Epstein-Barr Virus miR-BART6-3p Inhibits the RIG-I Pathway

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Keywords

Epstein-Barr virus · RIG-I · miR-BART6-3p · Interferon · Innate immunity

Abstract

Recognition of viral pathogen-associated molecular patterns by pattern recognition receptors (PRRs) is the first step in the initiation of a host innate immune response. As a PRR, RIG-I detects either viral RNA or replication transcripts. Avoiding RIG-I recognition is a strategy employed by viruses for immune evasion. Epstein-Barr virus (EBV) infects the majority of the human population worldwide. During the latent infection period there are only a few EBV proteins expressed, whereas EBV-encoded microRNAs, such as BART microRNAs, are highly expressed. BART microRNAs regulate both EBV and the host's gene expression, modulating virus proliferation and the immune response. Here, through gene expression profiling, we found that EBV miR-BART6-3ps inhibited genes of RIG-I-like receptor signaling and the type I interferon (IFN) response. We demonstrated that miR-BART6-3p rather than other BARTs specifically suppressed RIG-I-like receptor signaling-mediated IFN- β production. RNA-seq was

used to analyze the global transcriptome change upon EBV infection and miR-BART6-3p mimics transfection, which revealed that EBV infection-triggered immune response signaling can be repressed by miR-BART6-3p overexpression. Furthermore, miR-BART6-3p inhibited the EBV-triggered IFN- β response and facilitated EBV infection through targeting the 3'UTR of RIG-I mRNA. These findings provide new insights into the mechanism underlying the strategies employed by EBV to evade immune surveillance.

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Introduction

Epstein-Barr virus (EBV) was first isolated from Burkitt lymphoma tissues of African children by Epstein et al. [1] in the 1960s. It belongs to the γ -subfamily of the herpes virus and possesses a high infection rate. Approximately 95% of the world's population sustains an asymptomatic life-long EBV infection [2]; however, the seemingly harmless infection status cannot conceal its potential pathogenicity. High titers of EBV antibodies are detected in the patients of various diseases, such as Burkitt

lymphoma, Hodgkin lymphoma, lymphoproliferative diseases, nasopharyngeal carcinoma (NPC), gastric cancer, and infectious mononucleosis [3–11]. EBV became the first confirmed tumor virus by the International Union against Cancer. Most EBV-associated patients are under either immune suppression or immunodeficiency conditions, which suggests that inhibition of the host's immune response is crucial for the pathogenesis of EBV-associated diseases.

EBV possesses an approximately 184-kb-long linear double-stranded DNA genome with more than 80 open reading frames, and can express more than 85 genes [12]. Transcription of its genome varies from different states of infection [13]. EBV has developed multiple strategies to evade the host's antiviral response. During latent infection it expresses only a few genes (such as *EBNA1* and *LMP1/2*), while noncoding RNAs are highly expressed, including *EBERs* and BART microRNAs [14].

Recent studies demonstrated that BART microRNAs are very abundant in NPC [15], which is in latency II form. BART microRNAs can regulate both the EBV gene and host gene expression, playing an important role in NPC tumorigenesis. Several BART microRNAs have been involved in the host immune response and became a strategy employed by EBV for immune evasion during EBV infection [16]. For instance, BART20-5p, BART2-5p, and BART3 are involved in immune evasion by targeting TBX21/T-bet, MICB, and IPO7, respectively [17–19]. However, the roles of BART microRNAs in regulating host immune responses are still unclear. In the current study, we chose BART6-3p, which is highly abundant in NPC tissues [15], to explore its pathophysiology function. We found that the expression of BART6-3p mimics in peripheral blood mononuclear cells (PBMCs) modulated the expression levels of genes of the RIG-I-like receptor signaling and type I interferon (IFN) response according to the gene expression profiling analysis.

