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

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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 pathogen (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

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TABLE 1 Primer sequences used for RT-PCR analysis in this study[a]

Name	Sequence (5′→3′)	Purpose or region
LGH4930	TTCGCCTGTTAGACGAAGC	KSHV RTA detection
LGH4929	GATTCGCAAGCTTCAGTCTCGGAAGTAATTACG	
Actin1	TTCTACAATGAGCTGCGTGT	Actin detection
Actin2	GCCAGACAGCACTGTGTTGG	
MyD88AF	CGGAATTCAATGGCTGCAGGAGGTCCCGGCGCGGGG	Region A: aa 1–75
MyD88AR	GGGGATCCCTTACAGCAGCCTGCCAGTGGGGTCCGCTTGT	
MyD88BF	CGGAATTCAGACGCCTGGCAGGGACGCCCT	Region B: aa 76–151
MyD88BR	GGGGATCCCTTAATCAAGTGTGGTGATGCCCGCCAGCTC	
MyD88CF	CGGAATTCAGACCCCCTGGGGCATATGCCTGAG	Region C: aa 152–225
MyD88CR	GGGGATCCCTTAATCAGAGACAACCACCACCATCC	
MyD88DF	CGGAATTCAGATTACCTGCAGAGCAAGGAA	Region D: aa 226–296
MyD88DR	GGGGATCCCTCAGGGCAGGGACAAGGCCTTGGCAAG	
TRIF1	GCCACGTCCCGCAGCGAGGCGGGGCAC	TRIF detection
TRIF2	CGGAATTCAATGGCCTGCACAGGCCCATCAC	

[a] For MyD88 primers, the amino acid (aa) coordinates of the regions covered are provided.

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 validity. RTA 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 μM. 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% CO_2 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. 301007) 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 recommendation. 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 (Virtis Virsonic cell disruptor; power level 3). Sonicates were diluted, and an aliquot was

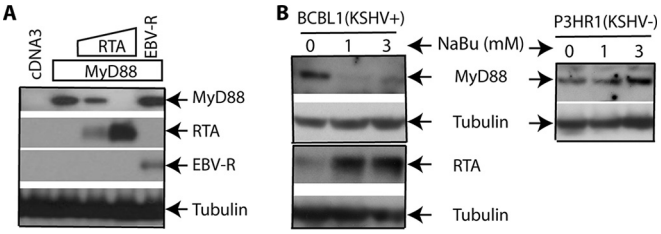

FIG 1 RTA modulates the expression of MyD88 protein. (A) RTA reduces the expression of MyD88 protein. 293T cells were transfected with cDNA3, RTA, EBV-RTA (EBV-R) (0.2 μg), and the MyD88 expression plasmid (0.1 μg) in various combinations, as shown on the top. E-RTA has significant homologies with KSHV 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°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. AM9515) 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 *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 MyB88 RNA was observed in KSHV lytic replications. Therefore, physiological levels of RTA were in-

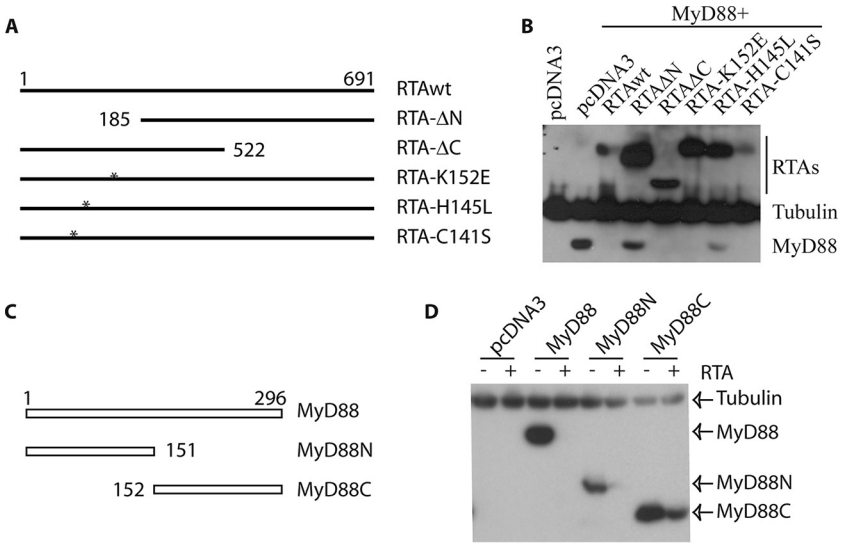

FIG 2 Domain analysis of RTA for MyD88 reduction. (A) Schematic diagram of RTA mutant constructs. The numbers denote the amino acid positions. The drawing is not to scale. (B) The N terminus of RTA is required for MyD88 regulation. 293T cells were transfected with vector pcDNA3 and with MyD88 (0.1 μg), 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.

