates were sonicated two or three times for 30 s each (Virtus Visonic cell disruptor; power level 3). Sonicates were diluted, and an aliquot was

*TABLE 1 Primer sequences used for RT-PCR analysis in this studya*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Purpose or region</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHG4930</td>
<td>TTGGCTCTTGTAGACGGAAGC</td>
<td>KSHV RTA detection</td>
</tr>
<tr>
<td>LHG4929</td>
<td>GATTCGCAAGCTTCAGTCTCGGAAGTAGTAAATTACG</td>
<td>Actin detection</td>
</tr>
<tr>
<td>Actin1</td>
<td>TTCTACAATGAGCTGGTGTTT</td>
<td></td>
</tr>
<tr>
<td>Actin2</td>
<td>GCCAGACAAGACTGGTGTTT</td>
<td></td>
</tr>
<tr>
<td>MyD88AF</td>
<td>CGGAATTCAATGGCCTGCACAGGCCCATCAC</td>
<td>Region A: aa 1–75</td>
</tr>
<tr>
<td>MyD88AR</td>
<td>GGGGATCCCTACAGGAGGACAAGGCCTTGGCAAG</td>
<td>Region B: aa 76–151</td>
</tr>
<tr>
<td>MyD88BF</td>
<td>CGGAATTCAGAGCTGGTGAGACGGGCTTGGCAAG</td>
<td>Region C: aa 152–225</td>
</tr>
<tr>
<td>MyD88BR</td>
<td>GGGGATCCCTTCCTAAACAGTGGTGAGACGGGCTTGGCAAG</td>
<td>Region D: aa 226–296</td>
</tr>
<tr>
<td>MyD88CF</td>
<td>CGGAATTCAGGCCCATCATATGCTGGTGAGACGGGCTTGGCAAG</td>
<td></td>
</tr>
<tr>
<td>MyD88CR</td>
<td>GGGGATCCCTTAATCACAGAAACCACACATCCT</td>
<td></td>
</tr>
<tr>
<td>MyD88DF</td>
<td>CGGAATTCATTACCTGCAAGAGCAAGGAAGGGCTTGGCAAG</td>
<td></td>
</tr>
<tr>
<td>MyD88DR</td>
<td>GGGGATCCCTACAGGAGGACAAGGCCTTGGCAAG</td>
<td></td>
</tr>
<tr>
<td>TRIF1</td>
<td>GCCAGCTCAGGCGAGGAGGGCCGAGGCGAC</td>
<td>TRIF detection</td>
</tr>
<tr>
<td>TRIF2</td>
<td>CGGAATTCATGGCCTGCAAGAGGGCTTGGCAAG</td>
<td></td>
</tr>
</tbody>
</table>

a For MyD88 primers, the amino acid (aa) coordinates of the regions covered are provided.
MyD88 protein expression occurred. Furthermore, we tested if E-RTA was unable to reduce the expression of MyD88 (KSHV RTA-mediated reduction seems to be specific, as E-RTA of RTA. EBV RTA (E-RTA) and KSHV RTA have significant homologies with KSHV RTA. Total DNA for transfection was kept the same with the use of vector DNA. The cell lysates were obtained 1 day later for Western blot analysis. The membrane was stripped and probed with another antibody. Images in the same box are derived from the same membranes. (B) MyD88 is downregulated during KSHV lytic replication. BCBL1 (KSHV\(^{+}\)) and P3HR1 (KSHV\(^{-}\)) cells were treated with sodium butyrate for 24 h. Cell lysates were made, and the expression of endogenous proteins was analyzed by Western blotting. The membrane was stripped and probed with another antibody. The same cell lysates were used, and images in the same box are derived from the same membranes.

preserved as an input sample and frozen at \( -80^\circ\)C until the reverse cross-linking step. Normal rabbit serum (NRS) or RTA antibodies and Protein A/G Plus agarose (Santa Cruz catalog no. 2003) were added to cell lysates, and the mixture rotated slowly at 4°C overnight. Immune complexes were collected, eluted, and placed at 65°C for 2 h to reverse the cross-linking. Proteinase K (Ambion catalog no. 25530-049) was added and incubated at 42°C for 45 min. Samples were subjected to phenol extraction and ethanol precipitation with GlycoBlue (Ambion catalog no. AM9513) as a carrier. DNA from the samples was removed by the use of DNase I (Ambion catalog no. AM1907). Sometimes DNase treatment was repeated one more time. Routine RT-PCR was carried out.

