Endoplasmic Reticulum-Shaping Atlastin Proteins Facilitate KSHV Replication

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Kaposi’s sarcoma-associated herpesvirus (KSHV) has two life cycle modes: the latent and lytic phases. The endoplasmic reticulum (ER) is the site for KSHV production. Furthermore, ER stress can trigger reactivation of KSHV. Little is known about the nature of the ER factors that regulate KSHV replication. Atlastin proteins (ATLs which include ATL1, ATL2, and ATL3) are large dynamin-related GTPases that control the structure and the dynamics of the ER membrane. Here, we show that ATLs can regulate KSHV lytic activation and infection. Overexpression of ATLs enhances KSHV lytic activation, whereas ATLs silence inhibits it. Intriguingly, we find that silencing of ATLs impairs the response of cells to ER stress, and ER stress can promote the lytic activation of KSHV. Our study establishes that ATLs plays a critically regulatory role in KSHV infection, thus expanding the known scope of biological processes controlled by ATLs to include KSHV infection.

Keywords: atlastin, endoplasmic reticulum, KSHV, lytic activation, ER stress

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8), is etiologically associated with KS, an angioproliferative malignancy of the human skin, and also with two angiolymphoproliferative disorders: body cavity-based B cell lymphoma (BCBL) (or primary effusion lymphoma [PEL]) and some forms of polyclonal B-cell proliferative multicentric Castleman’s disease (MCD) (Lange and Damania, 2020; Naipauer et al., 2020; Vega et al., 2020). An intensive research effort led to important progress regarding KSHV epidemiology, diagnosis, and potential therapeutic and prophylactic strategies (Gong et al., 2019; Holmes et al., 2020). The mechanisms involved in KSHV replication, host responses, and pathogenesis have also been extensively studied (Blumenthal et al., 2020; Yu et al., 2020; Zhao et al., 2020). However, the role of cellular proteins, in particular those present in the endoplasmic reticulum (ER), where viral production occurs, remains only partly characterized.
Like other herpesviruses, the replicative cycle of KSHV exists as latency and lytic replication. The transition between these states allows the establishment of a lifelong persistent infection, dissemination to sites of disease, and the spread to new hosts (Li et al., 2018; Ye et al., 2019; Juillard et al., 2020). KSHV mostly persists in the latent state during which it has a restricted latent gene expression program but can be reactivated and transitioned to the lytic state when triggered by stress conditions such as hypoxia or HIV coinfection (Gruffaz et al., 2020), or stimulated by other chemical signals such as 12-O-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate (NaB), and valproate (VPA) (Deng et al., 2007; Bellare and Ganem, 2009; Purushothaman et al., 2015; Gonnella et al., 2017). Furthermore, ER stress can trigger lytic reactivation of KSHV (Johnston et al., 2019). Lytic reactivation in response to ER stress is primarily due to XBP1(s). The ER stress-sensing mechanism involves the presence of XBP1(s) target sequences in the promoters of immediate early viral genes (Johnston et al., 2019). KSHV expresses the immediate early protein replication and transcriptional activator (RTA), which is essential and sufficient to induce lytic replication (Broussard and Damania, 2020). The RTA promoter in KSHV contains at least one XBP1(s) response element with an ACGT core motif.

Latency-associated viral proteins have been well characterized in transformation and tumourigenesis pathways (DiMaio et al., 2020); however, a number of studies have shown that abrogation of KSHV lytic gene expression impairs the oncogenesis of several cancers (Dai et al., 2018; Manners et al., 2018). Furthermore, several latently expressed proteins have been functionally tethered to the angiotensinogen and anti-apoptotic phenotypes of virus-infected cells. As a result, the investigation and therapeutic targeting of KSHV lytic cycles may be essential for the treatment of their associated malignancies (Chen et al., 2019; Gabaev et al., 2020).

The ER is the largest cellular organelle and is involved in many processes, namely, protein production and degradation, cell signaling, and the synthesis and distribution of lipids. Several proteins that shape the ER have been identified, namely, the membrane-bending reticulon (RTN) and receptor expression enhancing protein (REEP) and also atlastins (ATLs) and Lunapark (Chen et al., 2013). ATLs are large dynamin-related GTPases that dimerize in cis and trans to allow fusion of adjacent ER membranes. Humans have three ATLs (ATL1, ATL2, and ATL3), with redundant activities and various levels of expression in different cell types. Mutations in ATLs are associated with neurological diseases, such as hereditary sensory neuropathy and spastic paraplegia, and are characterized by axon and dendrite growth deficits (Fink, 2013). Therefore, ATLs are key cellular factors in regulating ER function, and important factors in human disease.