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). The effectiveness of lactacystin was confirmed by MDM2 expression (Fig. 4E) (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.

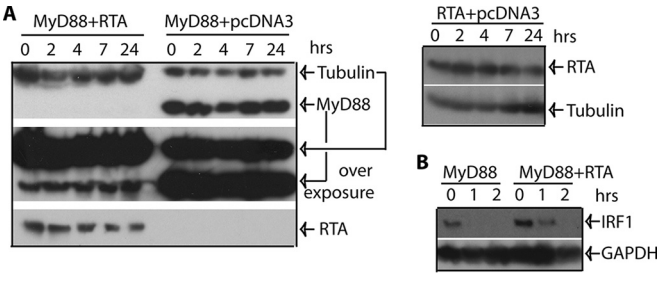

FIG 3 RTA did not affect the MyD88 protein stability. (A) MyD88 is a relatively stable protein. 293T cells in a 10-cm dish were transfected with MyD88 (0.4 μg) RTA, or MyD88 plus RTA (0.8 μg) expression plasmids. The amount of total DNA for transfection was kept the same with the use of vector DNA. At 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-transected cells.

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

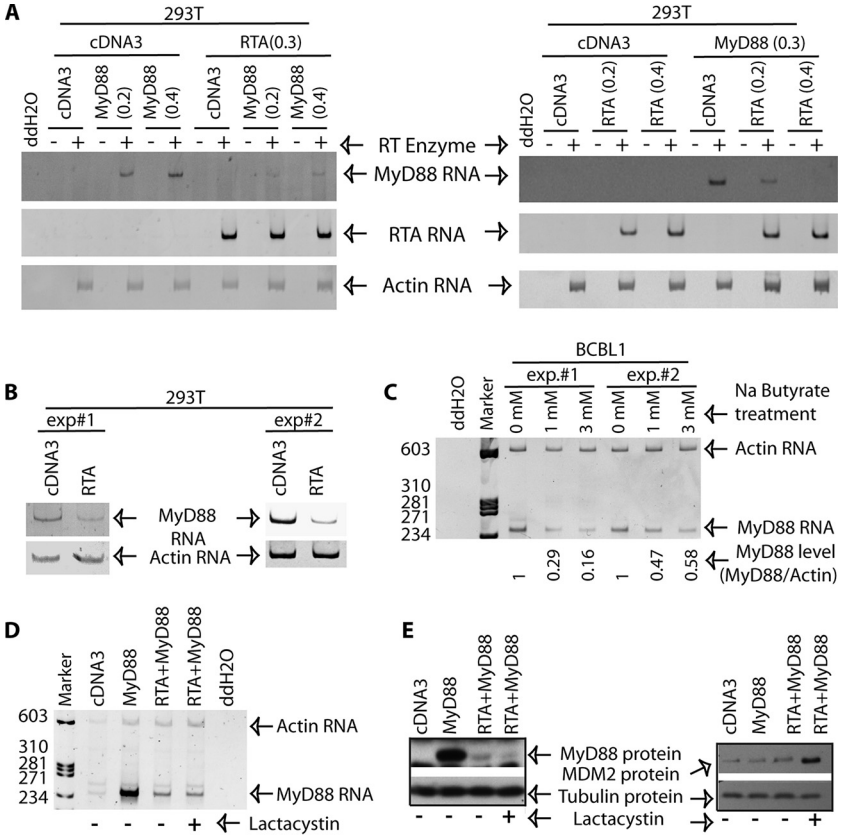

FIG 4 RTA reduces MyD88 RNA expression. (A) RTA reduces the RNA expression of MyD88 in transfected cells. 293T cells were transfected with a MyD88 or MyD88-plus-RTA expression plasmid. The amount of total DNA for transfection was kept the same with the use of vector DNA. Cell were collected 24 h after transfections, and RNA was isolated. Semiquantitative RT-PCR was carried out with specific primers. (B) RTA reduces endogenous MyD88 RNA expression. 293T cells were transfected with RTA and MyD88 expression plasmids. Total RNA were isolated 24 h after transfections. Semiquantitative RT-PCR was carried out with specific primers. Primers MyD88AF and MyD88BR for MyD88 were used for panels A and B. (C) MyD88 RNA is downregulated during KSHV lytic replication. BCBL1 (KSHV$^+$) cells were treated with sodium butyrate for 24 h. Total RNAs were isolated 24 h after treatments. Semiquantitative RT-PCR was carried out with specific primers at the same time. (D) Lactacystin did not modulate the expression of MyD88 RNA. 293T cells were transfected with various expression plasmids. At 4 to 6 h after transfection, cells were treated with lactacystin (10 μM). On the next day, total RNAs were isolated, and semiquantitative RT-PCR was carried out with specific primers. Primer MyD88BF and MyD88BR were used for panels C and D. (E) Lactacystin did not modulate the expression of MyD88 protein. Cell lysates were obtained from the experiment whose results are shown in panel D, and MyD88, tubulin, and MDM2 expression was examined by Western blotting.

