Epstein-Barr virus tegument protein BGLF2 in exosomes released from virus-producer cells assists *de novo* infection by enhancing viral gene expression

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Abstract

Viruses must adapt to the environment of their host cells to establish infection and persist. Diverse mammalian cells, including virus-infected cells, secrete extracellular vesicles such as exosomes containing proteins and miRNAs. These vesicles mediate intercellular communications, suggesting that they modulate viral infection by adapting cellular conditions. However, the roles of exosomes in viral infection remain unclear. Here we screened viral proteins to identify those responsible for the exosome-mediated upregulation of Epstein-Barr virus (EBV) infection. We found BGLF2 protein encapsulated in exosomes, which enhanced the EBV infection of Akata(-) B-cells. BGLF2 protein is a tegument protein that lines the space between the envelope and the nucleocapsid, and it is released shortly after infection into the cytoplasm. Therefore, tegument protein BGLF2 is encapsulated not only in viral particles, but also in exosomes secreted from infected cells, and plays crucial roles in establishing the EBV latent infection by modulating the cellular environment.

Importance

Tegument proteins that line the space between the envelope and nucleocapsid are released shortly after infection into the cytoplasm to modulate the cellular environment. In this study, we identified that tegument protein BGLF2, which is conserved in all herpesviruses, is incorporated into exosomes and transferred to neighboring cells. Exosomes containing BGLF2 enhanced the infectivity of EBV. These findings suggest that this and perhaps other tegument proteins support viral infection not only between the envelope and nucleocapsid, but also in extra-virus particles such as exosomes.

Introduction

Epstein-Barr virus (EBV), a member of the herpesviruses, mainly infects B cells. EBV infects about 90% of the world's population asymptomatically, but it can cause infectious mononucleosis and some malignancies such as Burkitt lymphoma, T/NK cell lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (1, 2). EBV demonstrates a biphasic life cycle that includes a latent phase and a lytic (productive replication) phase (3). After primary infection, EBV preferentially remains latent in host cells without viral production, although a limited number of viral genes are expressed. Under certain circumstances, latency is disrupted, and the virus shifts into the lytic phase. During this productive replication phase, all >80 viral genes are expressed, the viral genome undergoes replication, and progeny virions are produced (4, 5).

Similar to that of other herpesviruses, the EBV virion is composed of a viral genomic DNA-containing nucleocapsid surrounded by a viral envelope with glycoprotein spikes on its surface (6, 7). Located between the nucleocapsid and the outer viral envelope is the viral tegument. The tegument includes >10 viral proteins called tegument proteins (8) that are involved not only in morphogenesis, envelopment, and egress of progeny virions but also in primary *de novo* infection (9). Because they are released shortly after infection into the cytoplasm, tegument proteins play major roles in infectivity by modulating the intracellular environment, Indeed, we recently showed that the EBV tegument protein BGLF2 increases viral infectivity (10).

Extracellular vesicles such as exosomes are secreted from eukaryotic cells. These vesicles can contain proteins, RNA, microRNA, or DNA, and they serve as crucial mediators of intercellular communication involved in organismal homeostasis and disease states (11-13). The roles of extracellular vesicles have received considerable attention in recent years. Exosomes secreted by virus-infected cells contain viral materials (14). Interestingly, EBV-infected cell lines secrete a higher number of extracellular vesicles than uninfected cells (15), leading us to hypothesize that EBV utilizes exosomes to influence infection and pathogenesis by facilitating viral replication or activating immune evasion

strategies. For instance, the viral oncoprotein latent membrane protein 1 (LMP1) has been detected in exosomes (16-18). LMP1-positive exosomes activate the extracellular signal-regulated kinase and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways in the recipient cells (19). Although EBV encodes more than 80 genes, many of which are lytic proteins, the majority of viral proteins detected in exosomes are latent proteins (20), because these latent proteins have been identified using a forward genetics approach. Therefore, it is still unclear which EBV lytic proteins are encapsulated in exosomes and function during infection.

In this study, we use a reverse genetics approach to investigate the role of EBV protein-containing exosomes during infection. We performed a series of screenings and identified the tegument protein BGLF2, which enhanced *de novo* infection via exosomes. Our results suggest that this tegument protein functions via extracellular vesicle transfer like that of virus particles, ensuring efficient and robust modulation of the microenvironment for the infection.