The RIG-I-like receptor family (including RIG-I, MDA5, and LGP2) is an important member of pattern recognition receptors (PRRs), recognizing viral RNAs in the cytoplasm. RIG-I preferably recognizes short (approx. 300–1,000 bp) double-stranded RNAs with triphosphated motif at the 5' end [20] and can be activated by EBV-encoded *EBERs* [21]. Upon binding with specific RNA ligands, RIG-I performs a conformational change and transduces signals downstream through binding to the adaptor MAVS/IPS-1/CARDIF/VISA, leading to the translocation of the transcription factors NF- κ B and IRF3/IRF7 from the cytoplasm to the nucleus and induction of type I IFN. Interestingly, RIG-I is also one of the

IFN-stimulated genes (ISGs) and participates in amplifying the type I IFN response. Through inducing downstream ISG transcription, IFNs exert multiple functions, such as antiviral, antitumor, antiproliferation, and immune-regulation functions [22, 23], constituting the first defensive line of the human innate immunity.

In this study, we revealed that EBV BART6-3p inhibited the RIG-I-like receptor signaling and type I IFN response, thus facilitating EBV infection. Our findings will stimulate interest in focusing on correlations between EBV-encoded microRNAs and the host innate immune system.

Materials and Methods

Cell Culture and Treatment

HK-1 cells (EBV-negative NPC cells), C666-1 (EBV-positive NPC cells), BJAB (EBV-negative human B lymphoma cells), and B95.8 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum. The cells were transfected with RNAs and/or plasmids using Lipofectamine 3000 (Invitrogen).

EBV Virus Preparation, Infection, and Copy Number Detection

Purified EBV virions were obtained from the productive EBV B-cell lineage B95.8, as described in the literature [24]. The genome of EBV harvested from B85.8 cells does not have BART6 and a few of the other BART genes [14]. Viral titers were evaluated as described and expressed as transforming units per milliliter [24]. Virus preparations were assayed for EBV genome content by qPCR amplification for the BamHI-W fragment [25]. A total of 5×10^5 BJAB cells were seeded in 12-well plates and 50- μ L virion suspensions were added to each well for 2 h. To count the EBV copy number of the cells, the general DNA of the cells was extracted using a QIAmp DNA kit (Qiagen) and quantified. The EBV copy number was then detected using an EBV DNA Quantitative Fluorescence Diagnostic kit (Sansure Biotech, China) according to the manufacturer's instructions and as described previously [26].

Reagents

The following antibodies were commercially obtained: RIG-I pathway antibodies kit, cJun, phos-TBK1(Ser173), TBK1, phos-STAT1 (Tyr701), phos-IRF3(Ser396), and GAPDH (Cell Signaling Technology). Histone H3 came from Proteintech. EBV BARTs mimics, inhibitors, and control mimics were purchased from GenePharma (China). BART miRNA inhibitors are sequence specific and chemically modified and optimized nucleic acids designed to specifically target the microRNA molecules in cells. The cells were transfected with 100 pmol of BARTs mimics/inhibitors. 5'ppp-dsRNA was obtained from Invivogen (No. 15D10-MM) and IFN- α was obtained from R&D Systems.

Plasmids and Dual-Luciferase Reporter Assay

The 3'UTR of the *DDX58* gene (encoding RIG-I) was amplified and subcloned into the pMIR-Report luciferase vector (Ambion) to assay the effect of BART6-3p on firefly luciferase activity. The

ISRE luciferase reporter plasmid (purchased from Beyotime, China) is designed to monitor the activity of type I IFN-induced signal transduction pathways. Cells were seeded in 48-well plates and co-transfected with 200 ng of the luciferase reporter plasmid along with 20 pmol of BARTs mimics or negative control (NC) mimics. After 24 h of culture, firefly and *Renilla* luciferase activities were measured via a dual-luciferase reporter assay system (Promega). The data were expressed as the relative firefly luciferase activity normalized to the value of *Renilla* luciferase and were representative of 3 independent experiments.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and 2 µg of RNA was used to synthesize cDNA using a first-strand cDNA synthesis kit (Promega). qPCR was performed as described previously [27]. The primers are listed in online supplementary Table 1 (see www.karger.com/doi/10.1159/000479749 for all online suppl. material).

Western Blotting

Western blotting was carried out as described previously [27]. Cytoplasmic and nuclear fractions were separated using a nuclear and cytoplasmic protein extraction kit (Beyotime).

cDNA Microarray Analysis

A total of 1×10^7 PBMCs from a health donor were transfected with either NC mimics or BART6-3p mimics (150 µM) for 36 h. Total RNAs were extracted and detected by Affymetrix Human Genome U133 Plus 2.0 Array. The microarray analysis service was provided by CapitalBio (Beijing, China), as previously described [28]. These data have been deposited in the NCBI Gene Expression Omnibus (No. GSE94312). The differentially expressed genes between the NC and BART6-3p mimics were analyzed by GeneMANIA (<http://genemania.org>) [29] for functional analysis of the canonical pathways associated with the genes of interest.

