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Epstein-Barr virus miR-BHRF1-2 targets retinoic acid-inducible gene I and inhibits interferon release in primary B cells

Inauguraldissertation
zur Erlangung des Doctor scientiarum humanarum (Dr. sc. hum.)
an der
Medizinischen Fakultät Heidelberg
der
Ruprecht-Karls-Universität

vorgelegt von
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Yantai, China
2022

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1. Table of Contents

1. Table of Contents 1

2. List of Abbreviations 5

3. Introduction 8

3.1 Epstein-Barr virus 8

3.1.1 Discovery of Epstein-Barr virus (EBV) 8

3.1.2 Genome of EBV and its structure 9

3.2 EBV's replication cycles, latent infection and lytic replication 12

3.2.1 Latent infection 12

3.2.2 Lytic infection 14

3.2.3 Viral reactivation (from latency to lytic replication) 15

3.3 EBV-associated diseases 17

3.4 EBV microRNAs and their functions 23

3.4.1 Discovery and identification of the microRNAs 23

3.4.2 The classification of EBV miRNAs (BHRF1 miRNAs and BART miRNAs) 26

3.4.3 General functions of EBV BHRF1 miRNAs 29

3.4.4 General functions of EBV BART miRNAs 32

3.5 Innate immune responses induced by EBV and characteristics of the IFN-α signaling pathway 35

3.5.1 Innate immunity 35

3.5.2 Interferon-alpha and its functions 41

3.5.3 MiRNAs of EBV control innate antiviral immunity 43

3.6 Aim of the work 45

4. Materials and Methods 46

4.1 Materials 46

4.1.1 Bacteria 46

4.1.2 Eukaryotic cells 46

4.1.3 Primary cells 46
4.1.4 Cell culture media 46
4.1.5 Plasmids 47
4.1.6 Oligonucleotides 48
4.1.7 Recombinant EBVs (EBV and EBV-BAC) 49
4.1.8 Plasmids used for making recombinant EBV 50
4.1.9 Antibodies 51
4.1.10 Enzymes 51
4.1.11 Commercial Kits 52
4.1.12 Buffers and Media 52
4.1.13 Chemicals and Reagents 53
4.1.14 Consumables, equipment and software 54
4.2 Methods 55
4.2.1 Culture conditions 55
4.2.2 Transformation of bacteria 55
4.2.3 Eukaryotic cells culture and transfection 56
4.2.4 Construction of recombinant EBVs and related techniques 57
4.2.5 Construction of RIG-I 3'UTR reporter plasmid and luciferase reporter assays 57
4.2.6 Stable transfection of EBV into 293 cells and circle prep 58
4.2.7 Virus production and viral titers measurement 59
4.2.8 Virus infections 60
4.2.9 Immunofluorescence staining 61
4.2.10 RNA extraction and Real-time qPCR (RT-qPCR) 61
4.2.11 Western blots 63
4.2.12 ELISA (measurement of IFN-α and IL-6 productions) 64
4.2.13 Statistical analysis 64
5. Results 65
5.1 EBV miRNAs were involved in the regulation of innate immune responses 65
5.1.1 Construction of EBV (ΔmiR-ALL) recombinant virus strains and production

of EBV/ΔmiR-ALL viral producer cells 65
5.1.2 EBV miRNAs could inhibit the production of IFN-α and IL-6 in EBV-infected human primary B cells 68
5.2 The EBV miR-BHRF1 locus played an important role in the downregulation of IFN-α secretion 72
5.2.1 Constructions of EBV (ΔmiR-BHRF1 and ΔmiR-BART) recombinant virus strains 72
5.2.2 EBV miR-BHRF1 locus suppressed IFN-α secretion in EBV-infected primary B cells 74
5.2.3 The miR-BHRF1 homolog on the B95-8 EBV genome could also influence the IFN-α release in primary B cells 76
5.3 EBV miR-BHRF1-2 was mainly responsible for the inhibition of IFN-α production in EBV-infected primary B cells 78
5.3.1 The production of IFN-α was increased after the deletion of miR-BHRF1-2 in EBV-infected primary B cells 78
5.3.2 Rescue experiments by reintroducing miR-BHRF1-2 into LCLs 80
5.4 LMP1 was not involved in the inhibition of IFN-α secretion by EBV miR-BHRF1-2 in EBV-infected B cells 82
5.5 EBV miR-BHRF1-2 downregulated the production of IFN-α by targeting RIG-I, a dsRNA virus sensor 84
5.5.1 Proteomic analysis 84
5.5.2 Protein level of RIG-I was increased by the deletion of EBV miR-BHRF1-2 in EBV-infected B cells 87
5.5.3 Protein level of RIG-I was decreased after the reintroduction of EBV miR-BHRF1-2 in LCLs 89
5.5.4 EBV miR-BHRF1-2 downregulated RIG-I protein level by targeting its 3'UTR for degradation 91
6. Discussion 101
6.1 The connection between miRNAs and innate immunity 102
6.2 Characteristics of EBV miR-BHRF1 and their roles in IFN-α signaling

pathway 103
6.3 Interplay between LMP1 and miR-BHRF1-2 105
6.4 RIG-I plays an important role during EBV infection 106
7. Summary 110
8. Zusammenfassung 111
9. References 112
10. Acknowledgements 127
11. Eidesstattliche Versicherung 128

2. List of Abbreviations

Δ	Knockout or deletion
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Amp	Ampicillin
AP-1	Activator protein 1
ATM	Ataxia telangiectasia mutated
BAC	Bacterial artificial chromosome
BCR	B cell receptor
BHRF1	BamHI fragment H rightward open reading frame 1
BART	BamHI-A rightward transcripts
Cam	Chloramphenicol
CBP	CREB-binding protein
CTCF	CCCTC-binding factor
CREB	cAMP response element binding
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
EBER	Epstein-Barr virus encoded RNA
EBVaGC	Epstein-Barr virus-associated gastric carcinoma
EBNA	Epstein-Barr nuclear antigen
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
DLBCL	Diffuse large B cell lymphoma
GC	Gastric carcinoma

GFP	Green fluorescence protein
gp	Glycoprotein
HITS-CLIP	High-throughput sequencing of RNA isolated by cross-linking and immunoprecipitation
HRP	Horseradish peroxidase
IE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
IRF	IFN-regulatory factor
Kan	Kanamycin
Kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MEF2D	Myocyte-specific enhancer factor 2
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NGFR	Neuron growth factor receptor
OriLyt	Lytic Origin of replication
OriP	Latent Origin of replication
ORF	Open reading frame
PAGE	Polyacrylamide gel

PAR-CLIP	Photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PI3K	Phosphoinositide 3-kinases
Pol	Polymerase
PRRs	Pattern recognition receptors
PTLD	Post-transplant lymphoproliferative disorder
PML	Promyelocytic leukemia
qPCR	Quantitative polymerase chain reaction
RISCs	RNA-induced silencing complexes
RNA	Ribonucleic acid
rpm	Round per minute
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
STAT	Signal transducers and activators of transcription
Tet	Tetracycline
TGF-beta	Transforming growth factor-beta
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Terminal repeat
TRAF	Tumor necrosis factor receptor–associated factor
TRBP	Transactivation response element RNA-binding protein
Tris	Tris-Hydroxymethyl-Aminomethane
UTR	Untranslated region
UV	Ultraviolet
ZREs	Zta response elements

3. Introduction

3.1 Epstein-Barr virus

3.1.1 Discovery of Epstein-Barr virus (EBV)

Denis Burkitt took the critical first step in the discovery of the Epstein-Barr virus (EBV) in 1958 when suggesting to Anthony Epstein that he should seek for viruses in what became known as Burkitt's lymphoma. In 1964, Epstein, Achong and Barr succeeded in establishing culture cell lines *in vitro* and illustrated herpesvirus-like particles in biopsied cells derived from Burkitt's lymphoma by using electron microscopy (Figure 3.1) and EBV was then reported as the etiological factor of Burkitt's lymphoma (Epstein et al. 1964). Four year later, EBV was demonstrated to be one frequent etiological agent of infectious mononucleosis (IM) because Henle's lab found a strong correlation between EBV and IM (Evans et al. 1968). Since then, EBV has been shown to be associated with more human malignancy diseases such as nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma (HL), T cell and Natural Killer (NK) cell lymphomas, post transplantation-associated B cell lymphomas and some complications of the acquired immunodeficiency syndrome (AIDS) were also recognized as EBV-related diseases (Greenspan et al. 1985; Jones et al. 1988; Weiss et al. 1989).

EBV belongs to the gammaherpesvirus subfamily, and it is also named as human herpesvirus 4 (HHV-4). EBV infects more than 90% of the whole human population and is found as a mainly asymptomatic infection in the human population. Primary EBV infection mainly occurs during childhood and thereafter the virus remains in the host

body for the whole life (Liebowitz 1995). So far, EBV has been found to cause approximately 2% of all human tumors and its relationship to tumors in humans is still being explored (Young and Rickinson 2004).

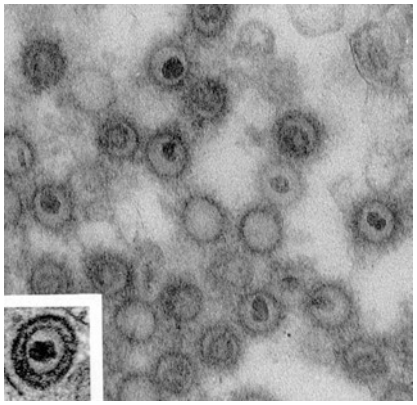


Fig. 3.1 The first electron micrograph of EBV. Immature virions assembling in the cytoplasm of a cultured EB1 lymphoma cell; *inset*, a mature enveloped particle (Epstein et al. 1964).