RESULTS

RTA reduces steady-state protein levels of MyD88. Previously, we found that RTA degrades TRIF protein (18). To assay for other TLR signaling molecules, we examined several other molecules involved in TLR signaling. 293T cells were cotransfected with target gene and RTA expression plasmids, and 1 day later, cell lysates were used to examine target gene expression. As shown in Fig. 1, we found that MyD88 protein levels were reduced in the presence of RTA. EBV RTA (E-RTA) and KSHV RTA have significant homologies, and both viruses are gammaherpesviruses. However, KSHV RTA-mediated reduction seems to be specific, as E-RTA was unable to reduce the expression of MyD88 (Fig. 1A). Other molecules, such as TRAM, were not affected in the presence of KSHV RTA (data not shown). Thus, the data suggest that RTA reduced MyD88 protein expression specifically.

The expression of RTA and that of MyD88 are inversely correlated in virus-infected cells. To examine whether RTA could affect MyD88 protein expression under physiological conditions, lytic replication of KSHV was induced in latently infected BCBL cells with chemicals. RTA is not expressed during the viral latency, but it is a key mediator for the switch from latency to lytic replication. When KSHV-positive BCBL1 cells were treated with sodium butyrate, RTA expression was significantly increased (Fig. 1B). In addition, a simultaneous downregulation of endogenous MyD88 protein expression occurred. Furthermore, we tested if MyD88 was degraded in KSHV-negative cell lines. P3HR1 is an EBV-positive but KSHV-negative Burkitt’s lymphoma cell line. With the sodium butyrate treatment, MyD88 steady-state levels did not change noticeably (Fig. 1B). These results suggest that the reduction of MyD88 was specifically associated with the induction of RTA under physiological conditions.

The N-terminal region of RTA is required for the modulation of MyD88. It is well known that RTA could serve as a ubiquitin ligase and degrade a protein through the proteasome pathway. To test whether the proteasome pathway was involved in reducing MyD88 levels, three RTA mutants defective in initiating the proteasome pathway were examined (Fig. 2A). Those mutant plasmids were transfected into cells along with the MyD88 expression plasmid. The expression of MyD88 was examined 1 day later. While RTA-H145L had a limited effect on MyD88 expression, RTA-K152E and RTA-C141S both reduced MyD88 expression significantly (Fig. 2B). In addition, RTAΔN, but not RTAΔC, was unable to decrease MyD88 expression effectively. Interestingly, the two mutants have very different molecular weights, and this may need further investigation. Those data suggest that the N terminus of RTA is involved in the modulation of MyD88 expression. The proteasome pathway may be partially employed, as the residue His-145 of RTA is involved in MyD88 regulation. In addition, a novel mechanism of MyD88 reduction is apparently present.

To localize the sequences of MyD88 that are important for RTA-mediated reduction, two mutants were made (Fig. 2C). As shown in Fig. 2D, the N terminus of MyD88 is the main region responsive to RTA, and the C terminus is still responsive but less efficient. The data suggest that RTA targets MyD88 expression through multiple regions.

RTA does not directly affect MyD88 protein stability. To study further if the proteasome pathway was involved in MyD88 protein reduction, we studied MyD88 protein stability in the presence of RTA. 293T cells were transfected with MyD88, with or without an RTA expression plasmid. One day later, cells were treated with cycloheximide to block \textit{de novo} protein synthesis. Cell lysates were made at various time points after cycloheximide treatment. As shown in Fig. 3A, while MyD88 protein was reduced in the presence of RTA, the stability of MyD88 protein was not noticeably changed in the presence or absence of RTA. RTA protein stabilities were also similar with and without MyD88. The half-life of IRF-1 protein was short, as expected (Fig. 3B) (45), suggesting that the protein degradation pathway was functional. These results suggest that protein stability might not be a major mechanism by which RTA regulates MyD88 levels.