Results and Discussion

Screening for EBV proteins that modulate infection via exosomal transfer

To explore the roles of exosome containing EBV proteins during infection, we generated an exosome library, from 293T cells expressing 71 different epitope-tagged EBV lytic proteins (10), and screened for EBV genes that both modulated infection and were detected in exosomes (Figure 1A). EBV-negative Akata(-) B-cells treated with each exosome from the library were experimentally infected with EBV, and the infectivity at 48 h post-infection (hpi) was compared (Figure 1B, upper panel). Additionally, the exosome library was subjected to immunoblot analysis with an anti-hemagglutinin (HA) antibody to detect EBV proteins in the exosomes (Figure 1B, lower panel). By integrating multiple screenings, we found BGLF2 and BLRF1 within some exosomes, and these enhanced EBV infection. Because BLRF1 encodes envelope glycoprotein N, which localized to the membrane, we here focus on the tegument protein BGLF2.

Exosome-mediated release of BGLF2 in culture supernatant

Because exosomes were purified by a precipitation-based method in our screening for easy handling, we cannot rule out the possibility of non-exosome contamination (21). To examine the incorporation of BGLF2 in the exosomes, we used an affinity-based method to isolate exosomes from the culture supernatant of 293T cells expressing BGLF2-HA. BGLF2 also was detected in exosomes purified by exosome-affinity resin (Figure 2A) or anti-CD9 antibody (data not shown). We further confirmed that detergent treatment of the culture supernatant impeded the detection of BGLF2 in exosomes, suggesting that BGLF2 was encapsulated within exosomes (Figure 2B).

BGLF2 stimulates the activator protein (AP)-1 signaling pathway (10, 22). To assess the function of BGLF2-containing exosomes, we added them to cells that were transfected with a pAP-1-Luc reporter and control plasmids. As shown in Figure 2C, BGLF2 was taken up by the recipient cells, and treatment with BGLF2-containing exosomes enhanced AP-1 reporter activity (Figure 2D). These findings indicate that functional BGLF2 is transferred via exosomes to recipient cells.

Orthologs of EBV BGLF2 in herpes simplex virus-1 and human cytomegalovirus wereincorporated into exosomes

To define the regions of BGLF2 responsible for packaging into exosomes, we created truncations of BGLF2 and then tested each for its incorporation (Figure 3A). Bortezomib, a proteasome inhibitor, was used for stabilizing deletion mutants. As shown in Figure 3A, the deletion of 98-158 amino acids (aa) precluded the incorporation of BGLF2 into exosomes, although deletion of the C-terminal domain did not. Thus, the incorporation of BGLF2 into exosomes required its central domain.

BGLF2 is a conserved tegument protein among all herpesviruses (22). Therefore, we tested whether herpes simplex virus type 1 (HSV-1) UL16 and human cytomegalovirus (HCMV) UL94 proteins (orthologs of EBV BGLF2) could be incorporated into exosomes. HSV-1 UL16 and HCMV UL94 proteins were detected in exosomes purified from cells expressing HSV-1 UL16 and HCMV

UL94 (Figure 3B). These findings indicate a conserved role of exosome containing BGLF2 or its orthologs among herpesvirus members.

Extra-particle BGLF2 enhances the infectivity of BGLF2-KO EBV

Next, we examined whether exosomal-transferred BGLF2 supports EBV infection using a BGLF2-knockout (BGLF2-KO) virus. The knockout of BGLF2 reduces EBV infectivity upon *de novo* infection of B-cells (10). Treatment with exosomes containing BGLF2 significantly enhanced the infectivity of BGLF2-KO EBV compared with a control treatment (Figure 4A).