RNA Sequencing

BJAB cells were transfected with NC (or BART6-3p) mimics for 48 h, and then infected with EBV virions for 2 h. RNAs were extracted by mirVana miRNA isolation kit (No. 1561, Ambion) and sequenced by the Solexa high-throughput sequencing service (Oebiotech, Shanghai, China). Data were extracted and normalized according to the manufacturer's standard protocol. The RNA-seq raw expression files and details have been deposited in NCBI GEO under accession No. GSE95634. Log-fold changes of up- or downregulated mRNAs between the NC and BART6-3p mimics were selected with a significance threshold of $p < 0.05$. The biological process pathway annotation was classified by DAVID bioinformatics resources (<http://david.abcc.ncifcrf.gov/>) [30].

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) [31] was used to compare the gene set differences between the 2 groups of primary gastric adenocarcinomas specimens (i.e., EBV positive and EBV negative) [32]. The RNA-seq data were obtained from the TCGA database of the gastric cancer genome atlas project. There were 26 EBV-positive and 269 EBV-negative gastric adenocarcinomas specimens in this cohort [32].

Statistical Analysis

Statistical analysis was determined by independent *t* test or ANOVA using SPSS17.0 and GraphPad Prism. Significance parameters were set at $p < 0.05$.

Results

BART6-3p Downregulates Genes of RIG-I-Like Receptor Signaling and IFN Response

To investigate the potential role of BART6-3p in regulating the host biological process, we transfected BART6-3p mimics into the human PBMCs and then analyzed the gene expression profile change of the cells (Fig. 1a). PBMCs are natural host cells of EBV. The cDNA microarray data (No. GSE94312) of PBMCs showed that 39 cellular genes were significantly upregulated (ratio >2.0) and 49 genes were downregulated (ratio <0.5) by BART6-3p overexpression compared to the NC group. The differentially expressed genes induced by BART6-3p are listed in online supplementary Table 2. We analyzed these downregulated cellular genes by means of the public database GeneMANIA, and found that they mainly cover the functions of response to virus and type I IFN signaling (Fig. 1b–d). In analysis of the gene-related pathway network, we found DDX58 and HERC5 strongly linked to each other, and both linked to ISG15 (Fig. 1e). DDX58 encodes the RIG-I protein, which belongs to the PRR family. HERC5 is a member of the HERC family of ubiquitin ligases, and functions as an IFN-induced E3 protein ligase. Both participate in the RIG-I/MDA-mediated induction of the IFN- α/β pathway. It is suggested that BART6-3p might suppress the RIG-I-like receptor signaling-mediated induction of type I IFN, thus influencing the host's immune response to EBV infection. There are limited reports on EBV infection-induced RIG-I signaling [21, 33–35].

EBV infection has been linked to carcinogenesis, such as lymphoma, NPC, gastric cancer, etc., and multiple mechanisms have been investigated. The Cancer Genome Atlas Research Network [32] sequenced a large cohort of gastric cancer specimens and found that about 9% of gastric cancer specimens are EBV positive. We further extended this finding by analyzing the gene expression data of those gastric cancer specimens of different entities, i.e., EBV-positive specimens versus EBV-negative specimens [32]. GSEA revealed gene sets strongly enriched in EBV-positive gastric cancer specimens compared with EBV-negative specimens. A few of the significant gene sets are RIG-I signaling and IFN- α/γ responses (online suppl.

Fig. 1. Thirty-seven genes were significantly downregulated (ratio <0.5) upon BART6-3p overexpression compared to the NC group in PBMCs. We analyzed these genes through the public database GeneMANIA. **a** PBMCs from a health donor were transfected with BART6-3p or NC mimics (150 μ M) for 36 h. The RNA level of BART6-3p was analyzed by qPCR. **b** Functional enrichment analysis of the downregulated genes. The coverage ratios are for the

number of annotated genes in the downregulated gene list versus the number of genes with that annotation in the human genome. **c, d** Function categories of the downregulated genes. Different genes have the same functions (**c**) and 1 gene may have different functions (**d**). **e** Pathway network categories of the downregulated genes. Two gene products are linked if they participate in the same reaction within a pathway.