ATLs enhance ZIKV replication and cytopathic effects (Neufeldt et al., 2019). But the role of ATLs in KSHV is uncharacterized. Here, we report that ATLs enhance KSHV replication and silencing of ATLs impairs KSHV infection. We further characterize the underlying viral and cellular mechanisms and report that ATLs affect KSHV activation by regulating ER stress. Beyond adding KSHV infection to the scope of biological processes regulated by ATLs, our study provides insights about potential therapeutic targets against KSHV infection and KSHV-related malignancies.

**MATERIALS AND METHODS**

**Reagents**

VPA, NaB, TPA, and tetracycline were purchased from Sigma-Aldrich Chemical Co. BFA was dissolved in DMSO at 1 M as a stock solution. VPA and NaB were dissolved in sterile ddH2O at 1 M as a stock solution. TPA was dissolved at 200 μg/ml concentration with sterile ddH2O. Tetracycline was dissolved in DMSO at 1 mg/ml as a stock solution.

**Cell Culture and Chemical Treatment**

iSLK.rKSHV.219 cells and HUVEC cells were cultured in DMEM medium (Gibco), and the body cavity-based KSHV+ lymphoma cell line BCBL-1 was maintained in RPMI1640 medium (Gibco). All these cultures were supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco).

iSLK.rKSHV.219 cells were treated with 1 μg/ml tetracycline plus 1 mM valproate (VPA) (Sigma) for 48 h to activate lytic replication. The BCBL-1 cells were subcultured at 3 × 10^5^ cells/ml and treated with 20 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) plus 0.3 mM NaB (Sigma) for 48 h to activate lytic replication. iSLK.rKSHV.219 cells were treated with 5 μg/ml brefeldin A (BFA) to induce ER stress.

**Cell Transfection**

iSLK.rKSHV.219, HUVEC, and BCBL-1 cells were transfected with Lipofectamine™ LTX (Invitrogen).

Specific siRNA oligonucleotides (GENERAL BIOSYSTEMS) for ATL1/2/3#1 were targeted against the following sequences: siATL1<sup>+</sup>: CAA UAA ACC UGA UGG UAA ATT; siATL2<sup>+</sup>: GGA GCU AUC CUU UUG AAC AUU CAU A; siATL3<sup>+</sup>: GCC CUG ACU UUG AUG GAA AAU UAA A. Specific siRNA oligonucleotides (Invitrogen) for ATL1/2/3#2 were targeted against the following sequences: siATL1<sup>−</sup>: UUU ACC AUC AGG UUU AUU GTT; siATL2<sup>−</sup>: UCC UGG UCU UAA AGU UGC AAC UAA U; siATL3<sup>−</sup>: GGG CUA CAU CAG GUA UGC UGA CAA A. Specific siRNA oligonucleotides (GENERAL BIOSYSTEMS) for CHOP were targeted against the following sequences: CHOP<sup>+</sup>: GCC UAG UAG GCU UAG A; CHOP<sup>−</sup>: GAA CUA GGA AAC GGA AAC A. Specific siRNA oligonucleotides (GENERAL BIOSYSTEMS) for BIP were targeted against the following sequences: BIP<sup>+</sup>: UAG GGU GUG UGU UCA UCC UGA; BIP<sup>−</sup>: GGA GCG CAU UGA UAC A. Specific siRNA oligonucleotides (GENERAL BIOSYSTEMS) for RTN2 were targeted against the following sequences: RTN2<sup>+</sup>: GUU CCA AUU UUG GAA UUG UCC, RTN2<sup>−</sup>: CCG AUA UGG GGA GUA AAG UGG. Specific siRNA oligonucleotides (GENERAL BIOSYSTEMS) for REEP2 were targeted against the
Infection of HUVEC Cells With KSHV
HUVEC cells were plated on 6-well plates at 5 x 10^5 cells/ml. The next day, cells were inoculated for 4 h at 37°C with 1.5 ml of cell culture media derived from iSLK.rKSHV.219 cells that have been treated with tetracycline plus VPA. This was followed by replacing with fresh DMEM media and cells were incubated at 37°C and 5% CO2 for 48 h.