To evaluate the effect of BGLF2-containing exosomes on EBV infection, we performed RNA-seq analysis (Figure 4B). Akata(-) cells were pre-treated with BGLF2-containing exosomes or control exosomes and then infected with BGLF2-KO EBV. Infected cells were collected by fluorescence-activated cell sorting (FACS) at 24 hpi according to EGFP-positivity, and transcriptome information of the infected cells was subsequently obtained by RNA-seq. As shown in Figure 4C and 4D, treatment with BGLF2-containing exosomes induced expression of EBV genes, although the expression level of EGFP, which was driven from a eukaryotic promoter (23), was not altered. This enhanced expression of EBV mRNAs was validated by qPCR (Figure 4E). LMP1 was one of the most differently expressed viral genes between the groups treated with exosomes with or without BGLF2 (Figure 4C). The EBV gene BHRF1 is highly expressed during the first two days after infection (24, 25). These findings imply a role for BGLF2 in viral gene expression in infected cells, consistent with previous reports that BGLF2 activates p38, JNK, and AP-1 to induce viral gene expression (10, 22).

Additionally, gene set enrichment analysis (GSEA) of RNA-seq data indicated that the inflammation response was significantly suppressed by treatment with BGLF2-containing exosomes (Figure 4F). This suppression is consistent with recent reports that BGLF2 protein impairs type I interferon (IFN) signaling through Tyk2 (26) and also dampens NF-κB activity (27), although these reports have focused on its roles during lytic infection. Because host innate immunity and its key mediator, type I IFN, control virus infection (28-30),

BGLF2 may protect infected cells from a type I IFN-mediated response during *de novo* infection. Indeed, we confirmed that type I IFN inhibited EBV infection in a dose-dependent manner (Figure 4G). Together, BGLF2 perturbed host signaling pathways on multiple levels to modulate the cellular environment to be suitable for establishing infection. Thus, we demonstrated that supplementation of BGLF2 via exosomes to infected cells contributes to EBV infection, similar to supplement via virus particles.

Virally infected cells released virions as infectious particles and non-infectious particles. From all herpesvirus-infected cells, L-particles are released in addition to virions (31-33). Because L-particles are composed of virus envelope and tegument proteins but lack viral genome and nucleocapsid proteins, these particles are non-infectious. Such conserved release of L-particles may shed light on an essential role of non-infectious particles in creating a microenvironment that facilitates their replication, spread, and persistence in the host cells. Recently, it has been demonstrated that L-particles mediate immune evasion by CD83 down-modulation on mature dendritic cells (34). Here, we demonstrated that tegument protein BGLF2 in exosomes was transferred to the target cells and then enhanced the infectivity of EBV by manipulating the cellular environment for viral gene expression. These findings suggest that this virus hijacks and utilizes extracellular vesicles such as exosomes.

Exosomes prepared from BGLF2-expressing 293T cells enhanced infectivity with the BGLF2-KO virus (Figure 4A), suggesting that exosomes serve as a cargo of tegument proteins to target cells. Although exosomes are released in exocytic bursts upon the fusion of the multivesicular bodies to the cell membrane (35), the final envelopment site for EBV originates from the Golgi apparatus (36), indicating that these non-infectious particles (L-particles and exosomes) are generated in different compartments. Furthermore, BGLF2 and its orthologs in HSV-1 and HCMV were incorporated into exosomes (Figure 3C), indicating that manipulation of the cellular environment by exosomes was a conserved mechanism among herpesvirus. The results of this study also suggest a

previously unforeseen essential role for non-infectious particles in viral protection.

Materials and Methods

Cells, plasmids and reagents

HEK293T (ATCC CRL-3216) and HEK293/EBV(dBGLF2) cells (10) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Akata(−) cells (37) were cultured in RPMI1640 medium containing 10% FBS. AGS/EBV-EGFP cells (a kind gift from Hironori Yoshiyama) (38) were grown in F-12 HAM's medium supplemented with 10% FBS and 400 μg/mL G418.

The BZLF1-expression plasmid, and HA-tagged EBV lytic protein-expression library were described previously (10, 39). For expression of C-terminal HA-tagged HSV-1 UL16 and HCMV UL94, constructs expressing UL16 and UL94 in pcDNA3 (Thermo Fisher Scientific, Waltham, MA, USA) were prepared by PCR and the In-Fusion cloning system (Takara Bio, Kusatsu, Shiga, Japan). The inserted DNA sequence of each vector was confirmed by direct DNA sequencing. The reporter plasmid pAP1-Luc and promoterless-RLuc (pGL4.70) were purchased from Promega (Madison, WI, USA).