3.1.2 Genome of EBV and its structure

EBV genome: The EBV genome is composed of double-stranded DNA and is around 172 Kb in length (Hiraki et al. 2001). Like most other lymphocryptovirus genomes, the EBV genome consists of two major domains: short and long unique sequence domains (US and UL). US and UL are separated by 6 to 12 tandem reiterations of a 3-kbp, internal direct repeat (IR1), UL is separated into four smaller segments (U2-U5) by three IRs (IR2-IR4) (Young and Rickinson 2004), as shown in Figure 3.2. EBV includes 2 to 5 tandem, 0.5-kbp, terminal repeats (TRs) and the number of repeats within an individual EBV genome is variable because of the random excision of the TRs after lytic DNA replication. Indeed, random recombination within the TRs happens during the circularization of its genome after EBV infection of the host. These TRs can serve as an indicator to determine whether infected cells arise from the same progenitor cell

(Bouvard et al. 2009). EBV B95-8 strain, derived from IM, was the first herpesvirus to have its genome cloned and sequenced in 1984 (Baer et al. 1984). Since the prototype B95-8 genome was sequenced from an EBV DNA BamHI fragment cloned library, the EBV open reading frames (ORFs) was then described according to their presence on BamHI fragments, in a descending order from A to Z based on their sizes (Bouvard et al. 2009). The ORFs of EBV are separated into lytic and latent genes (lytic and latent infections will be discussed in 3.2).

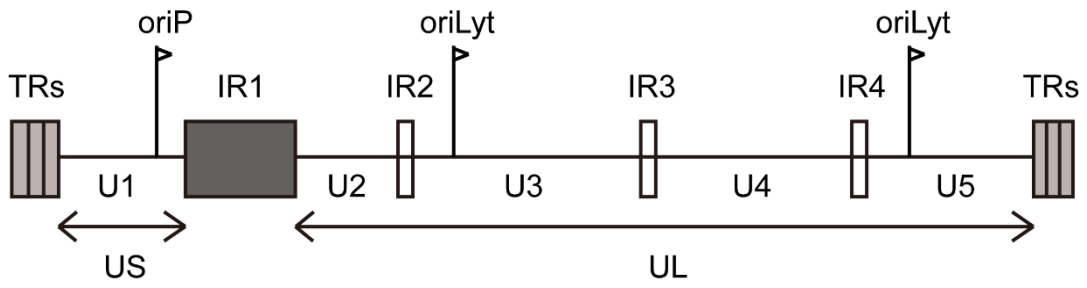


Fig. 3.2 The structure of EBV genome.

This graph shows a simplified EBV genome and it also shows the origin of replication in latent infection (oriP) and two origins of lytic replication (oriLyt), and their locations in EBV genome. This graph is adapted from Fig. 27.1 in (Young et al. 2007), with some modifications.

EBV structure: The innermost part of EBV virions is a linearized viral DNA and the inner layer that wraps the innermost part is the viral nucleocapsid. The space between outer envelope with glycoproteins and viral nucleocapsid is filled with protein complexes containing a large number of different tegument proteins (Figure 3.3). Viral

capsid proteins are important for wrapping the viral genome into the core of the nucleocapsid layer and for the delivery of the viral DNA at the nuclear membrane (Kieff et al. 1982). Tegument proteins are not only considerable for viral infection but also for the maturation of the virus (Feederle et al. 2006; Kalejta 2008). Viral glycoproteins can determine the tropism for host cells by interacting to different cellular surface molecules and then cooperate to mediate perfect attachment, fusion and entry into the host cells. B lymphocytes and epithelial cells are the major sites for EBV infection and EBV uses different glycoprotein combinations to infect B lymphocytes and epithelial cells. EBV glycoproteins gB and gH/gL complex are required for both B cell and epithelial cell fusion (Connolly et al. 2011). An additional glycoprotein, gp42, is also required for B cell fusion (Chen and Longnecker 2019). For viral attachment, EBV glycoproteins gp350/220 bind to CD21 (CR2) on B lymphocytes and BMRF2 may function similarly to gp350/220 and play a role in epithelial entry (Connolly et al. 2011; Fingeroth et al. 1984).

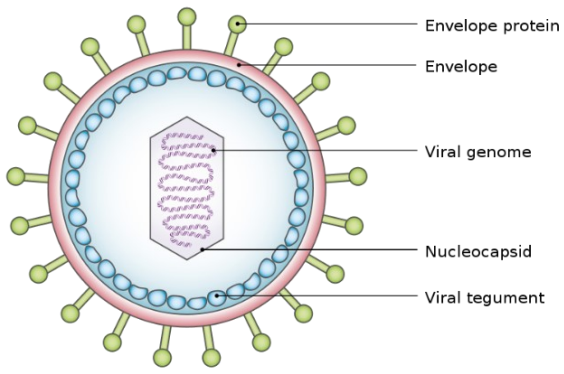


Fig. 3.3 Simplified diagram of the structure of EBV (Wikipedia).

3.2 EBV's replication cycles, latent infection and lytic replication

Similar to other herpesviruses, EBV exhibits two life cycles: latency and lytic replication phase. Although lytic replication phase is very important because the virus may produce virions during this phase and virus can be transmitted between host cells. EBV mainly remains in the latent infection phase in the majority of EBV-infected cells according to serological studies. Moreover, it is hard to detect EBV lytic proteins in healthy samples and even in the biopsies of EBV-associated carcinomas (Thompson and Kurzrock 2004). Intriguingly, EBV is able to downregulate or shut down latent transcript expression in order to escape from host immune surveillance and this process is essential for EBV immortalization (Kelly and Rickinson 2007). Such tricks played by EBV become the reason why more than 90% of the world human population are infected with EBV and many of them keep a long-term silent EBV infection.

3.2.1 Latent infection

EBV is a good model of herpesvirus latency and the epigenetic change that is associated with this process. How EBV establishes latency in its targeted cells is just partially understood. In comparison to EBV-infected epithelial cells, in almost EBV-infected B cells, the viral infection exists in a latent state, leading to B cell immortalization (Kerr 2019).

In latently infected cells, most viral genes are epigenetically repressed by cellular chromatin constituents and CpG methylation of viral DNA, but some certain viral genes escape and remain expressed to support the latent phase of EBV in its host cells (Buschle and Hammerschmidt 2020). In the latent state, the EBV genomic DNA exists

as a closed and stable extrachromosomal plasmid, which replicates via a viral enhancer oriP, and behaves like host chromosomal DNA. The latent EBV chromatin is also organized via the DNA-binding protein CTCF and cohesion subunits which have been reported to prevent the spreading of progressive CpG methylation of EBV viral DNA and keep latent viral promoters in an active state (Tempera et al. 2010).

After transferring across the mucosal epithelium, EBV infects B cells in secondary lymphoid tissues. EBV infection leads to the expression of EBNA2, EBNA3A-EBNA3C and EBNA-LP, leading to proliferation of infected B cells. However, some infected memory B cells can directly differentiate into latency 0 without any viral protein expression, this part of quiescent memory B cells can escape all possible immune detection (Babcock et al. 2000). EBV can drive naïve B cells into full latency III transformation (examples of malignancy: DLBCL and PTLD, EBV-associated tumors will be discussed in details in 3.3), which leads to the expression of nine EBV proteins: EBNA1, EBNA2, EBNA3A, 3B and 3C, EBNA-LP, LMP1 and LMP2, two EBV-encoded small RNAs EBERs and 44 mature miRNAs derived from 25 pre-microRNAs. After activation from EBV latency III, B cells enter the germinal center reaction and only three EBV latent proteins, EBNA1 and LMP1, LMP2 were expressed and turns into latency II (examples of malignancy: HL and NPC from epithelial cells). This process was thought to help EBV-infected B cells to survive germinal center reaction and get access to memory B cells pool (Babcock et al. 1998). Moreover, a much more restricted program latency I (examples of malignancy: BL) with only EBNA1 expression happens when the activated B cells turn into memory B cells, and

EBNA1 itself is enough to support the basic latent infection in replicating memory B cells.

3.2.2 Lytic infection

During lytic phase of infection, EBV genome can be amplified up to around 1000-fold by the viral replication machinery. Compared to the episomal state during latency, lytic replication is for the generation of progeny virus. EBV genome will be packed into viral particles and be spread to neighboring cells.

BZLF1 and *BRLF1*, encode viral transcription factors that modulate the transition from viral latency to lytic infection. Compared to BRLF1, BZLF1 (also called EB1, ZEBRA or Zta), acts as a key switch regulator to induce the lytic phase of EBV's life cycle and it initially activates the transcription of *BRLF1* gene during the viral reactivation. And BRLF1 is indispensable for the expression of all viral lytic genes in the lytic phase (Feederle et al. 2000). Moreover, early transcription factor BZLF1 working together with the BRLF1 transcription factor prefers to bind to promoters containing methylated CpG and promotes the transcription of early lytic viral genes. EBV lytic replication is organized by the early lytic proteins that allow viral DNA replication and the expression of late lytic proteins (Rickinson 2001). BZLF1 makes contributions to the EBV DNA replication that is also important for the production of late lytic proteins by binding to many essential ZRE sites located in the lytic origin of replication, oriLyt. In lytically replicating cells treated with phosphonoacetic acid (PAA, an inhibitor specific to the viral DNA polymerase), the late lytic protein gp350 will not be expressed (Summers and Klein 1976).