Fig. 2. BART6-3p suppresses RIG-I and IFN- β expression. **a** HK-1 cells were transfected with NC mimics or 5'ppp-dsRNA (in different concentrations) and cultured for 24 h. The protein levels of RIG-I were detected by Western blotting. **b** HK-1 cells were transfected with BART6-3p or NC mimics (final concentration 100 pmol) for 48 h. The RNA level of *BART6-3p* was analyzed by qPCR. **c** HK-1 cells were transfected with BART6-3p or NC mimics (final concentration 100 pmol) and cellular RNAs were harvested at the indicated time points. The mRNA level of RIG-I was analyzed by qPCR. The gene expression level at 0 h was set as 1. **d** Group 1, HK-1 cells were transfected with either NC mimics or BART6-3p mimics (6-3p) for 48 h; group 2, cells were transfected with either

NC mimics or BART6-3p mimics (6-3p) for 24 h initially, and then transfected with 0.75 μ g/mL 5'ppp-dsRNA for another 24 h; group 3, cells were transfected with NC mimics or BART6-3p mimics (6-3p) for 44 h initially, and then 500 U/mL of IFN- α for stimulation was added for another 4 h. The 3 groups of cells were harvested at the same time point and the expression levels of the indicated proteins were detected by Western blotting. **e, f** The treatment of HK-1 was similar as described in **d**. **e** Cytoplasmic and nuclear fractions were isolated and the expression levels of indicated proteins were assayed by Western blotting. **f** RNAs were extracted and detected by qPCR. Data are shown as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ compared with NC.

Fig. 1). The complicated nature of tumor tissues made this result hard to explain by only 1 single factor, such as EBV infection; however, the GSEA result suggests the possibility that the RIG-I signaling and IFN response are hyperactivated in the EBV-positive cancer specimens compared with EBV-negative specimens.

BART6-3p Is a Negative Regulator of the RIG-I-IFN- β Signaling

RIG-I is a cytosolic pattern recognition receptor that especially senses short blunt double-stranded RNAs with uncapped 5'triphosphate moiety and becomes activated. Here, we transfected an EBV-negative NPC cell line HK-1 with a synthetic ligand 5'ppp-dsRNA. RIG-I expression can be stimulated by 5'ppp-dsRNA in a dose-dependent manner (Fig. 2a), and we chose 0.75 μ g/mL of 5'ppp-dsRNA as the optimal stimulus concentration. In order to identify the effect of BART6-3p on RIG-I-like receptor signaling, we transfected BART6-3p mimics into HK-1 cells in the presence or absence of 5'ppp-dsRNA stimulation. Figure 2b confirmed the transfection efficiency of BART6-3p mimics in HK-1 cells. We also performed time course analysis on the RIG-I expression levels upon BART6-3p mimics transfection, and Figure 2c showed that the RIG-I mRNA levels were gradually decreased in the 60-h period posttransfection. In group 1 (Fig. 2d), BART6-3p mimics decreased the expression of RIG-I and phos-TBK1 (lane 2 vs. lane 1). As a serine/threonine kinase, phos-TBK1 can associate with TRAF3 and TANK, and phosphorylates IFN regulatory factors (IRFs) IRF3 and IRF7. This activity allows the subsequent homodimerization and nuclear translocation of the IRFs, leading to the transcriptional activation of proinflammatory and antiviral genes, including IFNA and IFNB [36]. Under the stimulation of either 5'ppp-dsRNA stimulation or IFN- α , BART6-3p can still decrease the expression of RIG-I and phos-TBK1 (Fig. 2d, group 2, 3). We noticed that the TBK1 phosphorylation was markedly reduced by BART6-3p. Thus, we suspect that the reduction of phos-TBK1 induced by BART6-3p may be through RIG-I-dependent or independent pathways. It is well established that the translocation of IRF3 and STAT1 from the cytoplasm to the nucleus is a key determinant of the RIG-I-like receptor signaling-mediated type I IFN response. Therefore, we detected expression levels of phosphorylated IRF3 and STAT1 in the cytoplasm and nucleus via Western blotting. Most of the RIG-I proteins are located in the cytoplasm. Upon 5'ppp-dsRNA stimulation, the nucleus phos-STAT1 and IRF3 proteins are significantly increased, and under this stimulation BART6-3p can still de-

crease nucleus phos-STAT1 and IRF3 protein levels (Fig. 2e). Figure 2e also showed that under the stimulation of either 5'ppp-RNA or IFN- α , RIG-I and IFN- β mRNA expression levels are increased, and BART6-3p can inhibit the expression of the 2 genes (lane 2 vs. lane 1).