Quantitative Reverse Transcription-PCR (qRT-PCR)
Total RNA was extracted from cells using Trizol (life technologies) according to the manufacturer’s protocol. RNA was converted to cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer’s protocol.

Relative transcript levels of selected cellular and genes were determined with gene-specific primers plus SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa) by 7500 fast real-time PCR system (Applied Biosystems). The sequences of the primers used are shown in Table 1.

Relative expression levels were calculated using the ΔΔCT method after normalization to actin. Individual samples were assayed in triplicates.

Quantitative Analysis of KSHV Virions in Supernatant
Viral DNA was prepared from culture media by using the AxyPrep TM Body Fluid Viral DNA/RNA Miniprep Kit (AXYGEN) according to the manufacturer’s protocol. AxyPrep was used for the preparation of viral DNA. Viral DNA was prepared from culture media by using the AxyPrep TM Body Fluid Viral DNA/RNA Miniprep Kit (AXYGEN) according to the manufacturer’s protocol. Total RNA was extracted from cells using Trizol (life technologies) according to the manufacturer’s protocol. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer’s protocol.

Individual samples were assayed in triplicates.

| Gene      | Forward | Reverse |
|-----------|---------|---------|
| ORF50     | TATCCAGGAAGCGTCTCAT | GGTTAAAGGGGATGAATGCT |
| actin     | GGAGAACTGCGTGAGCAT | GTGAAGGCACCTGCTGACCTT |
| ORF68.1   | TGCTGCACGTTTCAGATGAA | GGCGGCCTAGAGAAATGGA |
| ORF59     | TTGGAGTGAGCGTCTGCCC | TCTTGAGCTCGCTGATAGATC |
| ORF9      | TAGGGCCCTGCTGCTGCTG | CCCAGATTGCGCCTGCTGTA |
| FAM106B   | GACACGTTCGAGAAGACCATC | TGGATGCGCTGCTGAGAAC |
| vLANA     | TCCAAAAGCTGCTGAGAAGCT | ATCAAGCTGACAGACACTG |
| vCyclin   | AAGCCCTCTTTCAAAAACG | TCTGTCAGCTGACACGAGG |
| hxBP1s    | AACCAACAGTTAAGACAGCGTCT | CCTTCTCTGGAGCAGTTGATT |
| BIP       | GGCCGCTGAGTGACGATGGA | TCACAACTGCGCTAAT |
| CHOP      | GGGCATGAAAGGAGAAAACG | CACCGACAGCGCTGGGCTCAAG |
| ATL1      | CAGATCACCGCTGTTTACCTGTA | CTCCCGGACAAACCTTCCTGCT |
| ATL2      | TGGTTTGTCCAGGAAATGAC | ATCAAGTTTGGAGCACAGA |
| ATL3      | AAGACTGTCCTCACCACAAAGTC | CCGTGTCAGGAGGAGAAAC |
| RTN2      | ATGGACATCTGCACCCCTGCTGAC | CGCGTGTCAGGAGGAGAAAC |
| REEP2     | TGGCTCATTTTGCG'CAACCGTGTA |
ATLs Overexpression Promotes VPA Induced KSHV Lytic Reactivation in iSLK.rKSHV.219 Cells

Most of KSHV infected cells are in latent state. Treatment with VPA can induce KSHV to enter the lytic phase, so as to better study the effect of ATL on the lytic reactivation of KSHV. To determine the role of ATLs in VPA induced KSHV lytic replication, we transfected iSLK.rKSHV.219 cells with an empty vector, ATL1, ATL2 or ATL3 respectively. Upon treatment with tetracycline and VPA to induce lytic reactivation, photomicrographs revealed more significant number of RFP + cells in ATLs-transfected cells than empty vector-transfected cells (Figure 2A). Consistently, there were more viral particles in media from ATLs-transfected cells than from empty vector-transfected cells (Figures 2B–D). In addition, we detected the expression of two representative KSHV latency genes vCyclin and vLANA related to KSHV DNA replication in host cells. The results showed that overexpression of ATL proteins could increase the expression levels of vCyclin (Figure S2A) and vLANA (Figure S2B), and knockdown of ATL proteins could inhibit the expression levels of vCyclin (Figure S2C) and vLANA (Figure S2D), which was consistent with the situation of viral DNA in the media.