RTA modulates MyD88 at RNA levels. Additional studies were performed to determine whether RTA regulated MyD88 at the RNA level. Cells were transfected with RTA and MyD88 expression plasmids with various combinations. RNA were isolated, and semiquantitative RT-PCR was used for determining the levels of MyD88 RNA. As shown in Fig. 4A, MyD88 RNA was degraded in the presence of RTA. Whether endogenous MyD88 RNA was affected by RTA was examined as well. As shown in Fig. 4B, endogenous MyD88 RNA levels in 293T cells were reduced when RTA was expressed. Moreover, whether endogenous RTA could inhibit endogenous MyD88 RNA under physiological conditions was examined. As described above, the lytic replication of KSHV was induced, and the increase of RTA was observed (Fig. 1B). RNA were isolated and processed for RT-PCR. As shown in Fig. 4C, reduced levels of endogenous MyD88 RNA was observed in KSHV lytic replications. Therefore, physiological levels of RTA were in-
versely correlated with MyD88 RNA expression following KSHV lytic replication.

Because RTA activates the proteasome pathway, we examined if RTA-mediated downregulation of MyD88 was affected by a proteasome inhibitor. MyD88 expression was analyzed in the presence of lactacystin, a potent inhibitor of the 26S proteasome. Lactacystin could not block the downregulation of MyD88 at both protein and RNA levels (Fig. 4D and E) (46, 47). In addition, we transfected TRIF, MyD88, and RTA together into cells. While both TRIF and MyD88 protein levels were reduced as expected, the level of MyD88 RNA, but not TRIF RNA, was reduced (data not shown) (18). These data suggested that RTA targeted MyD88 predominantly at the RNA level in transfection studies.

RTA decreases the MyD88 RNA synthesis rate. Once it was established that RTA inhibited MyD88 at the RNA level, we addressed whether RTA targeted MyD88 RNA synthesis and/or degradation. Ethynyl uridine (EU) is a special nucleotide that can specifically replace uridine and can be synthesized onto RNA during nascent-RNA synthesis. The newly synthesized RNA with EU can be isolated specifically from total RNA (see Materials and Methods for details). The relative rates of RNA synthesis could be calculated and compared. The transfected cells were labeled with EU for a short period of time, and de novo synthesized RNAs were isolated. The relative rates of MyD88 RNA synthesis were calculated. As shown in Fig. 5A and B, the rate of synthesis of new MyD88 RNA was lower in RTA- and MyD88-cotransfected cells than in cells transfected with MyD88 only. Next, the degradation of MyD88 RNA in the presence of RTA was examined. Actinomycin D was used to block de novo RNA synthesis, and RNAs were isolated at various times. Relative MyD88 RNA stability was measured using actin RNA as a standard. As shown in Fig. 5C, MyD88 RNA stability was not changed drastically in the presence or absence of RTA. Because actin RNA was also degraded accordingly, the relative amounts of MyD88 RNA (MyD88 RNA/actin RNA) did not show a linear reduction (Fig. 5C). Those studies revealed that RTA inhibited primarily the synthesis, not the degradation, of MyD88 RNA.

RTA may bind to MyD88 RNA. Because RTA modulates MyD88 expression in both ectopic (driven by the cytomegalovirus [CMV] promoter/enhancer) and endogenous (driven by the native MyD88 promoter/enhancer) settings (Fig. 1 to 4), and RTA-K152E, a DNA binding mutant (38), regulates MyD88 expression.

**FIG 2** Domain analysis of RTA for MyD88 reduction. (A) Schematic diagram of RTA mutant constructs. The numbers denote the amino acid positions. The drawing is not to scale. (B) The N terminus of RTA is required for MyD88 regulation. 293T cells were transfected with vector pcDNA3 and with MyD88 (0.1 μg), RTA (0.2 μg), and RTA mutant expression plasmids (0.2 μg) in various combinations, as shown at the top. The amount of total DNA for transfection was kept the same with the use of vector DNA. Cell lysates were made 1 day later, and Western blot analysis was performed with RTA, FLAG, and tubulin antibodies. (C) Schematic diagram of MyD88 mutant constructs. The numbers denote amino acid positions of MyD88. The drawing is not to scale. (D) Multiple regions of MyD88 are targeted by RTA. 293T cells were transfected with vector pcDNA3 and with MyD88 (0.1 μg), RTA (0.2 μg), or MyD88 deletion mutant plasmids in various combinations, as shown at the top. The amount of total DNA for transfection was kept the same with the use of vector DNA. Cell lysates were made 1 day later, and Western blot analysis was performed with FLAG and GAPDH antibodies.