Bortezomib (Cat# A10160; AdooQ BioScience, Irvine, CA, USA) was used at a concentration of 100 nM. Recombinant human type I IFN alpha (Cat# 11200-1) was purchased from PBL Assay Science (Piscataway, NJ, USA).

Antibodies and immunoblotting

Anti-HA (3F10) rat antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD63 (Ts63) mouse antibodies were obtained from Thermo Fisher Scientific. Rabbit anti-GAPDH (14C10), rabbit anti-CD9 (D8O1A), and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Immunoblot and signal detection were performed as described previously (39).

Virus preparation

EGFP-EBV was obtained from the 8-day-old cell-free supernatant of AGS/EGFP-EBV cells. The cell-free supernatant was filtered through 0.45 μm filters and then used as a virus stock. For the preparation of the BGLF2-KO virus, HEK293/EBV(dBGLF2) cells (10) were transfected with BZLF1 expression plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells and media were harvested and freeze-thawed, and cell debris was removed. The supernatant after centrifugation was filtered through 0.45 μm filters and then used as a virus stock. EBV-negative Akata(-) cells were infected with the virus, and EGFP-positive cells were counted by FACS to measure the viral titer.

Exosome purification

Exosome-containing conditioned media were centrifuged at 2000 × g to remove cells. Exosomes were purified with a Total Exosome Isolation Kit (Thermo Fisher Scientific) for screenings or exoEasy Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

Screenings

HEK293T cells were transfected with each plasmid expressing an EBV gene using Lipofectamine 2000 (Thermo Fisher Scientific). On the following day, media were changed to DMEM supplemented with 5% exosome-depleted FBS (System Biosciences, Palo Alto, CA, USA). After 48 h, exosomes were purified from the conditioned media. To identify exosomal proteins, we analyzed purified exosomes by immunoblot. For analyzing modulators of infectivity, EBV-negative Akata(-) cells were treated with purified exosomes in RPMI1640 supplemented with 5% exosome-depleted FBS (System Biosciences, Palo Alto, CA, USA). After 6 h, cells were infected with EGFP-EBV. At 48 h after infection, cells were fixed with 4% paraformaldehyde. The infectivity was determined using a flow cytometer (FACS Canto, BD Biosciences, San Jose, CA, USA).

Luciferase reporter assay

HEK293T cells were transfected with AP-1 reporter plasmid (pAP1-Luc) and promoterless-RLuc as an internal control using Lipofectamine 2000 (Thermo Fisher Scientific) and incubated in DMEM supplemented with 5% exosome-depleted FBS. After 48 h, cells were treated with purified exosome. Cells were lysed 24 h later and subjected to luciferase assays using the Dual-Luciferase Reporter Assay System (Promega).

RNA-seq

Akata(-) cells were pre-treated with BGLF2-containing or control exosome for 16 h, and then infected with BGLF2-KO EBV at room temperature for 2 h with agitation. After infection, cells were resuspended in medium containing exosomes. The top 30,000 EBV-infected cells expressing EGFP were sorted by a FACS Aria II Cell Sorter (BD Biosciences) at 24 hpi.

Total RNA was extracted using the RNeasy Mini kit (Qiagen). Evaluation of RNA, RNA-seq library preparation, Illumina sequencing, and data preprocessing were performed as described previously (40).

qRT-PCR

Total RNA was reverse-transcribed to cDNA using the PrimeScript II Reverse transcriptase kit (Takara Bio). The expression of mRNA was analyzed by qPCR using the 7500 Fast DX Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as previously described (18). The primer sequences used in this study are listed in Table 1.

Statistical analysis

Results are shown as the means ± standard error (SE) of at least three independent experiments. Statistical analyses were performed using Microsoft Excel. Welch's t-test was used to determine significance, and $P < 0.05$ was considered to be statistically significant.

Data availability

Data deposition of RNA-seq data set is progress. The RNA-seq dataset reported in this paper is available upon request.

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Conflict of interests

The authors declare no competing interests.

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Figure Legends

Figure 1: Combined screenings for the viral proteins that regulate the infectivity via exosomal transfer.