Furthermore, RT-qPCR showed that, compared to empty vector-transfected iSLK.rKSHV.219 cells, ATLs-transfected cells had significant increases in the mRNA levels of representative KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 and the expression level of RTA detected by western blot was consistent with that of mRNA (Figures 3A–C). These results indicate that ATL overexpression promotes VPA induced KSHV lytic reactivation in iSLK.rKSHV.219 cells.

Silencing of ATLs Impairs Spontaneous KSHV Lytic Reactivation in iSLK.rKSHV.219 Cells

We next used siRNAs to knock down ATLs expression in iSLK.rKSHV.219 cells. The results of RT-PCR showed that compared to control siRNA-treated cells, introduction of 2 different siRNA pairs targeting ATL1/2/3 all resulted in significant decreases in the mRNA levels of representative KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 (Figure 4A). Meanwhile, silencing of ATLs resulted in a decrease in viral particles in the cell media (Figure 4B). The efficiency of ATLs silence was verified by RT-qPCR (Figure 4C) and the expression level of RTA detected by western blot was consistent with that of mRNA (Figure 4D). These results indicate that silencing of ATLs impairs spontaneous KSHV lytic reactivation in iSLK.rKSHV.219 cells.

Silencing of ATLs Impairs VPA Induced KSHV Lytic Reactivation and Virus Particle Production in iSLK.rKSHV.219 Cells

To determine silencing of ATLs whether functions in VPA induced KSHV lytic replication, we transfected iSLK.rKSHV.219 cells with control siRNA or 2 different siRNA pairs targeting ATL1/2/3. Upon treatment with tetracycline and VPA to induce lytic reactivation, photomicrographs revealed less significant number of RFP + cells in siATL1/2/3-transfected cells than siCtrl-transfected cells (Figure 5A). Silencing of ATLs reduced mRNA levels of representative KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 (Figure 5B) and the expression level of RTA detected by western blot was consistent with that of mRNA (Figure 5C). Consistently, there were less viral particles in media from siATL1/2/3-transfected cells than from siCtrl-transfected cells (Figure 5D). Furthermore, we examined the effect of single ATL silencing on spontaneous (Figures S3A, B) or VPA induced lytic reactivation of KSHV (Figures S3C, D). The knockdown efficiency was ensured by RT-PCR (Figures S3E, F). The results showed that knockdown of single ATL protein could also inhibit the lytic reactivation of KSHV, but its inhibitory effect was significantly weaker than that of knockdown of three ATL proteins at the same time. We also rescued ATL1 (Figure S4A), ATL2 (Figure S4B), or ATL3 (Figure S4C) in our ATL1/2/3 knockdown experiments and the rescue efficiency was ensured by RT-PCR (Figures S4E, F). The experimental results show that the replenishment of single ATL protein can restore the lytic reactivation of KSHV to a great extent. These results show that silencing of ATLs impairs VPA induced KSHV lytic reactivation and virus particle...
production in iSLK.rKSHV.219 cells and ATL proteins may have a synergistic effect in regulating the lytic reactivation of KSHV.

**Silencing of ATLs Impairs Spontaneous and TPA Induced KSHV Lytic Reactivation and Virus Particle Production in BCBL-1 Cells**

To determine whether ATLs functions in viral reactivation in other KSHV-infected cells, we introduced siRNAs targeting ATL1/2/3 into BCBL-1 cells to greatly deplete ATLs mRNA levels (Figure 6A). BCBL-1 harbors latently-infected KSHV and is a B cell lymphoma cell line derived from the peritoneal effusion of a patient with primary effusion lymphoma. Silencing of ATLs impairs spontaneous KSHV lytic activation. There were decreased mRNA levels of representative KSHV lytic genes ORFK8.1 and ORF50 (Figure 6B). Viral particles in media were also reduced (Figure 6C) and the expression level of RTA detected by western blot was consistent with that of mRNA (Figure 6D).

Furthermore, the ATLs-silenced BCBL-1 cells were treated with NaB plus TPA induce KSHV lytic reactivation. RT-qPCR revealed that the mRNA levels of the lytic genes ORFK8.1 and ORF50 were significantly decreased in ATLs-silenced cells (Figure 6E). Consistently, there were less viral particles in media from siATL1/2/3-transfected cells than from siCtrl-transfected cells (Figure 6F). The protein expression of RTA was consistent with its mRNA expression (Figure 6G). These results indicate that silencing of ATLs impairs spontaneous and TPA induced KSHV lytic activation and virus particle production in BCBL-1 cells.