**FIG 3** RTA did not affect the MyD88 protein stability. (A) MyD88 is a relatively stable protein. 293T cells in a 10-cm dish were transfected with MyD88 (0.4 μg) RTA, or MyD88 plus RTA (0.8 μg) expression plasmids. The amount of total DNA for transfection was kept the same with the use of vector DNA. At 6 h after transfection, cells were split into a 6-well plate. Cycloheximide (100 μg/ml) was added after a 12-h incubation. Cell lysates were made at various times, as shown on the top, and Western blot analysis was performed. The membrane was stripped and probed with another antibody. Images in the same box are derived from the same membranes. (B) Detection of IRF1 stability. The lysates used for panel A were used. IRF1 protein stability was measured in MyD88 and MyD88 plus RTA-transfected cells.
we suspected that RTA might regulate MyD88 RNA synthesis through RNA binding. Whether RTA could bind to MyD88 RNA was examined by RNA-ChIP assay. Because there was no apparent target region(s) for RTA binding, we tested RNA binding activity covering the entire MyD88 coding sequences in four different regions (Fig. 6A; Table 1). In five independent experiments, while region B was consistently detected by RNA-ChIP, region A was difficult to detect. Regions C and D were not consistently detected. The authenticity of RNA-derived fragments was established (Fig. 6C). These experiments suggested that RTA may bind to region B of MyD88 RNA but not to region A. At the same time, RTA might bind to regions C and D weakly.

RTA downregulates the IL-1 signaling pathway. MyD88 is an essential component for IL-1 signaling as well as signaling pathways for several TLRs, such as TLR7 and TLR9. Due to the abundance of IL-1 in KS lesions, we tested if RTA blocked IL-1-mediated signaling pathway by assaying NF-κB activation. 293T cells were transfected with RTA and an NF-κB reporter construct, and transfected cells were treated with IL-1β. As shown in Fig. 7A, the NF-κB reporter was activated in response to IL-1β. However, in the presence of RTA, the activation was drastically reduced. We also tested if RTA blocked NF-κB activation induced by TNF-α: RTA did not inhibit TNF-α-mediated NF-κB activation (data not shown), in agreement with a previous report (19). Of note, TNF-α activates NF-κB through a different pathway, and MyD88 is not involved (48). In addition, RTAΔC, but not RTAΔN, could inhibit IL-1β-mediated activation of NF-κB (Fig. 7B). Finally, RTA, but not RTAΔN, directly inhibited MyD88-mediated activation of NF-κB (Fig. 7C). Those data showed a clear correlation between blockages of NF-κB activation and RTA’s ability to downregulate MyD88 (Fig. 2). Collectively, these data suggest that RTA blocks the IL-1β signaling pathway, possibly through MyD88.

DISCUSSION
KSHV needs to counteract host immune responses for a successful infection in vivo, and it has developed many mechanisms for the evasion of host innate immunity (17, 18, 33, 49–51). In this report, we have found that RTA may target MyD88 for inhibition of IL-1-mediated inflammation. First, we found that RTA reduces expression of MyD88 at both protein and RNA levels (Fig. 1A, 3, and...
Second, the regulation of MyD88 is apparently present under physiological conditions, as induction of lytic replication, and thus the RTA expression, reduces the expression of endogenous MyD88 at both protein and RNA levels (Fig. 1 and 4). This RTA-mediated reduction of MyD88 RNA apparently occurs through a novel mechanism. We have found that the rate of MyD88 RNA synthesis is reduced in the presence of RTA (Fig. 5A and B), and MyD88 RNA stability is not obviously influenced by RTA in the same system (Fig. 5C). Furthermore, RTA may bind to MyD88 RNA, especially to region B (Fig. 6 and Table 1). Based on all the data presented in this study, we propose the following mechanism by which KSHV modulates MyD88 expression. KSHV infection leads to RTA expression. RTA binds to newly synthesized MyD88 RNA, reduces its elongation function, and inhibits the rate of MyD88 RNA synthesis. The result is the reduction of MyD88 expression. Of note, due to the limitations of the RNA-ChIP assay, we cannot rule out the possibility that RTA may bind to MyD88 RNA indirectly through another molecule(s). In addition, RTA may still bind to MyD88 DNA directly or indirectly and modulate its transcription.