(A) The workflow of the screenings. Exosomes were purified from the conditioned media of HEK293T cells that were transfected with each plasmid expressing an EBV gene. For analyzing modulators of infectivity, EBV-negative Akata(-) cells were pre-treated with purified exosomes and then infected with EGFP-EBV. The infectivity was determined at 48 hpi by FACS analysis. For identifying exosomal proteins, purified exosomes were analyzed by immunoblot with an anti-HA antibody.

(B) Heatmap showing fold change (FC) of infectivity of EBV with Akata(-) cells in the presence of each exosome at 48 hpi. Infectivity changes (top panel) reflect the $\log^2$ FC relative to the control exosome purified from empty vector-transfected cells. p values are shown on the middle panel. The viral protein in purified exosomes is indicated as black on the bottom panel.

Figure 2: Functional BGLF2 proteins are transferred to the recipient cells via exosomes.

(A) BGLF2 protein-containing exosomes were secreted from BGLF2-expressing cells. HEK293T cells were transfected with HA-tagged BGLF2-expression or empty plasmid. Exosomes were isolated from the supernatant of the cells. CD63 and CD9 served as exosome markers.

(B) BGLF2 protein was incorporated into exosomes. Exosomes were purified from supernatants treated with or without 0.25% Triton-X 100 for 5 min at room temperature.

(C) BGLF2 was delivered to the recipient cells via exosomes. HEK293T cells were treated with BGLF2-containing exosomes for the indicated number of hours and then washed extensively and harvested. Cell lysates were applied to immunoblot analysis.

(D) BGLF2 transferred via exosomes activated the AP-1 promoter. HEK293T cells transfected with the pAP-1-Luc and promoterless-Rluc plasmids, and then

treatment with BGLF2-containing exosomes. Cell lysates were obtained at 24 h post-treatment and analyzed using luciferase reporter assays. Values (mean ± SEs) were calculated from three independent experiments. Double asterisks, $p<0.01$.

Figure 3: The orthologs of BGLF2 are incorporated into exosomes

(A) The C-terminal domain of BGLF2 was dispensable for incorporation into exosomes. HEK293 cells were transfected with a deletion mutant of BGLF2-expression plasmids and treated with or without 250 nM of Bortezomib for 24 h before harvest. Exosomes were isolated from the culture supernatant.

(B) HEK293T cells were transfected with expression plasmids containing HA-tagged BGLF2 or its HSV-1 or HCMV orthologs. Exosomes were isolated from the supernatant of the cells.

Figure 4: BGLF2-containing exosomes enhance the infectivity of BGLF2-KO EBV.

(A) Akata(-) cells were infected with BGLF2-KO EBV in the presence of BGLF2-containing exosomes. After two days, GFP positivity was determined by FACS. Results are presented as means ± SE of at least three independent experiments and are shown as relative infectivity to control treatment with PBS (infectivity value of 1). Double asterisks, $p<0.01$; n.s., not significant.

(B) The workflow of sample collection for RNA-seq/qRT-PCR analyses. Akata(-) cells were pre-treated with exosomes for 16 h, and then infected with BGLF2-KO EBV. The infected cells were collected via FACS at 24 hpi. Total RNA extracted from the sorted cells was subjected to RNA-seq and qRT-PCR.

(C) Gene expression levels were normalized as counts per million followed by $\log_2$-transformation with a pseudo-count of 1. Each bar indicates the $\log_2$ fold change of a viral gene expression between treatment with exosomes with or without BGLF2. Viral gene expression kinetics are categorized into five groups: latent, immediate early, early, leaky late, and late (41).

(D) Read counts of EBV genes in BGLF2-KO EBV-infected cells that were treated with BGLF2-containing or control exosomes by RNA-seq.

(E) Validation of enhanced EBV gene expression by qPCR. Data are presented as means ± SE. Samples were tested in duplicate. Asterisk, $p<0.05$; double asterisks, $p<0.01$; n.s., not significant.

(F) GSEA of RNA-seq data from BGLF2-KO EBV-positive cells treated with BGLF2-containing or control exosomes. NES; normalized enrichment score, FDR; false discovery rate.