**ATL Proteins Have Certain Specificity in the Regulation of KSHV Lytic Reactivation**

To argue for the specific effects of ATLs, we examined the effect of other ER-forming proteins, namely, RTN2 and REEP2, on lytic reactivation of KSHV. After knocking down RTN2 in iSLK.rKSHV.219 cells, the expression level of KSHV lytic genes was not significantly affected (Figure 7A). The efficiency of RTN2 silence was verified by RT-qPCR (Figure 7B). In additional, the results of RT-PCR also showed that knockdown of REEP2 did not significantly affect the lytic reactivation of KSHV (Figure 7C). And the efficiency of ATLs silence was verified by RT-qPCR (Figure 7D).

In summary, ATL proteins have certain specificity in the regulation of KSHV lytic reactivation.

**Silencing of ATLs Impairs KSHV De Novo Infection in HUVEC Cells**

We also speculated that ATLs may regulate de novo KSHV infection. To test this idea, we introduced siRNAs targeting ATLs into HUVEC cells to generate ATLs-silenced cell lines with greatly depleted ATLs. Then, we transfected ATLs-silenced HUVEC cells with empty vector or ATL3. After transfection, we infected these cell lines with rKSHV.219 and found that, compared to control or ATL3-replenishment cells, ATLs silence resulted in a significant decrease in the proportion of...
FIGURE 3 | ATLs overexpression promotes VPA induced expression of KSHV lytic genes in iSLK.rKSHV.219 cells. iSLK.rKSHV.219 cells were transfected with vector, ATL1, ATL2 or ATL3 respectively followed by treatment with tetracycline plus valproate (VPA) for 48 h to induce KSHV lytic reactivation. The effect of ATL1 (A), ATL2 (B), and ATL3 (C) on mRNA levels of KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01). Proteins were detected by western blot with indicated antibodies.

FIGURE 4 | Silencing of ATLs impairs spontaneous KSHV lytic reactivation in iSLK.rKSHV.219 cells. iSLK.rKSHV.219 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#2 respectively. Forty eight hours later, mRNA levels of KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method (A). Viral DNA in the media was quantified using qPCR, with normalization to an added plasmid FAM105B using the ΔΔCT method (B). Knockdown efficiencies of siATL1/2/3 in iSLK.rKSHV.219 cells were measured by RT-qPCR (C). Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01). (D) Proteins were detected by western blot with indicated antibodies.
FIGURE 5 | Silencing of ATLs impairs VPA induced KSHV lytic reactivation and virus particle production in iSLK.rKSHV.219 cells. iSLK.rKSHV.219 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#2 respectively followed by treatment with tetracycline plus valproate (VPA) for 48 h to induce KSHV lytic reactivation. (A) The cells were photographed for GFP and RFP fluorescence. (B) mRNA levels of KSHV lytic genes ORFK8.1, ORF9, ORF50, and ORF59 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (C) Proteins were detected by western blot with indicated antibodies. (D) Viral DNA in the media was quantified using qPCR, with normalization to an added plasmid FAM105B using the ΔΔCT method. Data are presented as means of three technical replicates (n = 3, group values are indicated by mean ± SEM; *p <0.05; **p <0.01).

FIGURE 6 | Silencing of ATLs impairs spontaneous and TPA induced KSHV lytic activation and virus particle production in BCBL-1 cells. BCBL-1 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#2 respectively. After 48 hours, knockdown efficiencies of siATL1/2/3 in BCBL-1 cells were measured by RT-qPCR (A). mRNA levels of KSHV lytic genes ORFK8.1 and ORF50 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method (B). Viral DNA in the media was quantified using qPCR, with normalization to an added plasmid FAM105B using the ΔΔCT method (C). BCBL-1 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#2 respectively followed by treatment with TPA plus NaB for 48 hours to induce KSHV lytic reactivation. (D) Proteins were detected by western blot with indicated antibodies. (E) mRNA levels of KSHV lytic genes ORFK8.1 and ORF50 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (F) Viral DNA in the media was quantified using qPCR, with normalization to an added plasmid FAM105B using the ΔΔCT method. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; **p<0.01). (G) Proteins were detected by western blot with indicated antibodies.
GFP + cells (Figure 8A). Furthermore, this decrease corresponded to the decrease in the mRNA levels of KSHV genes vLANA and vCyclin (Figure 8B). Interestingly, large ER-derived cytoplasmic vacuoles appeared in KSHV infected cells. In ATL-silenced KSHV-infected HUVEC cells, the formation of these vacuoles was strongly decreased at 24 h. Replenishment of ATL3 increased virus-induced vacuoles in ATL-silenced KSHV-infected HUVEC cells (Figure 8C). The efficiency of ATLs silence was verified by RT-qPCR (Figure 8D). These results indicate that silencing of ATL impairs KSHV de novo infection in HUVEC cells.