BZLF1 encoded by viral *BZLF1* gene is a basic leucine zipper transcription factor. It contains three domains: a basic domain that mediates sequence-specific binding to DNA genome, a transcriptional activation domain and a coiled-coil domain that is responsible for homodimerization (Sinclair 2003). In early lytic infection, BZLF1 binds strongly to methylated 5'-cytosine residues (meZREs, such as 5'-TGAGmeCGA-3', which are common in highly CpG methylated promoters) in a methylation-dependent manner, then induces the lytic amplification of EBV DNA genome. However, some key lytic viral promoters that contain meZRE sites cannot be expressed during the pre-latent phase because of the lack of DNA methylation, which prevents BZLF1 binding to meZRE targeting sequences and inhibits the synthesis of progeny virus (Bergbauer et al. 2010). To summarize, DNA methylation is necessary for the different phases of lytic replication.

3.2.3 Viral reactivation (from latency to lytic replication)

EBV reactivation is a coordinated process, the removal of repressive chromatin components and the access to viral and cellular factors are organized together to support the whole transcriptional program of EBV' lytic phase (Buschle and Hammerschmidt 2020). Compared to the observation of lytic replication after primary infection of B cells and epithelial cells, the process of lytic replication is rarely detected in EBV-associated tumor cells and healthy host cells. Although all EBV-associated cancers involve the latent cycle of EBV, the lytic cycle contributes a lot to the development and maintenance of EBV-associated malignancies (Li et al. 2016). However, how EBV itself switches from latency into lytic phase is still controversial, the mechanisms

underlying EBV reactivation have already been described.

Upon lytic reactivation, EBV goes through three subsequent lytic phases, immediate early (IE), early (E) and late (L) (Murata and Tsurumi 2014). As mentioned before, EBV reactivation can be triggered by two IE transactivators, BZLF1 and BRLF1, and the promoters of these genes (Zp and Rp, respectively) are initially activated by cellular transcription factors. However, many factors involved in the regulation of EBV reactivation have been identified, and the expression of the *BZLF1* and *BRLF1* genes can be activated by various physiological stimuli and chemical agents, such as TPA, BCR engagement, DNA damage and many more. Post-translational modification plays an important role in EBV reactivation. Phosphorylation of Ser173 and Ser186 of Zta can promote viral replication during lytic cycle, and phosphorylated Ser186 of Zta induced by TPA is essential for the full functional activity of Zta (Bhende et al. 2005; El-Guindy et al. 2007). Unlike phosphorylation, SUMOylation modification often negatively regulates Zta transcriptional activity (Hagemeier et al. 2010). However, protein-protein interactions positively or negatively affect Zta and Rta activities, CBP binds to both proteins; TORC2, C/EBP, Pax-5 and Oct-2 to Zta; Ikaros and retinoblastoma protein to Rta (Kenney and Mertz 2014; Li et al. 2016). Several signaling pathways, such as PKC, MAPK and PI3K, are activated after BCR antigen stimulation. The latent-lytic switch can be induced by NF-κB and AP-1 through PKC signaling pathway; the JNK signaling of MAPK family leads to the phosphorylation of c-Jun which then cooperates with Smads proteins to bind the AP-1 motif and the Smad4-binding element in the Zp promoter; some transcription factors such as MEF2D,

CREB and Sp1 that activate Zp or Rp can be activated in PI3K signaling pathway (Hagemeier et al. 2011; Li et al. 2016; Liu et al. 2013). DNA damage occasionally leads to the activation of ATM that induces EBV reactivation by a p53-dependent manner (Hagemeier et al. 2012).

Interestingly, EBV itself has the ability to regulate viral reactivation. For example, EBNA1 has been shown to play two roles: maintenance of latency and induction of lytic infection (Daikoku et al. 2004). And several EBV-encoded miRNAs have been found to inhibit the transition from latency to lytic viral replication, such as miR-BART2, miR-BART18 (Barth et al. 2008; Qiu and Thorley-Lawson 2014). Overall, there are many possibilities to induce the switch from latency to lytic replication in EBV positive cells.

3.3 EBV-associated diseases

Most people in the world are EBV-positive and EBV plays a role in many human diseases and causes approximately 1.5% of human cancer in the world (Table 1). Infectious mononucleosis (IM) is the most common EBV-associated disease, the main problem that it causes is lymphocytic proliferation and it is also accompanied with fever, sore throat and fatigue (Gershburg and Pagano 2005). The large majority of IM sufferers can recover fully within six months. In rare cases, the symptoms of acute IM persist continuously for years as chronic fatigue, but the cause of this condition is still unknown. Although, EBV infection is ubiquitous, many of the EBV-associated malignancies are geographically restricted. This means that local factors may be involved in tumor

development (Crawford 2001).

(a) Burkitt's lymphoma (BL) was first reported as a tumor of the jaw and is associated with EBV infection (Burkitt 1958). BL is the commonest childhood tumor in areas such as tropical Africa, Brazil and Papua New Guinea, where malaria is holoendemic (Molyneux et al. 2012). However, the epidemiological maps of malaria and BL overlap with each other. BL can be a fast-growing tumor and is fatal if being untreated. Intensive chemotherapy has provided a good long-term life survival in children, but the outcome in elderly adults is poor (Molyneux et al. 2012). Adjuvant monoclonal antibody therapy with rituximab has been reported to improve outcome (Molyneux et al. 2012). The WHO classification of Burkitt's lymphoma describes three clinical variants: endemic, sporadic (the predominant type found in some non-malarial regions), and immunodeficiency-related (Jaffe 2009). For sporadic BL, the most common symptom is abdominal pain, but patients with endemic BL most present with jaw or periorbital swellings. EBV, malaria and HIV infection are mainly recognized as cofactors for BL. c-MYC is one of the most essential transcriptional factors that regulate multiple cellular functions, such as cell proliferation, growth and apoptosis (Nguyen et al. 2017). The role of EBV in BL is to block apoptosis in B cells with an MYC translocation through EBNA1, BHRF1, EBERs and LMP1 (Paschos et al. 2009). The translocation t(8;14)(q24;q32) is a characteristic of BL which happens in around 80% BL cases. Other translocations, t(2;8)(p12;q24) and t(8;22)(q24;q11) are found in around 10-15% of patients (Bernheim et al. 1981; Bertrand et al. 1981). The consequence of translocations is MYC oncogene dysregulation, but dysregulation of MYC is not exclusive for BL, it

also happens in aggressive lymphomas such as diffuse large B-cell lymphoma (DLBCL) (Nguyen et al. 2017). Nevertheless, the sequence of events between MYC translocation and EBV infection in BL is still unclear.

(b) Hodgkin's lymphoma (HL) accounts for 15% to 25% of all lymphomas and is also one of the commonest lymphomas in children and adolescents in the world (Mottok and Steidl 2018). The commonest clinical presentation of HL is a painless chronic lymphadenopathy. The treatment is usually combination chemotherapy, radiation and PD-1 inhibitors are highly efficacious both for HL and relapsed/refractory HL. Hodgkin's lymphoma is characterized by the presence of Reed-Sternberg cells (HRS) in an inflammatory background (Shanbhag and Ambinder 2018). The etiology of HL is actually not well understood, EBV can be detected in HRS cells, the only truly malignant cells in HL specimens. According to published articles, the frequency of correlation between EBV and HL is much higher in developing countries (Ambinder et al. 1993). The viral gene expression in EBV-positive HL is the latency type II pattern, with positivity for EBERs, EBNA1 and LMP1, 2. LMP1 can interact with TRAF molecules, which contributes to the activation of the nuclear factor κB (NF-κB) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathways. High level constitutive activation of NK-κB and JAK-STAT signaling pathways are characteristic of HL (Mosialos et al. 1995).

(c) Post-transplant lymphoproliferative disorder (PTLD) and HIV-associated lymphoma are a group of conditions that cause an uncontrolled lymphoid proliferation which is also observed in other EBV-associated tumors. PTLD shows many similarities

to classic lymphomas and is characterized by an abnormal proliferation of lymphoid cells in the background of iatrogenic of immunosuppression after transplantation (Dharnidharka et al. 2016). According to the WHO classification definition, PTLD includes four main types: early lesions, polymorphic PTLD, monomorphic PTLD and classic Hodgkin lymphoma-like PTLD. The clinical symptoms of PTLD are fever, weight loss, allograft dysfunction and lymph node enlargement. Most PTLDs are EBV-related and EBV-positive B lymphocytes resemble latency III B lymphoblasts. Chemotherapy, B cell-specific antibodies and EBV-specific T cells therapy are efficient for the treatment of PTLD although the survival rate is only 50% (Knight et al. 2009). However, the treatment efficacy is limited. Immunosuppression and coinfection with oncogenic viruses, such as EBV and HHV8, contribute to HIV lymphomagenesis (Carbone et al. 2009). In the pre-antiretroviral therapy (ART) period, the risk of developing lymphoma was up to 150-fold higher in HIV patients than in the general population (Noy 2019).