(Fig. 2), 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

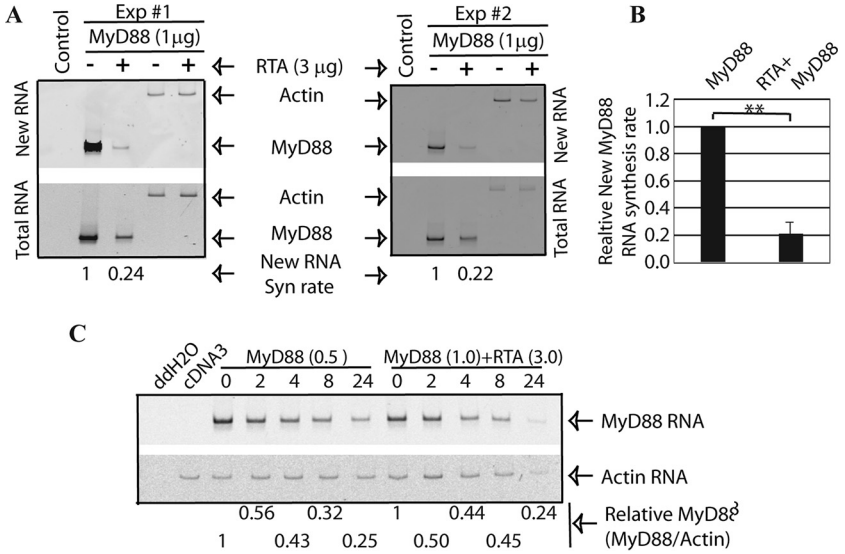

FIG 5 RTA reduces the rate of MyD88 RNA synthesis. (A) RTA reduces the relative RNA synthesis rate for MyD88. 293T cells were transfected with various plasmids, as shown at the top. The cells were labeled with ethynyl uridine (EU) for 30 to 60 min, and total RNAs were isolated. The EU-containing RNA (newly synthesized) was isolated from the total RNA with the use of a Click-iT nascent-RNA capture kit as described in Materials and Methods. RT-PCR were carried out with the appropriate primers. The PCR products were separated in a polyacrylamide gel. The relative MyD88 RNA levels were determined (MyD88 RNA/actin RNA). The relative new synthesis rate was calculated as relative newly synthesized MyD88 RNA (EU-labeled MyD88/EU-actin) versus relative total MyD88 RNA (total MyD88/total actin). (B) Comparison of relative MyD88 synthesis rates. The average relative synthesis rates with and without RTA from five independent experiments are shown. Statistical calculation was done with Microsoft Excel. **, $P < 0.01$. (C) MyD88 RNA stability is not affected drastically by RTA. RTA and MyD88 plasmids were transfected into 293T cells. More MyD88 plasmid was transfected in the presence of RTA (shown at the top) in order to achieve similar MyD88 RNA levels. One day after transfection, the cells were labeled with actinomycin D to inhibit RNA synthesis. Total RNAs were isolated at the various times (hours posttreatment), and MyD88 RNA levels were detected by semiquantitative RT-PCR. Actin levels were used as a control. Results of one representative experment of three independent experiments are shown.