BART6-3p decreased the nucleus phos-IRF3 and phos-STAT1 (Fig. 2e), which is consistent with the decrease of RIG-I and IFN- β mRNA expression (Fig. 2f). These results demonstrated that BART6-3p negatively regulated the RIG-I-like receptor signaling and type I IFN response.

BART6-3p Specifically Inhibits the Expression of RIG-I and the Activation of IFN- β

As BART microRNA expressions are highly active in NPC, it was of great interest whether the inhibition effect of BART6-3p on RIG-I-like receptor signaling and IFN- β is specific. Therefore, we separately transfected 6 different miR-BARTs mimics in HK-1 cells. Only BART6-3p mimics decreased the expression of both RIG-I and phos-STAT1 (Fig. 3a). We then examined the activation of IFN- β by ISRE luciferase reporter assay, and found that only BART6-3p, but not other miR-BARTs, can significantly inhibit the activation of ISRE, i.e., the transcription of the *IFNB1* gene (Fig. 3b). We also assayed the effect of BART6-3p mimics on STAT3 and RIG-I signaling downstream targets, and Figure 3c shows that the expression levels of *IL-1B*, *IL-6*, *IL-12A*, *ISG15*, and *c-MYC* were inhibited by BART6-3p. To further investigate whether BART6-3p have a direct effect on RIG-I, we knocked down the expression of BART6-3p in an EBV-positive NPC cell line, C666-1, which highly expressed endogenous BART6-3p (Fig. 3d). The expression of RIG-I and IFN- β were significantly increased when BART6-3p was inhibited (Fig. 3e, f). Collectively, these results revealed that BART6-3p, rather than other miR-BARTs, specifically inhibited the expression of RIG-I and the activation of IFN- β .

BART6-3p Suppresses EBV-Triggered IFN- β Production and Facilitates EBV Infection

To evaluate the underlying mechanisms of BART6-3p in regulating the cellular antiviral response, we infected BJAB cells with EBV virions derived from the B95.8 cell line. The EBV virus derived from B95.3 cells does not possess BART6 and other BART genes, but it has the *BART3* gene [14]. EBV infection led to an increased transcriptional level of BART3-3p but not the BART6-3p gene (Fig. 4a). Also, we could see that the transfection of BART6-p mimics was successful in BJAB cells, as mea-

Fig. 3. BART6-3p but not other BARTs specifically suppress RIG-I expression and IFN- β activation. **a** HK-1 cells were transfected with NC mimics and 6 different BART mimics separately for 48 h. The indicated protein levels were detected by Western blotting (above), and the expression levels of the individual BARTs were assayed by qPCR (below). **b** HK-1 cells were cotransfected with ISRE luciferase reporter (400 ng each) and different BARTs mimics separately for 24 h before luciferase reporter assays were performed. **c** HK-1 cells were transfected with BART6-3p or NC mimics for 24 h. The mRNA expression levels of the indicated genes were analyzed by qPCR. The gene expression level of NC was set

as 1. **d**, **e** The endogenous level of BART6-3p was detected by qPCR after C666-1 cells (EBV-positive NPC cell line) were transfected with BART6-3p inhibitor for 48 h (**d**), and the RIG-I protein level was detected by Western blotting (**e**). **f** C666-1 cells were transfected with NC, BART6-3p mimics, RIG-I siRNA, and BART6-3p inhibitor separately for 48 h and the mRNA levels of *RIG-I* and *IFN- β* were detected by qPCR. Both the mean mRNA expression and mean luciferase activity were calculated from 3 independent experiments and data are shown as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ compared with NC.