(G) Akata(-) cells were infected with EBV-EGFP in the presence of type I IFN (100 or 500 Unit/mL). After two days, GFP positivity was determined by FACS. Results are presented as means ± SE of three independent experiments. Double asterisks, $p<0.01$; n.s., not significant.

Table 1: Oligonucleotide primers used for qRT-PCR

Primer name	Sequence (5' to 3')
BHRF1 forward	AAATGGTACCCTGCATCCTG
BHRF1 reverse	CCACATGTTCGGTGTGTGTT
LMP1 forward	CTGATGATCACCCTCCTGCT
LMP1 reverse	CTAAGACAAGTAAGCACCCGAAG
EGFP forward	ACGTAAACGGCCACAAGTTC
EGFP reverse	AAGTCGTGCTGCTTCATGTG
GAPDH forward	CCTCCAAGGAGTAAGACCCC
GAPDH reverse	TGTGAGGAGGGGAGATTCAG

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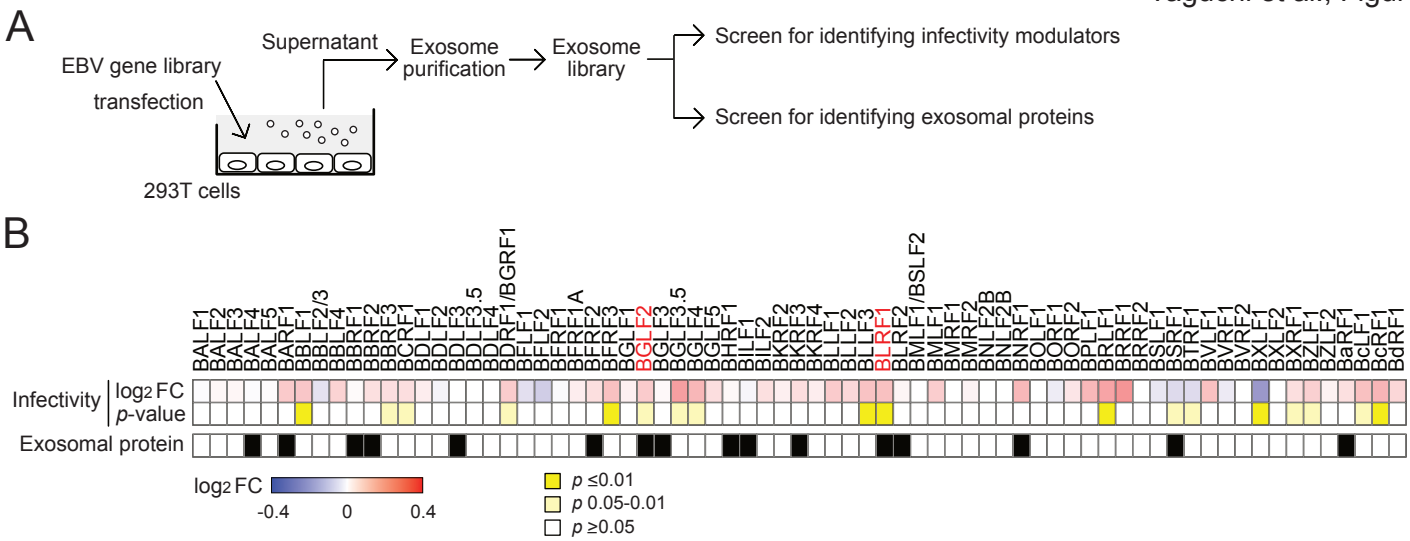
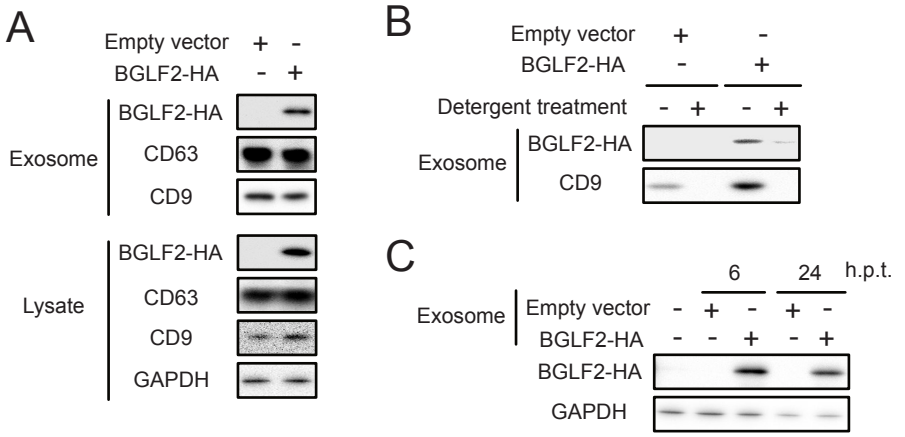
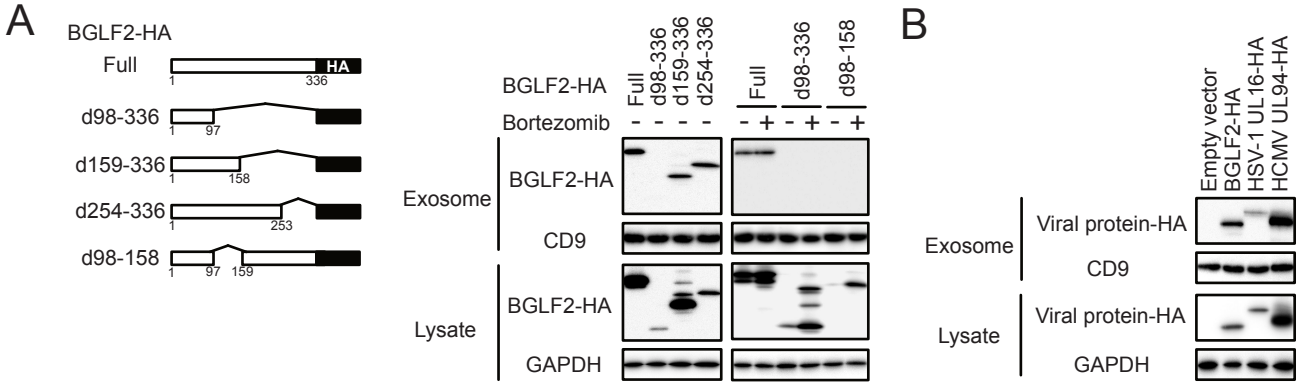
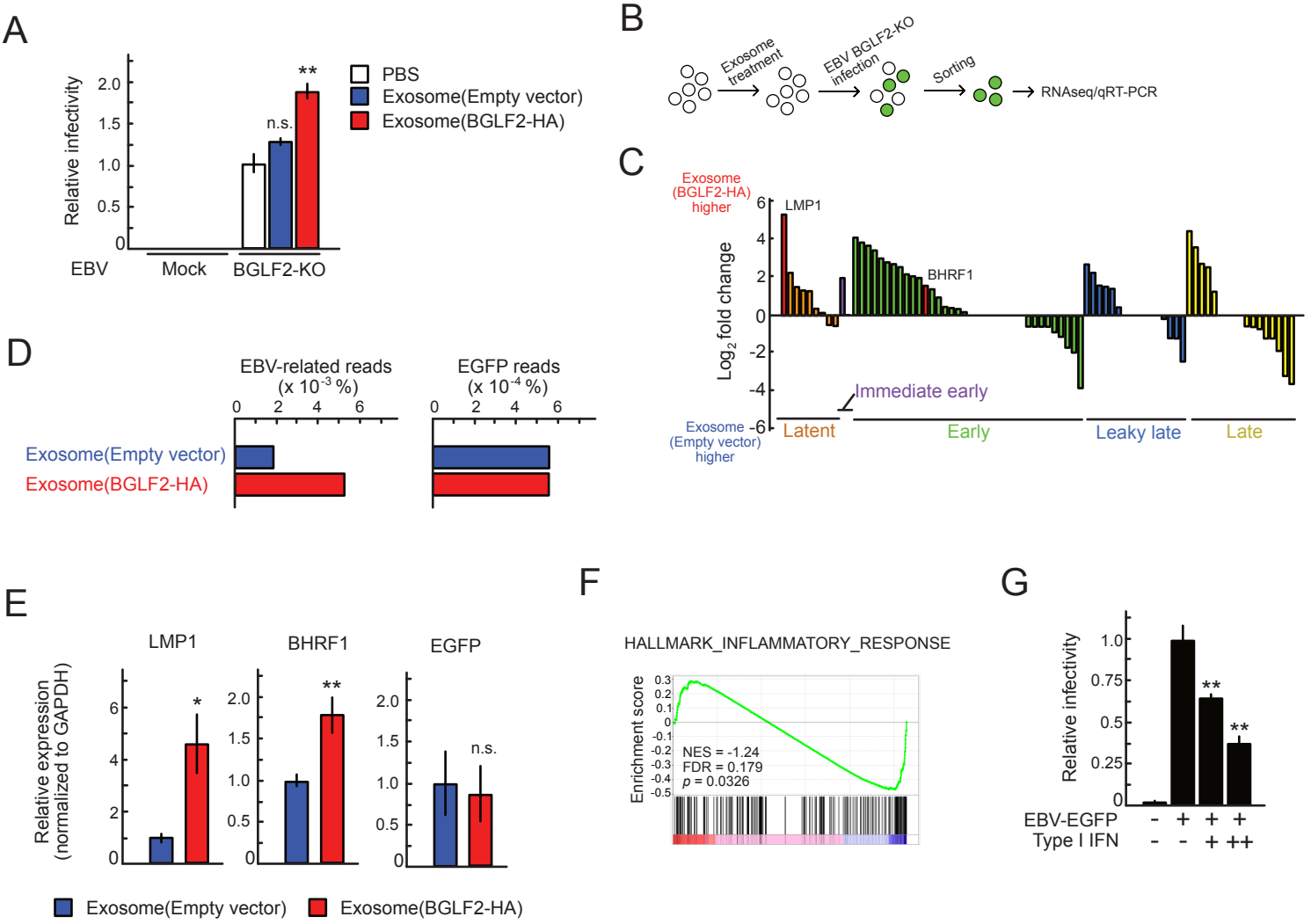