(d) Nasopharyngeal carcinoma. EBV is tightly associated to nasopharyngeal carcinoma (NPC) and can infect about 10% of gastric carcinomas (GC). NPC is a type of tumor which occurs in the squamous epithelium of the post-nasal space. The WHO has classified NPC as keratinizing and non-keratinizing squamous carcinomas. NPC that is endemic to Southern China and Southeast Asia is almost the undifferentiated histological type and 100% associated with EBV infection (Tsao et al. 2017). Nasal obstruction, bleeding and deafness are caused by the primary tumor and node enlargement is caused by the primary tumor metastasis. Radiotherapy and

immunotherapy are the treatment of choices. EBV infection of epithelial cells needs cell-to-cell contact which is different from EBV infection in B cells. Recently, studies have shown that the non-muscle myosin heavy chain IIA (NMHC-IIA) and the novel receptors neuropilin 1 (NRP1) collaborated to mediate the entry of EBV into epithelial cells (Xiong et al. 2015). Decreased expression of p16 and increased expression of CyclinD1 can inhibit the senescence phenotype of nasopharyngeal epithelial cells after stable EBV infection. NPC displays an EBV gene expression known as type II latency, with EBERs expression, BARTs, EBNA1, LMP1 and LMP2A are all detected. Intriguingly, LMP1 can induce the expression of IL-6 by activation of NF-κB signaling, IL-6 then activates IL-6R and STAT3 signaling to support the growth of nasopharyngeal epithelial cells (Kieser and Sterz 2015).

(e) Gastric carcinoma. EBV-associated gastric cancer comprises around 10% of all gastric carcinoma cases. LMP2 plays an important role in GC tumorigenesis by upregulating cellular oncogenes and downregulating the Wnt pathway. LMP1 is a key EBV-encoded oncoprotein that was linked with EBV-associated lymphomas and nasopharyngeal carcinomas. However, LMP1 is not detected in GC. BARF1 can stimulate GC cell proliferation and survival via NF-κB/cyclinD1 pathway (Naseem et al. 2018). For the GC therapy, a recent meta-analysis has shown GC with EBV infection is most likely to overexpress PD-L1. Therefore, in parallel the traditional treatments, immune checkpoint therapy may present an opportunity for a better and effective treatment in GC.

(f) Natural killer/T cell lymphoma (NKTCL). The highly aggressive Natural killer/T

cell lymphoma is most commonly an extranodal lymphoma. NKTCL is common in Asia and in South and Central America. However, in the United States and Europe, the percentage of NKTCL is around 0.2%-0.4% of newly diagnosed non-Hodgkin lymphomas. According to published articles, more than 80% of NKTCLs arise from Natural Killer (NK) cells and around 15% develop from T cells (van Doesum et al. 2021). EBV is the major etiology of NKTCL, EBV-encoded small RNAs are very important to differentiate NKTCLs from reactive inflammatory processes. In NKTCL host genome, SNVs (single nucleotide variants) and indels of EBV genome were tested. An average of 1152 SNVs and 44.8 indels (<50 bp) of EBV per sample was detected in 27 EBV genomes derived from NKTCL tumor biopsies (Peng et al. 2019). Some deletions of EBV BART miRNAs were also identified, but scientists have found some BART miRNAs, such as miR-BART1-5p, miR-BART7-3p and miR-BART13-3p are higher in NKTCL patients' serum samples compared to healthy control groups (Komabayashi et al. 2017). The treatment of NKTCL changes according to disease stage, chemotherapy and radiotherapy are the most important therapeutic therapies. Hemopoietic stem cell transplantation and targeted therapy are commonly combined.

Table 1: EBV-associated malignancies

Type of tumors	Proposed cells of origin	% EBV positive	Viral protein expression	Endemic regions
Burkitt's lymphoma	Centroblast	100%	EBNA1, LMP2A	Equatorial Africa, Papua New Guinea
Hodgkin's lymphoma	Centrocytes	lymphocytes depleted >95% mixed cellularity 70%	EBNA1, LMP1, LMP2	Worldwide
Nasopharyngeal carcinoma	Squamous epithelial cells	100%	EBNA1, LMP1, LMP2	China, Southeast Asia
Gastric carcinoma	Epithelial cells	average 10%	EBNA1, LMP1, LMP2	Largely different between countries
NK/T cell lymphoma	NK/T lymphocytes	100%	EBNA1, LMP1, LMP2	Asia, South and Central America
AIDS-associated lymphoma	Many types	30-90%	EBNA1, EBNA2, EBNA3A-C, LMP1, LMP2	Worldwide
PTLD	All	>90%	EBNA1, EBNA2, EBNA3A-C, LMP1, LMP2	Worldwide

Table 1. The table structure and the data are adapted and modified from a review paper (Crawford 2001).

3.4 EBV microRNAs and their functions

3.4.1 Discovery and identification of the microRNAs

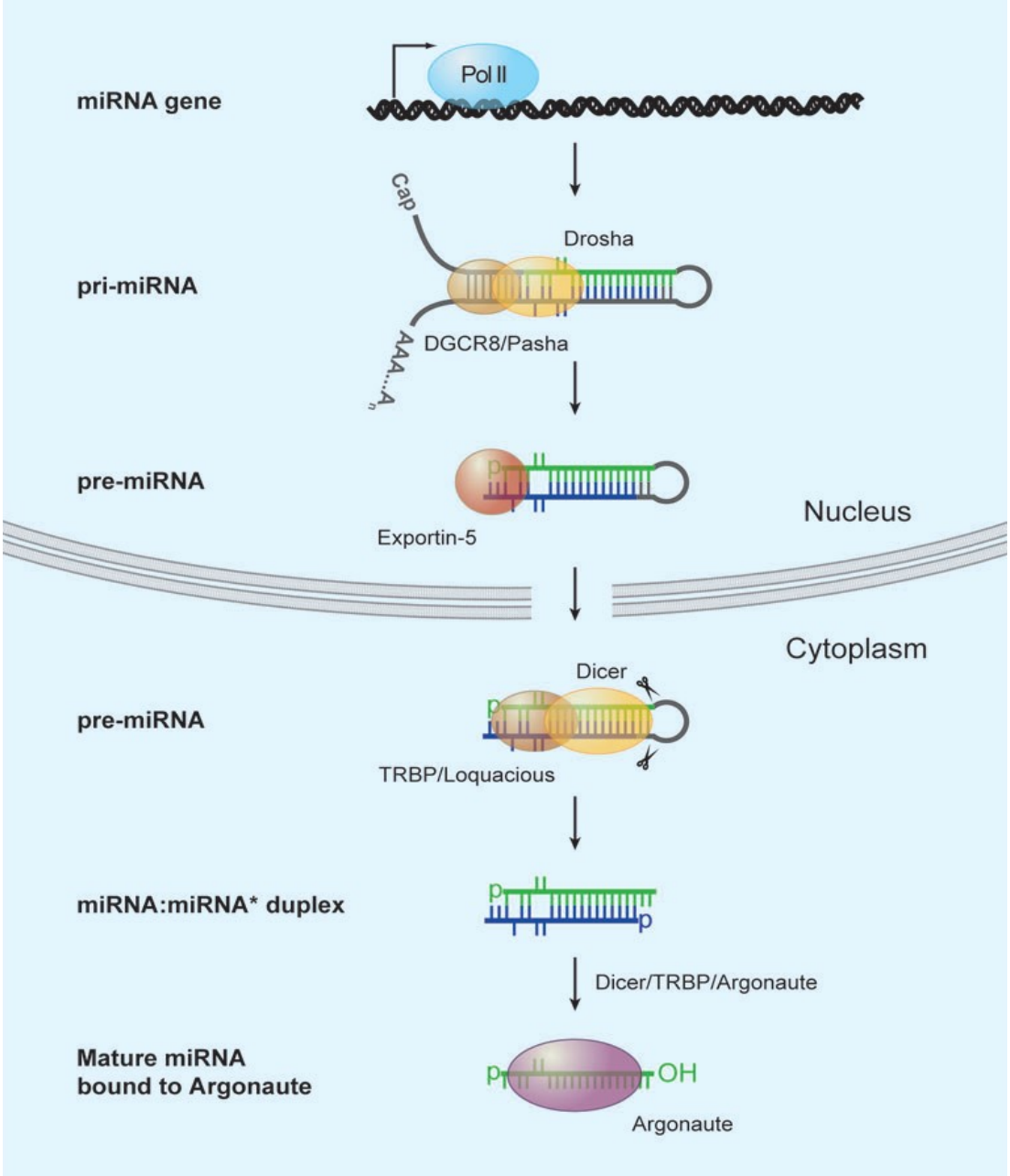
MicroRNAs (miRNAs) are a class of small non-coding and evolutionarily conserved RNAs consisting of approximately 19-24 nucleotides (nt). The miRNAs were initially discovered in 1993 when *lin*-4 gene was found to produce two small RNAs that did not encode functional proteins (Lee et al. 1993). Since 1993, hundreds of miRNAs have

been identified in plants, animals and viruses. MiRNAs were subsequently found to downregulate gene expression by base pairing with the 3' untranslated regions (UTR) of targeted messenger RNAs. It was estimated that around 60% of protein coding genes in mammals were regulated by miRNAs (Friedman et al. 2009).