sured by qRT-PCR in Figure 4a (left side). EBV infection was sufficient to trigger RIG-I-like receptor signaling and an IFN- β response, whereas BART6-3p mimics could repress EBV infection-induced RIG-I and IFN- β expression (Fig. 4b). We next detected the amount of cellular

EBV copy numbers to assess the effect of BART6-3p on EBV proliferation. As shown in Figure 4c, EBV-infected BJAB cells possessed significantly high EBV copy numbers, while it was not detectable in the mock cells. Raji cells were used as an EBV-positive control cell line. Here,

Fig. 4. BART6-3p suppresses the EBV infection-triggered IFN- β response and facilitates EBV infection. **a–c** BJAB cells were transfected with either NC or BART6-3p mimics for 48 h and then infected by EBV virions for 2 h in EBV infection groups. Transcriptional levels of BART6-3p and BART3-3p were detected by qRT-PCR (**a**). Also, the cellular RNAs harvested from this experiment were used for RNA sequencing, and the result is presented in Figure 5. The host cellular genes *RIG-I* and *IFN- β* were detected by qRT-PCR (**b**). The EBV genome copy numbers were detected, and Raji cells were used as an EBV-positive control cell line (**c**). **d, e** BJAB cells were cotransfected with ISRE luciferase reporter (400 ng each) and NC (or BART6-3p) mimics for 48 h, and then infected by EBV virions for 2 h in the EBV infection group. Cell lysate was used for the luciferase reporter assay (**d**) and Western

blotting (**e**). Both of the mean mRNA expression and mean luciferase activity was calculated from 3 independent experiments and are presented with the standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group. Mock, BJAB cells with no infection and treatment; EBV, cells were infected with EBV virions for 2 h; NC+EBV, cells were transfected with NC mimics for 48 h and then infected with EBV virions for 2 h; 6-3p+EBV, cells were transfected with BART6-3p mimics for 48 h and then infected with EBV virions for 2 h. BJAB are EBV-negative cells, and Raji is an EBV-positive cell line. Purified EBV virions were obtained from the productive EBV B-cell lineage B95.8. The genome of EBV harvested from B85.8 cells does not have *BART6* and some other *BARTs* genes, but *BART3* is present [14].

we used an EBV DNA quantitative fluorescence diagnostic kit (as described previously [26]) to assay the EBV copy numbers of the cells. Compared with the NC+EBV group, the BART6-3p+EBV group possessed higher EBV copy numbers. This suggested that BART6-3p mimics could facilitate EBV infection in BJAB cells in vitro.

We then tested ISRE reporter activity in BJAB cells upon EBV infection, which is designed to monitor the activity of type I IFN-induced signal transduction pathways. Figure 4d demonstrated that EBV infection was sufficient to induce ISRE activity, whereas BART6-3p repressed this effect. Consistent with this, phosphorylated

STAT1 was increased by EBV infection but was decreased by BART6-3p mimics (Fig. 4e). These results demonstrated that BART6-3p facilitated EBV infection in BJAB cells, probably by suppressing the IFN- β response, the most important antivirus response of the host.

BART6-3p Suppresses EBV-Triggered Immune Responses

To further investigate the role of BART6-3p in EBV-induced host responses, we analyzed the global transcriptome change of BJAB cells upon EBV infection by means of an RNA-sequencing technique. BJAB cells were treated

Fig. 5. BART6-3p suppresses EBV infection-triggered immune responses. BJAB cells were treated as described in Figure 4a, and cellular RNAs were isolated and sequenced. **a** 408 genes were significantly upregulated upon EBV infection (EBV vs. mock); 246 genes were significantly downregulated by BART6-3p mimics (6-3p+EBV vs. NC+EBV). **b–e** The DAVID bioinformatics resource was used

to investigate the biological pathways related to these differentially expressed genes. The 408 gene-related GO pathways and KEGG pathways are listed in **b** and **c**; the 246 gene-related GO pathways and KEGG pathways are listed in **d** and **e**. An asterisk (*) indicates the pathways that are activated by EBV infection and repressed by BART6-3p mimics.