It is interesting that KSHV RTA could degrade MyD88 but not its EBV counterpart, EBV-R (Fig. 1). Both EBV and KSHV are...
gammaherpesviruses, and they encode RTAs with significant homologies. However, they have slight differences in cell tropism, and the roles of the two RTAs seem to be different in different viruses. In EBV, BZLF1 is a major player in inducing EBV lytic replication, and EBV-R is a minor player. In contrast, KSHV RTA is the major player and critical for KSHV lytic replication, and it has several mechanisms to block the host’s innate immunity. The differences in their ability to regulate MyD88 may represent their general roles in different viruses.

The ability of KSHV RTA to bind to RNA is novel. Some herpesvirus proteins can bind to RNA. EBV EBNA1 has been shown to be an RNA-binding protein that plays a role in viral genome replication (52, 53). In addition, EBV Mta and KSHV ORF57 also bind to RNA to facilitate RNA export (54, 55). It is unique, though, that RTA may bind to RNA specifically and modulate the RNA synthesis. However, the nature of the RNA binding activity and of the detailed mechanism for the regulation of MyD88 RNA synthesis requires further investigation.

Recently there was a report that RTA degrades MyD88 through the proteasome pathway (56), which apparently fits with the ability of RTA to function as an E3 ligase. This is apparently contradictory to the results we report here. Under the conditions of our studies, the proteasome pathway does not appear to be the predominant mechanism. First, the half-life of MyD88 protein was not changed drastically in the presence of RTA (Fig. 3). Second, proteasome inhibitors (lactacystin) could not restore MyD88 protein expression in the presence of RTA (Fig. 4E). However, we could not eliminate the possibility that proteasome may play certain roles, as the His-145 residue in the putative E3 ligase domain of the RTA failed to modulate MyD88 expression (Fig. 2) (17, 21). We speculate that the different cell types, transfection conditions, and gene dosages used in these two studies may collectively contribute to the apparent discrepancies. In any case, RTA may modulate MyD88 with multiple mechanisms under different circumstances, including the proteasome pathway.

Because MyD88 is critically involved in IL-1 and TLR signaling, the results here suggest another novel pathway by which KSHV blocks host innate immunity as well as inflammation. Because IL-1 inhibits viral replication at least in endothelial cells, and IL-1 is abundant in the microenvironment of the KS, the blockage of the IL-1 signaling pathway (Fig. 7) may help KSHV to counteract inflammation-mediated damaging effects (28–31). In addition, TLR4 has been identified as an important antiviral molecule against KSHV infection, and KSHV has developed a mechanism for rapid suppression of TLR4 expression (33). TLR4 activation would lead to activation of both TRIF and MyD88, which further leads to the induction of immunity against the virus. Therefore, this RTA-mediated degradation of MyD88 and TRIF may be used by KSHV to escape TLR4-mediated innate immunity against KSHV (56). Finally, as the reduction of MyD88 was observed in KSHV-infected cells under physiological concentrations in native environments (Fig. 1 and 4), this phenomenon might naturally occur in individuals who have KSHV infection in vivo.

MyD88 is a multifunctional adaptor protein, mediating activation of several transcription factors, including NF-κB, a key mediator for proinflammatory cytokines (for a review, see reference 25). Additionally, TLR may induce apoptosis, and MyD88 is required in certain situations (24). The reduction of MyD88 may apparently alleviate the potential apoptosis process. It is tempting to speculate that RTA-mediated MyD88 regulation may not be limited to the blockage of host inflammation and immunity but may also include other cellular activities for the benefits of KSHV replication in vivo.

ACKNOWLEDGMENTS

We thank Ren Sun for providing RTA expression plasmid and Clinton Jones for critical reading of the manuscript.

This work was supported in part by grants from the National Institutes of Health (CA138213, RR15635), Department of Defense (W81XWH-12-1-0225), and National Multiple Sclerosis Foundation (PP3446) (L.Z.).

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Luwen Zhang under grant numbers CA138213 and RR15635. National Multiple Sclerosis Society (NMSS) provided funding to Luwen Zhang under grant number PP3446. DoD | Congressionally Directed Medical Research Programs (CDMRP) provided funding to Luwen Zhang under grant number W81XWH-12-1-0225.

REFERENCES