In the canonical miRNAs maturation pathway, described in Figure 3.4, miRNA genes are transcribed by RNA polymerase II (Pol II) to generate a stem-loop containing primary miRNA (pri-miRNA) (Bushati and Cohen 2007). The primary transcripts contain 5' cap structures which are polyadenylated and 3' poly (A) tails, like to that found in mRNAs (Lee et al. 2004). After that, a microprocessor complex which contains RNAse type III Drosha and DGCR8, a double-stranded RNA binding protein, cleaves the folded pri-miRNAs around 11 bp far away from the junction part between the single- and double-stranded RNAs at the end of hairpin structure and produces a 60-100 nucleotide long, stem-loop structured miRNA precursor (pre-miRNA) that has a 2 nt overhang at its 3' terminal end and a terminal loop of minimal 10 nt (Denli et al. 2004; Han et al. 2004; Lee et al. 2006). Exportin 5 and Ran-GTP work together and transport pre-miRNA from the nucleus to the cytoplasm where RNAse type III enzyme Dicer, PACT, TRBP together with Argonaute (Ago) protein then cleave off the pre-miRNAs' terminal loop to generate mature double-stranded miRNAs. The mature miRNA will then be unwound, one strand is incorporated to form the RNA-induced silencing complex (RISC), this strand is named as the miRNA guide strand; the other is degraded and this complementary strand is called passenger strand or miRNA*. Ago proteins usually have two RNA-binding domains, one is the PIWI domain that can bind

to the miRNA 5' end, the other is the PAZ domain that binds to the 3' terminal end of the miRNA (Yang and Yuan 2009). As an important member of Ago protein family, Ago2 has endonuclease activity and can directly cleave targeted transcripts, but the cleavage efficiency depends on the level of miRNA complementarity (Pillai et al. 2007). In most cases, miRNA-loaded RISC subsequently targets the mRNA 3' UTR to post-transcriptionally downregulate gene expression through the partially complementary sequences' binding between RISC and mRNA. And the fully assembled RISC, which minimally contains an Ago family protein and a mature miRNA, inhibits protein production by direct inhibition of translational initiation or through deadenylation of the targeted mRNA, which leads to final degradation of the targeted mRNA by a cytoplasmic exonuclease, called XRN1 (Wu et al. 2006). The sequence complementary to 2-8 nt of the mature miRNA is termed as the miRNA "seed" sequence. However, scientists have found miRNAs often have a moderate impact on the protein production of the targeted mRNA, but each miRNA can target multiple genes and most genes can be targeted by several miRNAs, which finally induces a more efficient effect. In addition to the crucial role of miRNAs in development and differentiation processes, miRNAs are now considered to contribute substantially to the development of several diseases and different types of tumors (Mohr and Mott 2015).

Fig. 3.4 Biogenesis and processing of microRNAs (Bushati and Cohen 2007).



3.4.2 The classification of EBV miRNAs (BHRF1 miRNAs and BART miRNAs)

Many miRNAs are encoded by genomes of different virus families. EBV was the first virus that was discovered to encode miRNAs among the herpesvirus family (Pfeffer et al. 2004). Several articles have identified 25 EBV precursor miRNAs: three BHRF1

pre-miRNAs and twenty-two BART pre-miRNAs, by using bioinformatics analysis in wild type EBV-transformed LCLs and EBV-positive NPC patient samples (Chen et al. 2010; Grundhoff et al. 2006). The total of 25 pre-miRNAs will finally produce 44 mature miRNAs with four BHRF1 mature miRNAs and forty BART mature miRNAs. Interestingly, scientists have found EBV miRNAs are highly conserved among other lymphocryptoviruses. For example, the Grundhoff lab found that the closely related rhesus LCV could encode thirty-four pre-miRNAs in which twenty-one of them shared sequence identity with EBV miRNAs, it means some miRNAs among different types of viruses are evolutionarily conserved (Walz et al. 2010).

All three BHRF1 pre-miRNAs are encoded around the *BHRF1* gene' ORFs, which encode a viral Bcl2 homolog. BART pre-miRNAs are located within introns of the *BART* gene region, two large clusters together contain twenty-one BART miRNAs and one isolated BART miRNA, miR-BART2 is located antisense to the EBV *BALF5* gene that encodes the viral DNA polymerase, as shown in Figure 3.5. By using High-throughput sequencing and diverse PCR-based miRNA arrays, differences in EBV miRNAs expression in virally infected tumors and tissues has been revealed. EBV miRNAs expression depends on the particularly viral latency program. EBV latency III takes place in LCLs in vitro and in some EBV positive B cell tumors and high expression levels of BHRF1 miRNAs are detected in all those cell types. On the other hand, BART miRNAs can be detected in all forms of EBV latency. BART miRNAs are thought to share expression pattern of the BART noncoding RNAs and are especially abundant in EBV latency II (Cai et al. 2006; Edwards et al. 2008). However, both

BHRF1 miRNAs and BART miRNAs are expressed during the lytic cycle in EBV-positive tumors. For example, scientists have confirmed that miR-BHRF1-2 and miR-BHRF1-3 located in the 3'UTR of the early lytic transcript of BHRF1 and BART mRNAs can be induced when lytic reactivation occurs. In generally, EBV miRNAs' expression is upregulated during EBV lytic replication.

Exosomes are extracellular membrane vesicles, which contains miRNAs, mRNAs and proteins. They can protect miRNAs from nuclease degradation, so miRNAs are relatively stable in serum and plasma samples. Circulating EBV miRNAs can be used as biomarkers for some EBV-associated tumors. The levels of miR-BART2-5p, miR-BART6-5p and miR-BART17-5p in NPC patients' serum correlate with NPC prognostic status (Wong et al. 2012). Although EBV infection is highly associated with the development of several tumors, the role played by the viral miRNAs in this process is just beginning to emerge. It has become clear that EBV miRNAs contribute substantially to viral latency by modulating viral and cellular genes involved in cell growth, apoptosis and signaling pathways. More details of EBV miRNAs' function will be discussed in 3.4.3 and 3.4.4.

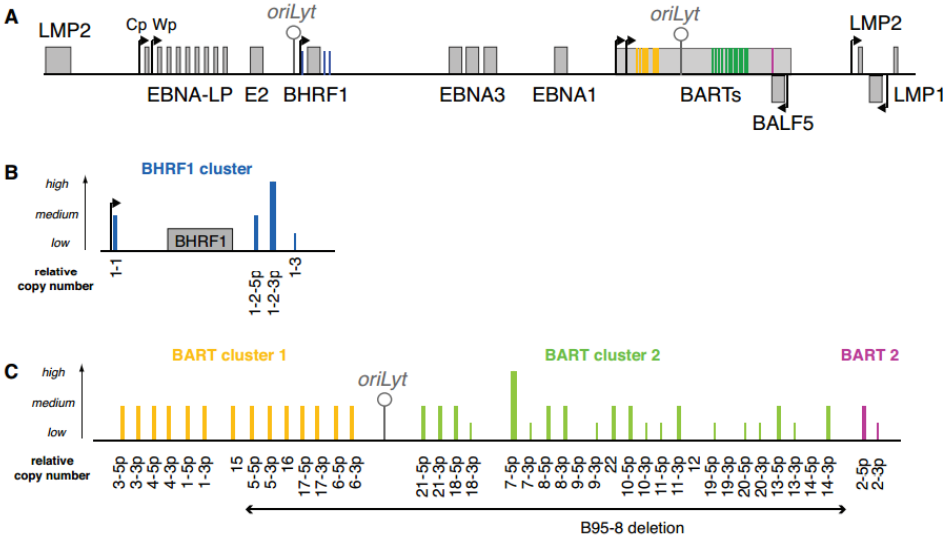


Fig. 3.5 Location and expression levels of EBV miRNAs. (A) Schematic of the EBV genome. Gray boxes represent the latent EBV genes and colored lines are the EBV pre-miRNAs. EBV miRNA are derived from the transcripts of BHRF1 and BART. (B, C) The detailed location and structures of the mature BHRF1 miRNAs and BART miRNAs are shown (Klinke et al. 2014).

3.4.3 General functions of EBV BHRF1 miRNAs

Three BHRF1 pre-miRNAs are located around *BHRF1* gene, miR-BHRF1-1 is located in 5'UTR region of the *BHRF1* gene, and also overlaps the TATA box of *BHRF1* gene promoter. The others, miR-BHRF1-2 and miR-BHRF1-3 are located in *BHRF1* gene 3'UTR region. Transcriptional promoters for BHRF1 and BART miRNAs are different. For BHRF1 miRNA cluster, the major latency promoters are Cp and Wp, whereas P1 and P2 promoters are responsible for the BART miRNA cluster. So, the expression level of EBV miRNAs varies with the nature of infected cells. BHRF1 miRNA expression is

mainly restricted to latency III, it is abundant in LCLs but hardly detectable in BL, NPC or NK/T cell lymphoma. However, BHRF1 miRNAs can be detected in Wp-restricted BL cell lines. While BART miRNA expression is seen in all latency stages, their expression levels also change a lot between different infected cell lines. For example, LCLs lack the expression of miR-BART9-5p and miR-BART20-3p (Qiu et al. 2011). Both BHRF1 miRNAs and BART miRNAs can be upregulated during lytic reactivation (Amoroso et al. 2011). Even though the BART miRNAs are expressed as clusters, the expression levels of individual BART miRNAs in each cluster vary strikingly (Marquitz and Raab-Traub 2012). It has been found that BHRF1 and BART miRNAs can be detected on two days post infection and the expression of miR-BHRF1-1 and miR-BHRF1-2 peaks on three days post infection, whilst the expression of miR-BHRF1-3 peaks after five days during primary infection of B cells (Amoroso et al. 2011). Since of EBV miRNAs identification, they have been intensively studied. The most efficient way to know their functions is to remove each miRNA from its genome. Scientists have found that the prominent role played by the EBV BHRF1 miRNAs is to mediate B cell transformation and immortalization after early phase of EBV infection by using different mutants that lack only one of the BHRF1 miRNAs (Feederle et al. 2011a; Feederle et al. 2011b). In our lab, analysis of viral mutants lacking each of the BHRF1 miRNAs respectively showed all of them are essential for maximal transformation efficiency (Feederle et al. 2011b). Whereas, miR-BHRF1-1 appeared to be a little bit less important for B-cell transformation and the expression of miR-BHRF1-3 was dependent on miR-BHRF1-2 expression, suggesting that miR-BHRF1-2 plays an

important role in EBV-mediated cell transformation (Feederle et al. 2011a).