as described in Figure 4a, and RNAs were isolated and sequenced. In Figure 5a, the RNA-seq data (No. GSE95634) showed that 408 genes were significantly upregulated upon EBV infection (EBV vs. mock; see online suppl. Table 3), whereas BART6-3p mimics significantly inhibited the expression of 246 genes (6-3p+EBV vs. NC+EBV; see online suppl. Table 4). We used DAVID bioinformatics resources [27] to investigate the biological pathways related to the 408 and 246 genes. The 408 genes that are upregulated by EBV are involved in the immune response, inflammatory response, response to cytokines,

IFN- γ -mediated signaling, and cellular response to lipopolysaccharide (Fig. 5b), as well as NF- κ B signaling, EBV infection signaling, TLR signaling, and B-cell receptor signaling (Fig. 5c). The 246 genes that are inhibited by BART6-3p mimics are involved in the immune response, cellular response to lipopolysaccharide, and TLR signaling (Fig. 5d), as well as NF- κ B signaling and B-cell receptor signaling (Fig. 5e). The transcriptome analysis result suggested that EBV infection triggered the host cells' immune responses, whereas BART6-3p mimics can repress these immune responses.

Fig. 6. RIG-I is a target gene of BART6-3p.
a Predicted BART6-3p binding sites in the 3'UTR of RIG-I mRNA according to the computational algorithms of RNAhybrid.
b HK-1 cells were cotransfected with RIG-I 3'UTR dual-luciferase reporter plasmid and NC (or BART6-3p) mimics for 24 h before the luciferase reporter assay was performed.
c A schematic model of BART6-3p functioning in the RIG-I-mediated antiviral response (IFN- β response) through inhibition of its target gene RIG-I. IFN, interferon; IFNAR, interferon- α receptor; ISRE, interferon stimulated response element; IRF3, interferon regulatory factor 3.

BART6-3p Targets RIG-I

To further understanding the regulatory mechanism of BART6-3p in RIG-I expression, RNAhybrid, an online tool for microRNA target prediction, was used to search for potential BART6-3p target genes. Two BART6-3p-binding sites were found in the 3'UTR of RIG-I mRNA, demonstrating perfect base pairing between the seed sequence of mature BART6-3p and the 3'UTR of RIG-I mRNA (Fig. 6a). We subcloned the full-length RIG-I 3'UTR into a dual-luciferase reporter vector. As shown in Figure 6b, BART6-3p significantly inhibited the RIG-I 3'UTR luciferase activity. This suggests that RIG-I is a target gene of BART6-3p.

Discussion

Viral infection is an important pathogenic factor of epidemic diseases, leading to a constant threat to organisms. The recognition of pathogen-associated molecular patterns by PRRs becomes critical to initiate the host innate

antiviral response, which consists of the first line for defending against viral invasion. Among many PRRs, RIG-I is one of the most important cytosolic RNA sensors and expresses ubiquitously. It can recognize a panel of viruses, including vesicular stomatitis virus, Sendai virus, influenza virus, Ebola virus, and EBV, etc. [37–39]. It has been demonstrated that short dsRNAs with a triphosphate- or diphosphate-motif at the 5' end are major ligands for RIG-I. Upon binding with RNA ligands, RIG-I becomes activated and induces type I IFN production. Type I IFNs then trigger thousands of ISGs to potentially exert their intrinsic functions through JAK/STAT signaling, including preventing virus invasion, disturbing viral replication, transcription, and translation during the virus life cycle [40].

Samanta et al. [21] reported that RIG-I detects EBV-encoded RNAs, EBERs, and subsequently triggers the type I IFN response. In the current study, we also showed that EBV infection significantly increased RIG-I and IFNB1 expression (Fig. 4b). Using GSEA software, we discovered that EBV-positive cancer specimens have hyperactivated RIG-I signaling and the IFN response com-

pared to EBV-negative cancer specimens (see online suppl. Fig. 1), although the complicated nature of tumor tissues compromised this conclusion. EBERs are highly abundant during latent infection in all EBV-infected cell types. Similarly, the BARTs represent other abundant RNAs. Although the levels of their transcripts differ dramatically from cell types, they are consistently highly abundant in latency II cancer specimens and thought to be involved in tumorigenesis [41]. An increasing number of studies have found BARTs to contribute to immune evasion by targeting cellular factors related to the immune system. BART20-5p targets TBX21/T-bet, leading to decreased IFN- γ production, IL-2, and Th2 cytokine production [19]. BART15-3p targets NLRP3 and subsequently inhibits NLRP3 inflammasome activation and IL-1 β cytokine production [42]. These discoveries strongly suggest it is worth further study to demonstrate more EBV-encoded microRNAs which can target the host's immune system, thus offering a better understanding of the immune evasion strategy of EBV [16].