A
Empty vector + -
BGLF2-HA - +
Exosome
BGLF2-HA
CD63
CD9
Lysate
BGLF2-HA
CD63
CD9
GAPDH
B
Empty vector + -
BGLF2-HA - +
Detergent treatment - + - +
Exosome
BGLF2-HA
CD9
C
6 24 h.p.t.
Exosome
Empty vector - + - + -
BGLF2-HA - - + - +
BGLF2-HA
GAPDH
D
**
Fold activation
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Exosome
Empty vector
BGLF2-HA


A
BGLF2-HA
Full
HA
1
336
d98-336
1
97
d159-336
1
158
d254-336
1
253
d98-158
1
97
159
BGLF2-HA
Bortezomib
Full
d98-336
d159-336
d254-336
Full
d98-336
d98-158
- - - - - + - + - +
Exosome
BGLF2-HA
CD9
Lysate
BGLF2-HA
GAPDH
B
Empty vector
BGLF2-HA
HSV-1 UL16-HA
HCMV UL94-HA
Exosome
Viral protein-HA
CD9
Lysate
Viral protein-HA
GAPDH


A
Relative infectivity
2.0
1.5
1.0
0.5
0
**
n.s.
PBS
Exosome(Empty vector)
Exosome(BGLF2-HA)
EBV
Mock
BGLF2-KO
B
Exosome treatment
EBV BGLF2-KO infection
Sorting
RNAseq/qRT-PCR
C
Exosome (BGLF2-HA) higher
Exosome (Empty vector) higher
Log2 fold change
LMP1
BHRF1
Immediate early
Latent
Early
Leaky late
Late
D
EBV-related reads (x 10-3 %)
EGFP reads (x 10-4 %)
Exosome(Empty vector)
Exosome(BGLF2-HA)
E
LMP1
BHRF1
EGFP
Relative expression (normalized to GAPDH)
*
**
n.s.
Exosome(Empty vector)
Exosome(BGLF2-HA)
F
HALLMARK_INFLAMMATORY_RESPONSE
Enrichment score
NES = -1.24
FDR = 0.179
p = 0.0326
G
Relative infectivity
**
**
EBV-EGFP - + + +
Type I IFN - - + ++