Several programs, such as RNA hybrid, are usually used to predict miRNA targets. In order to facilitate the identification of the miRNA binding sites of targeted mRNAs, EBV-infected cells are subjected to photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) and high throughput sequencing (HITS-CLIP). However, due to the homologous degree between miRNA seed region and its target is variable, validated experiments of this interaction are needed when one mRNA has been identified as a potential target. For example, luciferase reporter assay is considered as solid evidence for the miRNA-mRNA interaction. In this experiment, the 3'UTR of miRNA targets are cloned behind the luciferase ORF. After the transfection of luciferase plasmid with or without the putative miRNA, the luciferase activity will be measured.

Here, EBV miR-BHRF1 targets that have been validated, were briefly summarized in Table 2 and functions of these cellular targets were also described.

Table 2: Cellular targets of EBV miR-BHRF1

MiRNAs	Cellular targets	Functions	References
miR-BHRF1-1	GUF1	GTPase	(Skalsky et al. 2012)
miR-BHRF1-1	SCRN1	Exocytosis	(Skalsky et al. 2012)
miR-BHRF1-1	NAT12	Acetyltransferase	(Skalsky et al. 2012)
miR-BHRF1-1	p53	Apoptosis	(Li et al. 2012; Xu et al. 2020)
miR-BHRF1-1	RNF4	SUMO-targeted ubiquitin ligase	(Li et al. 2017)
miR-BHRF1-2	BACH1	Oxidative stress	(Skalsky et al. 2012)
miR-BHRF1-2	KDM4B	Histone demethylase	(Skalsky et al. 2012)
miR-BHRF1-2	ZNF451	PML bodies	(Skalsky et al. 2012)
miR-BHRF1-2	PD-L1/PD-L2	Immune checkpoint ligands	(Cristino et al. 2019)
miR-BHRF1-2	PRDM1/Blimp1	B-cell terminal differentiation	(Ma et al. 2016)
miR-BHRF1-2	MALT1	Immune homeostasis	(Wang et al. 2017)
miR-BHRF1-2	IL1R1	Immune response	(Skinner et al. 2017)
miR-BHRF1-2	CTSB	Lysosomal enzyme	(Tagawa et al. 2016)
miR-BHRF1-3	TAP2	MHC class I-mediated presentation	(Albanese et al. 2016)
miR-BHRF1-3	CXCL11	Immune response	(Xia et al. 2008)
miR-BHRF1-3	VAV2	Immune response	(Bouvet et al. 2021)

3.4.4 General functions of EBV BART miRNAs

For BART miRNAs, two miRNA clusters are located within the intron of the *BART* gene at the 3' terminal, whilst miR-BART2 is found between the *BILF1* and the *BALF5* genes. Although BART miRNAs can be detected in all latent stages, their expression levels vary strongly between different infected cell types because they share the expression pattern of the BART ncRNA (Qiu et al. 2011). And it has also been shown that the level of the BART miRNAs was correlated with the level of the promoter

methylation. For example, BL cell lines with high BART promoter methylation express relatively low levels of BART miRNAs. On the other hand, LCLs express high levels of BART miRNAs but its level of promoter methylation is relatively low (Skalsky and Cullen 2015). And the production of BART miRNAs in EBV-infected B cells is inhibited by the treatment with DNA methyltransferase inhibitors (Kim et al. 2011). However, compared to EBV M81 strain, EBV B95-8 strain has a 12 kb deletion in the *BART* gene region, so it lacks several EBV BART miRNAs expression.

EBV BART miRNAs can be detected on two days post infection and their expression levels will continue to increase in the first week. Comparing to miR-BHRF1, which mainly contributes to cell growth and survival in primary B cells, BART miRNAs can enhance the growth of EBV-infected epithelial cells by inhibiting the production of pro-apoptotic and tumor suppressor genes and can also increase the risk of metastasis in epithelial tumors (Marquitz et al. 2011). While in B cells, BART miRNAs play an important role in protecting early-phase LCLs and BL cell lines from apoptosis. Additionally, part of BART miRNAs can influence multiple signaling pathways in innate or adaptive immunity (Bouvet et al. 2021).

EBV BART miRNAs can not only target cellular mRNAs but also target multiple viral protein-coding mRNAs. Here, EBV transcripts targeted by BART miRNAs and cellular transcripts targeted by BART miRNAs were briefly summarized in Table 3 and Table 4 respectively.

Table 3: EBV transcripts targeted by EBV miR-BART

MiRNAs	Viral targets	Functions	References
miR-BART20-5p	BZLF1/BRLF1	Lytic replication	(Jung et al. 2014)
Multiple BART miRNAs	LMP1	EBV-mediated growth transformation	(Albanese et al. 2016)
miR-BART22	LMP2A	Block tyrosine kinase signaling	(Albanese et al. 2016)
miR-BART10-3p	BHRF1	Apoptosis	(Riley et al. 2012)
miR-BART2-5p	BALF5	DNA polymerase	(Barth et al. 2008)
miR-BART5-5p,17-3p	BNRF1	Major tegument protein	(Skalsky et al. 2014)
miR-BART1-5p	BALF2	ssDNA binding protein	(Skalsky et al. 2014)

Table 4: Cellular transcripts targeted by EBV miR-BART

MiRNAs	Cellular targets	Functions	References
miR-BART1	IRF9	Immune response	(Bouvet et al. 2021)
miR-BART1	OAS2	Immune response	(Bouvet et al. 2021)
miR-BART1-5p	SP100	PML bodies	(Skalsky et al. 2012)
miR-BART1-5p	LY75, CLIP1	Immune response	(Skalsky et al. 2012)
miR-BART2	IFNAR1	Immune response	(Bouvet et al. 2021)
miR-BART2	JAK2	Immune response	(Bouvet et al. 2021)
miR-BART2-5p	OTUD1, PELI1	NF-κB signaling	(Skalsky et al. 2012)
miR-BART2-5p	MICB	Immune response	(Nachmani et al. 2009)
miR-BART3	CAND1	NF-κB signaling	(Skalsky et al. 2014)
miR-BART3	JAK1	Immune response	(Bouvet et al. 2021)
miR-BART3	FBXW9	Adaptor protein	(Skalsky et al. 2014)
miR-BART3-3p	p53	Tumor suppressor	(Wang et al. 2019)
miR-BART3-3p	DAZAP2	Wnt signaling	(Skalsky et al. 2012)
miR-BART3-5p	DICE1	Tumor suppressor	(Kang et al. 2015)
miR-BART3, 16	TOMM22, IPO7	Transport	(Dolken et al. 2010)
miR-BART3, 22	MAP3K2	Immune response	(Bouvet et al. 2021)
miR-BART3, 6, 19	RIG-I	Immune response	(Bouvet et al. 2021), (Lu et al. 2017)
miR-BART5	ZCCHC3	Immune response	(Bouvet et al. 2021)
miR-BART6-5p	DICER	MiRNA biogenesis	(Kang et al. 2015)
miR-BART7	CHUK	Immune response	(Bouvet et al. 2021)

miR-BART8-3p	RNF38	E3 ligase	(Lin et al. 2018)
miR-BART9	CDH1	Cell migration	(Hsu et al. 2014)
miR-BART10	FYN	Immune response	(Bouvet et al. 2021)
miR-BART10	VAMP3	Immune response	(Bouvet et al. 2021)
miR-BART13-3p	CAPRIN2	Wnt signaling	(Riley et al. 2012)
miR-BART14	YWHAZ	Protein modifier	(Grosswendt et al. 2014)
miR-BART14	RAC1	Immune response	(Bouvet et al. 2021)
miR-BART15-3p	NLRP3	Inflammasome	(Haneklaus et al. 2012)
miR-BART15-3p	BRUCE	Apoptosis	(Choi and Lee 2017)
miR-BART16	CBP	Immune response	(Bouvet et al. 2021)
miR-BART17	IKBKB	Immune response	(Bouvet et al. 2021)
miR-BART18-5p	MAP3K2	MAPK signaling	(Qiu and Thorley-Lawson 2014)
miR-BART19	Viperin/RSAD2	Immune response	(Bouvet et al. 2021)
miR-BART19-3p	WIF1	Wnt signaling	(Wong et al. 2012)
miR-BART20-5p	TBX21/T-bet	Immune response	(Lin et al. 2013)
miR-BART22	CASP3	Apoptosis	(Harold et al. 2016)
miR-BART22	IRAK2	Immune response	(Bouvet et al. 2021)
Multiple BART miRNAs	APC/NLK	Wnt signaling	(Wong et al. 2012)
Multiple BART miRNAs	BCL2L11/Bim	Apoptosis	(Marquitz et al. 2011)
Multiple BART miRNAs	PTEN	Tumor suppressor	(Cai et al. 2015)