Silencing of ATLs Impairs the Response of Cells to ER Stress, and ER Stress can Promote the Lytic Reactivation of KSHV

ATLs are large dynamin-related GTPase that dimerize in cis and trans to allow fusion of adjacent ER membranes. ER stress can trigger reactivation of EBV, KSHV, and MHV68. In order to find out whether the lytic activation of KSHV caused by ATLs is related to ER stress, we first detected the effect of ATLs silence on ER stress. The results of RT-PCR showed that mRNA levels of ER stress related genes BIP, CHOP, and hXBP1(s) were decreased in BFA-treated ATLs-silenced iSLK.rKSHV219 cells when compared with control cells (Figure 9A). In other words, silencing of ATLs impairs the response of cells to ER stress. Furthermore, treating iSLK.rKSHV219 cells with VPA or brefeldin A (BFA) can increase mRNA levels of ER stress related genes BIP, CHOP, and hXBP1(s) (Figure 9B). Then we treated iSLK.rKSHV219 cells with DMSO or BFA for 24 h. Photomicrographs revealed more significant number of RFP + cells in BFA-treated cells than DMSO-treated cells (Figure 9C).

Consistently, there were increased mRNA levels of representative KSHV lytic genes ORF8.1, ORF9, ORF50, and ORF59 in BFA-treated cells (Figure 9D).

Knockdown of CHOP or BIP can Antagonize the Promoting Effect of ATL Proteins on the Lytic Reactivation of KSHV to a Great Extent

To further confirm whether ATL proteins had a direct effect on viral replication or an indirect effect by acting on the ER stress response. We examined the effect of CHOP or BIP knockdown on the expression of ATL proteins and the lytic reactivation of KSHV which was promoted by ATLs overexpression. The results showed that knockdown of CHOP did not affect the expression of ATL proteins (Figure 10A). In additional, compared with wild-type cells, overexpression of ATL1 (Figure 10B), ATL2 (Figure 10C), or ATL3 (Figure 10D) in CHOP knockdown cells significantly reduced the promotion of KSHV lytic reactivation.

Similarly, knockdown of BIP could not significantly affect the expression of ATL proteins in cells (Figure 10E). After knocking down BIP in iSLK.rKSHV.219 cells, ATL1 (Figure 10F), ATL2 (Figure 10G), or ATL3 (Figure 10H) was overexpressed, and its promoting effect on the lytic reactivation of KSHV was significantly weakened. That is, CHOP or BIP should be involved in the regulation of ATL proteins on KSHV lytic reactivation and ATL proteins have an indirect effect on KSHV lytic reactivation by acting on the ER stress response. In additional, we detected the effect of ATL1 (Figure S5A), ATL2 (Figure S5B), or ATL3 (Figure S5C) single depletion on BFA induced ER stress. The results showed that knockdown of single ATL protein had no significant effect on BFA induced ER stress.
FIGURE 8 | Silencing of ATL impairs KSHV de novo infection in HUVEC cells. HUVEC cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#1 plus ATL3 respectively followed by infection with rKSHV219 for 24 h. (A) The cells were photographed for GFP fluorescence. (B) mRNA levels of KSHV latent genes vCyclin and vLANA were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (C) Virus-induced vacuoles were observed by light microscopy. (D) Knockdown efficiency of siATL1/2/3 and replenishment efficiency of ATL3 in HUVEC cells were measured by RT-qPCR. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p < 0.05; **p < 0.01).