In this study, we chose BART6-3p as our study object, which is highly expressed in latency II malignancy and plays a role in tumorigenesis [43–46], but has no clear functions related to immune regulation. By using a cDNA microarray technique, we found that 49 genes were downregulated upon BART6-3p mimic transfection in PBMCs, which are natural host cells of EBV infection, and most of them participated in the type I IFN virus response, as well as the RIG-I-like receptor signaling. We demonstrated that BART6-3p rather than other BARTs specifically suppressed the RIG-I-like receptor signaling mediated by IFN- β production either with or without RIG-I stimuli in vitro. We infected the BJAB cells with EBV virions after the cells were transfected with BART6-3p (or NC) mimics, and found that 6-3p mimics can improve the EBV infection efficacy compared to NC mimics (Fig. 4c). RNA sequencing was used to analyze the global transcriptome change upon EBV infection and BART6-3p transfection. Since these EBV virions were from B95.8 cells, the endogenous *BART6* gene and other *BART* genes were absent from the EBV genome. Through GO and KEGG pathway classification of the genes upregulated by EBV infection, as well as genes downregulated by BART6-3p mimics, we clearly saw an immune response triggered by EBV infection (Fig. 5b, c), and we also noticed that BART6-3p mimics significantly reversed this immune response (Fig. 5d, e). An EBV-triggered immune response certainly implies the host cells' innate immune system is activated in order to defend against the virus invasion; however, the EBV-encoded BART6-3p can suppress the immune or inflammation response, which

will benefit the virus to establish a more friendly environment inside the host cells. We noticed that BART6-3p can repress the expression of RIG-I and IFN- β , as well as phospho-STAT1, which are induced by EBV infection (Fig. 4b, d, e), suggesting BART6-3p could reduce the virus immunogenicity through this mechanism. Furthermore, we showed that BART6-3p inhibited the EBV-triggered IFN- β response and facilitated EBV infection through directly targeting the 3'UTR of *RIG-I* mRNA.

EBV persists as a life-long infection in >95% of the global human adult population, suggesting it is perfectly immune controlled in most infected individuals [47]. EBV-associated molecular patterns can be recognized by the immune system, such as the TLRs and RIG-I-like receptors, in order to induce immune/inflammation responses to protect the host cells from EBV invasion. However, EBV has also developed multiple strategies to evade the immune attacks from the host cells. EBV expresses very limited viral proteins at the latency infection status, which only excite a minor immune response. The EBV-encoded proteins BNLF2A, BILF1, and BGLF5 can reduce human leukocyte antigen expression [48–50]. BART2-5p targets MICB and thus impairs the killing ability of NK cells [51]. BART3 targets IPO7 expression in tumor-infiltrating leukocytes, impairing their cytotoxic function [52]. Recognition of viral pathogen-associated molecular patterns by PRRs is the first step in the initiation of an innate immune response. RIG-I serves as one of the cytoplasmic PRRs and primarily detects either viral genome RNA or replication transcripts. Avoiding RIG-I recognition is a strategy employed by viruses for immune evasion. BART6-3p is a new player added into this category through targeting RIG-I-IFN signaling. As illustrated in Figure 6c, EBER can stimulate RIG-I, whereas BART6-3p can suppress RIG-I expression. BART6-3p can also inhibit the downstream signaling of RIG-I, thus reducing IFN production, which in turn results in less STAT phosphorylation and reduced ISG transcription. It is well known that the IFN response is the most important antiviral immune response of the host cells.

Considering the highly restricted patterns of viral protein expression in most EBV-associated tumors, the abundance of BART miRNAs suggests that they could be major factors in the contribution of EBV to tumorigenesis and evasion of immune control. The evidence provided in this study suggests the hypothesis that EBV carefully chooses multiple ways (or players) to control the immune balance, and the directed therapies of miR-BARTs will be developed in the near future to evoke the host immune system to prevent virus-induced inflammation and diseases.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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