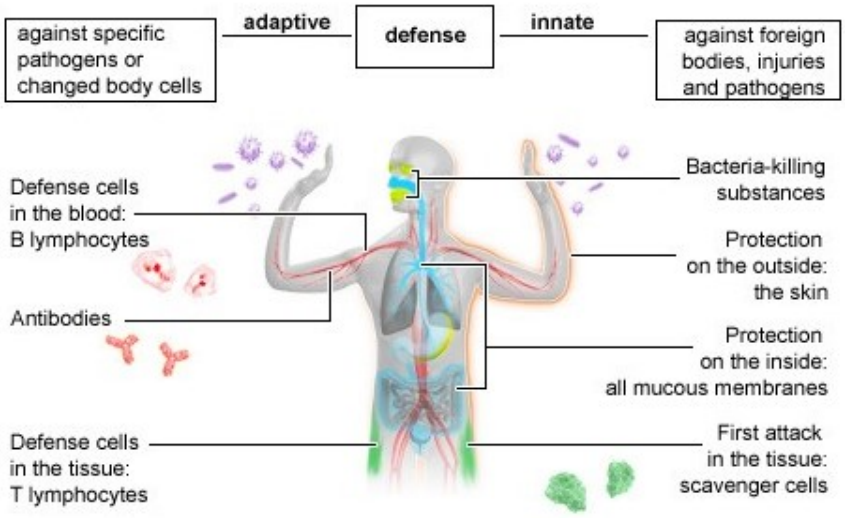
3.5 Innate immune responses induced by EBV and characteristics of the IFN-α signaling pathway

3.5.1 Innate immunity

Human bodies are exposed to millions of potential pathogens everyday but our bodies have the ability to avoid infection, which depends on innate and adaptive immune systems, as shown in Figure 3.6. Compared to the adaptive immunity, the innate immunity is the host first line to defense against pathogens entering into the body. It

can respond to all germs and foreign substances, which makes the innate immune responses “non-specific”. By contrast, our adaptive immune system can remember previous encounters with “specific” pathogens and recognize, destroy them when these pathogens enter the body again. Although the adaptive immune system is theoretically much more powerful, the development of adaptive immune responses is slow when they face a new pathogen for the first time and it takes around one week for the activation and expansion of specific B-cell and T-cell clones. Therefore, we rely on our innate immune system to protect us during the early days of exposure to a new pathogen.

Fig. 3.6 Innate and adaptive immune systems (InformedHealth.org).



The field of innate immunity made its beginnings more than 100 years ago. Innate immune responses have been discovered in both vertebrates and invertebrates (plants, fungi and insects), whereas the adaptive immune systems are only confined to vertebrates (Riera Romo et al. 2016). In vertebrates, the efficient activation of adaptive

immune responses needs cooperation with innate immune responses.

Innate immune responses are “non-specific” and depend on protection offered by the skin and other epithelial surfaces, and protection offered by a group of proteins and immune cells. The skin and all mucous membranes can form a physical barrier against pathogens and protect them from entering the body. Additionally, physical movements like the hair-like structures in the intestine muscles or in the bronchi, and chemical secretions like acid and enzymes in stomach and vagina also prevent pathogens from gaining a foothold.

In vertebrates, innate immune responses are predominantly dependent upon white blood cells (also known as leukocytes and include: neutrophils, basophils, eosinophils, mast cells, NK cells, macrophages and dendritic cells), a group of professional immunocytes that engulf and kill pathogens (Beutler 2004). Additionally, some organs’ epithelial cells are also equipped with a wide repertoire of innate immune receptors that lead to the activation of signaling pathways upon pathogen recognition (Pott and Hornef 2012). Most innate immune leukocytes cannot reproduce on their own, they originate from the hematopoietic system in the bone marrow.

In the Table 5, our current understanding of functions of different innate immune cells was summarized.

Table 5: Innate immune cells

Name	Characteristics	Functions
Neutrophils	Make up to 40%-70% of all white blood cells, short-lived and highly motile	Phagocytosis, chemotaxis, degranulation and neutrophil extracellular traps (NETs)
Basophils	Make up to 0.5%-1% of the circulating white blood cells, the largest type of granulocytes	Anti-parasitic infection and allergies
Eosinophils	Make up to 1%-3% of white blood cells, "acid-loving"	Anti-parasitic and anti-bacterial infection, immediate allergic reactions
Mast cells	Resident cells of connective tissue and contain granules rich in histamine and heparin	Homeostasis and tissue repair, angiogenesis, anti-parasitic and anti-bacterial infection, allergies, nervous system
NK cells	Cytotoxic lymphocytes, which are analogous to cytotoxic T cells and represent 5%-20% of all circulating lymphocytes	Cytolytic granule mediated cell apoptosis, ADCC, self-tolerance, adaptive immune responses, tumor cell surveillance
Macrophages	Have various names according to their anatomical location, antigen-presenting cells and a type of white blood cells that engulf and digest pathogens	Phagocytosis, adaptive immune responses, tissue homeostasis, muscle regeneration and wound healing
DCs	Professional antigen-presenting cells, the most common division of DCs is "conventional" vs. "plasmacytoid" DCs	Antigen presentation in adaptive immune responses, IFN-α production, cross-presentation

How microbes are sensed or how these immune cells are activated, largely depends on pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira 2010). Recent evidences have indicated that PRRs also recognize endogenous molecules released from some damaged cells, these molecules are then termed as damage-associated molecular patterns (DAMPs) (Takeuchi and Akira 2010). The activation of PRRs triggers cascades of intracellular

signaling pathways, which lead to the production of type I interferons, proinflammatory cytokines, chemokines and other anti-pathogen proteins that work together to eliminate pathogens and infected cells (Taguchi and Mukai 2019). Currently, several different categories of PRR families have been determined and include: Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), cyclic GMP-AMP (cGAMP) synthase and stimulator of interferon genes (STING) (Taguchi and Mukai 2019).

The cellular localizations and ligands of PRRs vary a lot. For example, TLRs, CLRs and STING are transmembrane proteins; RLRs and NLRs, they are cytoplasmic proteins. Due to the variable localizations of different PRRs, they recognize different molecular patterns of microbes and self-components. Several good reviews have already made a list of PRRs and their localization, origins of their ligands, so I summarized part of all publications in Table 6.

Table 6: PRRs and their ligands

Name	Localizations	Origins of the ligands	Cell types
TLRs (TLR11-13 are not found in human beings)			
TLR1	Plasma membrane	Triacyl lipoprotein of bacteria	Macrophages, DCs and B lymphocytes
TLR2	Plasma membrane	Lipoprotein of bacteria, viruses and parasites, zymosan of fungi	Macrophages, neutrophils, DCs and mast cells
TLR3	Endosome	Double-stranded RNA of viruses	DCs and B lymphocytes
TLR4	Plasma membrane	LPS of bacteria	Macrophages, neutrophils, DCs, B lymphocytes (only in mice) and intestinal epithelial cells
TLR5	Plasma membrane	Flagellin of bacteria	Macrophages, intestinal epithelial cells, DCs and B lymphocytes
TLR6	Plasma membrane	Diacyl lipoprotein of bacteria and viruses	Macrophages, plasmacytoid DCs and B lymphocytes
TLR7, TLR8	Endosome	Single-stranded RNA of bacteria and viruses	Macrophages and DCs (TLR7: plasmacytoid DCs)
TLR9	Endosome	CpG-DNA of bacteria, viruses and protozoa	Macrophages, plasmacytoid DCs and B lymphocytes
TLR10	Plasma membrane	Triacylated lipoproteins	Macrophages, intestinal epithelial cells and B lymphocytes
RLRs			
RIG-I	Cytoplasm	Short dsRNA of viruses	Almost all cell types
MDA5	Cytoplasm	Long dsRNA of viruses	Almost all cell types
LGP2	Cytoplasm	Blunt-ended dsRNA of viruses	Almost all cell types
NLRs			
NOD1	Cytoplasm	IE-DAP, bacteria	Epithelial cells, stromal cells and endothelial cells
NOD2	Cytoplasm	MDP, bacteria	Various epithelial cell types
CLRs			
Dectin-1	Plasma membrane	Beta-Glucan, fungi	DCs, monocytes, macrophages and B lymphocytes
Dectin-2	Plasma membrane	Beta-Glucan, fungi	Macrophages and DCs

MINCLE	Plasma membrane	SAP130, self and fungi	Macrophages, monocytes, DCs and B lymphocytes
cGAS-STING			
cGAS, STING	Cytosolic, endoplasmic reticulum	Double-stranded DNA of viruses	Both immune and non-immune cells

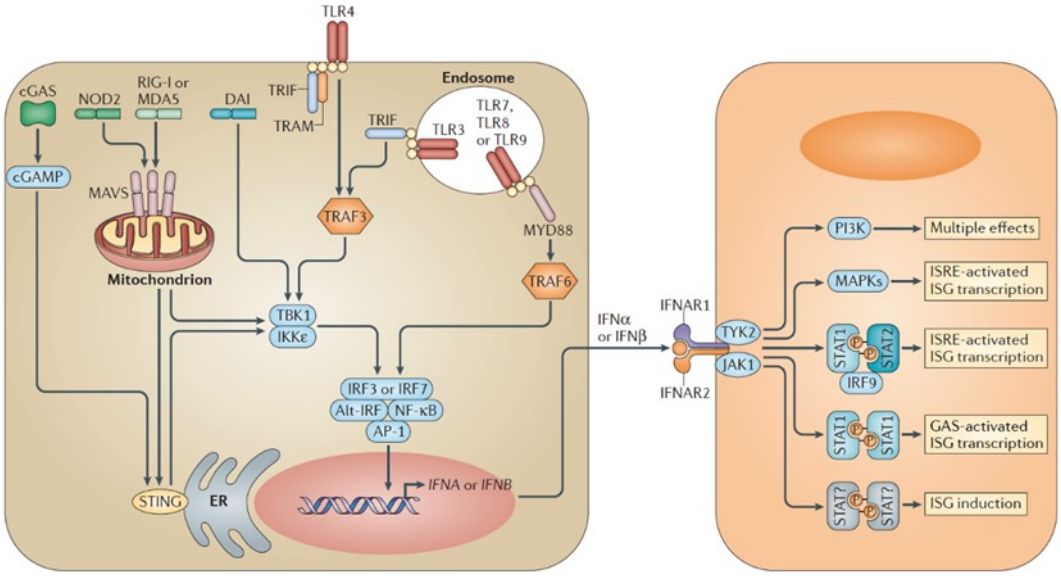
3.5.2 Interferon-alpha and its functions

After the sensing of PAMPs or DAMPs, PRRs will upregulate the transcription of genes, which encode proinflammatory cytokines, type I interferons and other antimicrobial components, such as chemokines and complements. The proinflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-alpha, are mainly involved in the inflammatory response, a process that regulates the cell death of damaged tissues and initiates tissue repair. Chemokines are a family of small signaling proteins that can induce directional movement of leukocytes or other cell types. Complements are involved in the complement system that can enhance the ability of antibodies and phagocytic cells to clear damaged cells or extracellular microbes.