FIGURE 9 | Silencing of ATLs impairs the response of cells to endoplasmic reticulum (ER) stress, and ER stress can promote the lytic reactivation of KSHV. (A) iSLK.kSHV.219 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#1 respectively followed by treated with BFA for 24 hours. mRNA levels of ER stress related genes BIP, CHOP and hXBP1(s) were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (B) iSLK.kSHV.219 cells were treated with tetracycline plus VPA or BFA for 24 hours, mRNA levels of ER stress related genes BIP, CHOP and hXBP1(s) were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (C) iSLK.kSHV.219 cells were treated with DMSO or BFA for 1 hour followed by treatment with tetracycline plus VPA for 48 hours to induce KSHV lytic reactivation. The cells were photographed for GFP and RFP fluorescence. (D) mRNA levels of KSHV lytic genes ORFK8.1, ORF50 and ORF59 were measured by RT-qPCR. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; **p < 0.01).
Moreover, ER stress had no obvious effect on the expression of ATL proteins (Figures S5D–F). This suggests that ATL proteins may also have a synergistic effect on the regulation of ER stress, which is consistent with the synergistic regulation of KSHV lytic reactivation. This further proves that ATL proteins affect the lytic reactivation of KSHV through the regulation of UPR.

**DISCUSSION**

The ER is the largest cellular organelle which links many cellular organelles and is involved in numerous processes. Several proteins that shape the ER have been identified, including ATLs, RTNs, REEPs, and Lunapark (Chen et al., 2013). Mutations in ATLs have been linked to neurodegenerative diseases, namely, hereditary spastic paraplegia (Fink, 2013). Furthermore, ATLs enhance ZIKV replication and cytopathic effects (Neufeldt et al., 2019). But the role of ATL proteins in KSHV is uncharacterized. In this study, we revealed that ATL overexpression promotes spontaneous and induced KSHV lytic reactivation in KSHV-harboring cells. Opposing this process, silencing of ATL impairs KSHV lytic reactivation. In the process of further exploring the mechanism of ATLs, we found that silencing of ATLs impairs the response of cells to ER stress, while ER stress can promote the lytic reactivation of KSHV. These data indicate that ATLs may be a potential treatment target for aggressive PEL and KSHV infection.

In order to ensure the smooth progress of the experiment, we first detected the expression of ATL proteins in iSLK.rKSHV.219 and BCBL-1 cells, and detected the effect on their mutual expression level (Figure S1). Subsequently, we have demonstrated that spontaneous KSHV lytic reactivation is promoted by ATLs in KSHV-harboring cells; consistently, lytic activation of KSHV is suppressed in ATLs-silence cells (Figures 1, 4). Moreover, because a single ATL protein knockdown cannot inhibit the lytic reactivation of KSHV to a great extent (Figure S3), and supplementing a single ATL protein in ATL proteins knockout cells can significantly restore the lytic reactivation of KSHV (Figure S4), it can be inferred that several ATL proteins should have a synergistic effect on the regulation of KSHV lytic reactivation.

Although in our experiment, we detected that RTN2 and REEP2 had no significant effect on the lytic reactivation of KSHV (Figure 7), which could explain the specificity of ATL proteins on...
the regulation of KSHV to a certain extent. However, according to the existing studies, there are several proteins that shape the ER. So, it will be interesting to determine whether other ER related proteins have effects on KSHV lytic activation. In additional, as ATLs can enhance virus production, our next step will be to explore whether it also affects the infection efficiency of these viruses.

Since KSHV can also be activated by some stimuli such as hypoxia, HIV infection, chemical reagents VPA, NaB, and TPA (Manners et al., 2018), we also tested the effect of ATLs on KSHV lytic reactive induction by VPA or TPA. The results showed that ATLs also enhanced lytic reactive induction of KSHV induced by VPA or TPA (Figures 2–6). These results suggested that ATLs might affect a common event in KSHV reactive or virion assembly processes induced by various stimuli. Moreover, other γ-herpesviruses also have lytic activation events similar to KSHV, it will be interesting to determine whether ATLs can modulate other γ-herpesviruses infection.

In our study, silencing of ATLs impaired KSHV de novo infection and virus-induced vacuoles (Figure 8). Many signaling pathways have been reported to be involved in the regulation of KSHV de novo infection, such as MAPK, NF-κB, antiviral and inflammation signaling pathways (Lee et al., 2016; Uppal et al., 2018; Wei and Lan, 2018; Zhao et al., 2018; Golas et al., 2020). Thus, it will be fascinating to determine whether ATLs have effects on a series of events involving growth and development, inflammation and antiviral.