4). 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

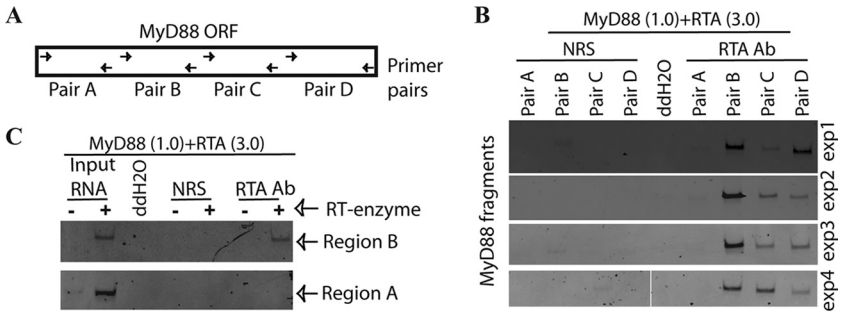

FIG 6 RTA may bind to MyD88 RNA. (A) Schematic diagram of primers used for detection of MyD88 RNA. The four pairs of primers are used to detect different regions of MyD88 RNA (Table 1). All four products are approximately the same length, around 230 bp. The drawing is not to scale. (B) RTA may bind to MyD88 RNA. 293T cells were transfected with expression plasmids as shown on the top. One day after transfection, cells were fixed with formaldehyde for 30 min and sonicated briefly to avoid extensive damage to RNA. Normal rabbit serum (NRS) and RTA antibody were used for the RNA-ChIP assay. After digestion with DNase, RT-PCR was carried out with the appropriate primers, shown in panel A. Results from four independent experiments are shown. (C) RTA may selectively bind to MyD88 RNA. RNA-ChIP were done as above, and primer pairs A and B were used for detection. Input RNA (1:100) was used as a control. Results of one representative experiment are shown.

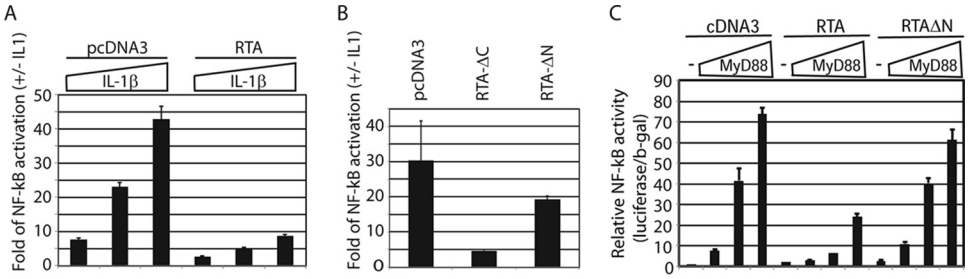

FIG 7 RTA blocks IL-1 signaling. (A) RTA blocks IL-1-mediated NF-κB activation. 293T cells were transfected with cDNA3 or RTA along with the NF-κB reporter construct and β-galactosidase expression plasmids. Total DNA for transfection was kept the same with the use of vector DNA. After 4 to 6 h transfection, the cells were washed and then treated (+) with IL-1β (0.5, 1, and 5 ng/ml) or not treated (−). One day later, the cells were collected, and luciferase and β-galactosidase assays were used for detection of reporter activation. The relative activation of NF-κB reporter (with or without IL-1β) is shown. (B) RTAΔC blocks IL-1-mediated NF-κB activation. Various plasmids were transfected into 293T cells as shown on the top. The cells were then treated (+) with IL-1β (5 ng/ml) or not treated (−). The relative activation of NF-κB reporter (with or without IL-1β) is as shown. One set of representative results is shown. (C) RTA blocks MyD88-mediated activation of NF-κB. Various plasmids were transfected into 293T cells as shown on the top. Luciferase and β-galactosidase activities were measured 1 day later. The relative activation of NF-κB reporter is shown. One representative set of results is shown.

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*.

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