We determined that silencing of ATLs impairs the response of cells to ER stress, and ER stress can promote the lytic reactive induction of KSHV (Figure 9). Knockdown of CHOP or BIP can antagonize the promoting effect of ATL proteins on the lytic reactive induction of KSHV to a great extent (Figure 9). From this we speculate that ATL proteins may have an indirect effect by acting on the ER stress response. Knockdown of a single ATL protein cannot significantly affect ER stress (Figure S5), so it suggests that ATL proteins may also have a synergistic effect on the regulation of ER stress, which is consistent with the synergistic regulation of KSHV lytic reactive induction. These results further prove that ATL proteins affects the lytic reactive induction of KSHV through the regulation of UPR. It has been reported that excessive ER stress can inhibit the lytic reactive induction of KSHV, while an appropriate amount of ER stress can promote the lytic reactive induction of KSHV (Johnston et al., 2019). ATLs can control the ER stress in KSHV-harboring cells at a level that can just promote the lytic reactive induction of KSHV without causing it to be inhibited.

In conclusion, ATLs overexpression promotes while ATLs silently suppress KSHV replication and reactive induction in KSHV-harboring cells. These finding suggests that ATLs can be expected to be a potential target for the treatment of KSHV or other similar virus infection related tumors and other diseases.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

W-YL and G-HZ conceived and designed the study, analyzed all the data and wrote the manuscript. W-YL performed all the experiments. YW helped with cell culture and some reporter assays. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.790243/full#supplementary-material

**Supplementary Figure 1** | The overexpression of single ATL had no significant effect on the expression of other two ATL proteins. (A) The physiological expression of the ATL1/2/3 proteins in iSLK.KSHV.219 cells and BCBL-1 cells. (B–D) iSLK.KSHV.219 cells were transfected with different amounts of ATL1, ATL2, or ATL3 respectively, 48 hours later, protein levels of ATL1, ATL2, and other ER-forming proteins were measured by western blot with indicated antibody.

**Supplementary Figure 2** | ATL proteins alter viral DNA replication in the cell. (A, B) iSLK.KSHV.219 cells were transfected with vector, ATL1, ATL2 or ATL3 respectively, 48 hours later, mRNA levels of KSHV latency genes vCyclin and vLANA were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (C, D) iSLK.KSHV.219 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#2 respectively, 48 hours later, mRNA levels of KSHV latency genes vCyclin and vLANA were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01).

**Supplementary Figure 3** | The knockdown of single ATL had no significant effect on the expression of KSHV lytic genes. (A, B) iSLK.KSHV.219 cells were transfected with siCtrl, siATL1, siATL2, or siATL3 respectively. 48 hours later, mRNA levels of KSHV lytic genes ORF9 and ORF50 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (C, D) iSLK.KSHV.219 cells were transfected with siCtrl, siATL1, or siATL3 respectively followed by treatment with tetracycline plus valproate (VPA) for 48 hours to induce KSHV lytic reactive induction. mRNA levels of KSHV lytic genes ORF9 and ORF50 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (E) Knockdown efficiencies of siATL1/2/3 in iSLK.KSHV.219 cells were measured by RT-qPCR. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01).

**Supplementary Figure 4** | Rescue of single ATL in ATL1/2/3 knockdown cells could antagonize the inhibitory effect of ATL1/2/3 knockdown on the lytic reactive induction of KSHV. iSLK.KSHV.219 cells were transfected with siCtrl, siATL1/2/3#1 or siATL1/2/3#1#1 siATL1 (A), siATL2 (B), or siATL3 (C) respectively, 48 hours later, mRNA levels of KSHV lytic genes ORF9 and ORF50 were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. (D–F) Knockdown and rescue efficiencies of ATL1/2/3 in iSLK.KSHV.219 cells were also measured by RT-qPCR. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01).

**Supplementary Figure 5** | The knockdown of single ATL had no significant effect on the ER stress. (A–C) iSLK.KSHV.219 cells were transfected with siCtrl, siATL1,
siATL2, or siATL3 respectively followed by treated with BFA for 24 hours. mRNA levels of ER stress related genes BiP and CHOP were measured by RT-qPCR, with normalization to actin using the ΔΔCT method. Data are presented as means of three technical replicates (n=3, group values are indicated by mean ± SEM; *p<0.05; **p<0.01). (D–F) The corresponding protein expression was detected by western blot with the indicated antibody.

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