The type I interferons family consists of 13 partially homologous IFN-alpha subtypes in humans, one single IFN-beta and some other gene products, such as IFN-kappa, IFN-delta, IFN-epsilon, etc. Type I interferons (IFNs) have been regarded as key contributors to efficient antiviral responses. On the other hand, it was also reported that type I interferons were involved in the modulation of proinflammatory responses (Honda et al. 2006), but I will just discuss their roles in antiviral responses in this chapter. Upon antiviral responses, type I interferons can induce apoptosis of virally

infected cells, protect uninfected cells or bystander cells from virus infection, stimulate hematopoietic stem cell proliferation, enhance action of immune cells and finally activate acquired immune responses (Katze et al. 2002). In the induction of innate signaling pathways, secreted IFNs bind to cellular interferon receptors, IFNAR1 and IFNAR2, to activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Best and Ponia 2019). It will lead to the phosphorylation and nuclear translocation of IFN-stimulated gene factor 3 (ISGF3), ISGF3 is a complex that is composed of STAT1, STAT2 and IRF9, the complex binds to IFN-stimulated response elements (ISREs) in gene promoters in the nucleus, as shown in Figure 3.7. Finally, the pathway will induce expressions of hundreds of IFN-stimulated genes (ISGs), which are responsible for the biological functions of type I interferons in viral defense.

Fig. 3.7 Several signaling pathways of type I interferons' induction (McNab et al. 2015).



3.5.3 MiRNAs of EBV control innate antiviral immunity

Since EBV miRNAs have been discovered, functions of viral miRNAs were subsequently confirmed. EBV can encode at least 44 miRNAs, which can theoretically regulate hundreds of genes. It means that the identification of all cellular targets is just beginning. Until now, there are only several published papers clarifying the relationship between innate immune responses and EBV miRNAs. EBV miR-BART2-5p represses the expression of the stress-induced Natural Killer (NK) cell ligand, MICB, to evade recognition and elimination of NK cells (Nachmani et al. 2009). EBV miR-BART16 can directly target CREB-binding protein, which is a crucial transcriptional coactivator in IFN signaling pathway, to facilitate the establishment of latent infection and enhance viral replication (Hooykaas et al. 2017). In NPC cells, EBV miR-BART6-3p can inhibit the production of IFN-beta and facilitate EBV infection by targeting the 3'UTR of RIG-I mRNA (Lu et al. 2017). Meanwhile, a recently published research paper from Prof.

Hammerschmidt's lab has documented that many EBV miRNAs are involved in the regulation of type I IFN secretion and ISGs release in both EBV-infected primary B cells and plasmacytoid dendritic cells (pDCs) through directly binding to cellular targets associated with type I IFN signaling pathways at the early infection phase (Bouvet et al. 2021).

However, more research work is still necessary in order to reveal uncanny tricks of EBV miRNAs, which can help viral evasion in the surveillance of innate immunity.

3.6 Aim of the work

Viral miRNAs have been indicated to play an important role in the innate and adaptive immune evasions. Multiple miRNAs encoded by EBV have been shown to control dozens of cellular genes that contribute to antiviral innate immune responses. However, the mechanism of how EBV miRNAs regulate the production and secretion of proinflammatory cytokines and type I IFNs at different infection phases of EBV-infected B cells, still remains unknown.

Therefore, the aim of my PhD project was:

- To understand the contribution of the EBV miRNAs in EBV-infected B cells at different infection phases.
- To explain the underlying mechanism of how cellular genes, involved in innate immune responses, are regulated by EBV miRNAs.

4. Materials and Methods

4.1 Materials

4.1.1 Bacteria

E. coli DH5 alpha and *E. coli* DH10B

4.1.2 Eukaryotic cells

Human embryonic kidney (HEK) 293 cells are a specific immortalized cell line obtained by human embryonic epithelial kidney cells with adenovirus.

4.1.3 Primary cells

Peripheral blood CD19-positive primary B cells are isolated from fresh buffy coats by using Ficoll density gradient method, followed by positive selection with anti-CD19 PanB Dynabeads and detachment of the beads (Invitrogen).

The license to use human primary cells: The Ethics Committee of the University of Heidelberg approved the study (approval 392/2005).

4.1.4 Cell culture media

Name	**Usage**	**Source of supply**
RPMI-1640	For regular cell culture	Invitrogen
Fetal bovine serum	Supplement for the culture medium	Biochrom AG

4.1.5 Plasmids

Vector Plasmids

Name	Description
pcDNA 3.1	It contains a CMV promoter (Invitrogen)
pRK5	It puts the insert behind an early gene promoter from CMV
B1249	It contains a minimal CMV promoter controlled by a bidirectional Tet operator and a tetracycline transactivator protein (Tet-On) driven by a chicken beta-actin promoter with CMV enhancer (CAGp). One site of this bidirectional promoter includes the human neuron growth factor receptor gene (NGFR) with a truncated cytoplasmic tail and GFP (Bornkamm et al. 2005). The other site is available for cloning. The plasmid contains the latent EBV origin of replication oriP derived from B95-8 genome, and a rat CD2 gene derived by the hPGK promoter.

Expression Plasmids

Name	Vector	Description
P509	pRK5	BZLF1 gene derived from EBV/B95-8 controlled by a CMV promoter
pRA	pRK5	BALF4 (=gp110=gB) gene derived from EBV/B95-8, this plasmid is co-transfected with BZLF1 to increase the virus titers
B2092	B1249	Co-expression of NGFR gene and BHRF1-2 miRNA derived from EBV/B95-8; controlled by a tetracycline inducible promoter

4.1.6 Oligonucleotides

Name	Aim	Sequence
BHRF1-1 fwd	Cloning primers used for generation of BHRF1 miRNA mutants	GGTCTCTAGACTTCTTTTATCCTCTTTTTGG
BHRF1-1 rev		GCATCTAGAGTGAAATATCTCTAAAAATAC
BHRF1-2 fwd		CTTTTAAATTCTGTTGCAGCAGATAGCTGATAC CCAATGTAACAGCTATGACCATGATTACGCC
BHRF1-2 rev		ATCCCACCTAGGACACCCAATTGTAGATATGG CCAGCACTCCAGTCACGACGTTGTAAAACGA C
BHRF1-3 fwd		CAATTGGGTGTCCTAGGTGGGATATACGCCTG TGGTGTTCAACAGCTATGACCATGATTACGCC
BHRF1-3 rev		ATTTTAACGAAGAGCGTGAAGCACCGCTTGC AAATTACGTCCAGTCACGACGTTGTAAAACGA C
BHRF1-2 fwd	Stem-loop RT-qPCR	ACACTCCAGCTGGGTATCTTTTGCGGCAGA
BHRF1-2 rev		CTCAACTGGTGTCGTGGAGTCGGCA
BHRF1-2* fwd		ACACTCCAGCTGGGAAATTCTGTTGCAGCA
BHRF1-2* rev		CTCAACTGGTGTCGTGGAGTCGGCA
Mut 1 fwd	Cloning primers used for generation of RIG-I 3’UTR	GCAGTCGACTGTCCTTTCTACTTGCCTCTATA GAATATGTATCCAAATGGCATTCACG
Mut 1 rev		CGTGAATGCCATTTGGATACATATTCTATAGAG GCAAGTAGAAAGGACAGTCAGACTGC
Mut 2 fwd		AGCAAGGTTTGCTGATGCTTCCTCAAGCTTAG

	mutants	TTTGCGGTTTCCTGG
Mut 2 rev		CCAGGAAACCGCAAACTAAGCTTGAGGAAGC ATCAGCAAACCTTGCT
Mut 3 fwd		CTTTGGCTTTTCTTCATCTGTAAAACACTAGTA ATACTGAACTGTAAGGGTTAGTGGAG
Mut 3 rev		CTCCACTAACCCTTACAGTTCAGTATTACTAGT GTTTTACAGATGAAGAAAAGCCAAAG
Mut 4 fwd		GTCAAGGGAGGGACCTGACGCCTATCGATTG GATCACGGGGGCAG
Mut 4 rev		CTGCCCCCGTGATCCAATCACCTCCCGTCAGG TCCCTCCCTTGAC
Mut 5 fwd		CAGTTGTCCCCATGCTGTTCTTCTCCACGTGA GTTAGTTCTCATGAGATCTGATGG
Mut 5 rev		CCATCAGATCTCATGAGAACTAACTCACGTGG AGAAGAACAGCATGGGGACAACTG
Mut 6 fwd		CACACACTCTCTCTCTCATCTGCACACATGTT CTCCTTGCCTGCTTCCCCTTCTG
Mut 6 rev		CAGAAGGGGAAGCAGGCAAGGAGAACATGT GTGCAGATGAGAGAGAGAGTGTGTG

4.1.7 Recombinant EBVs (EBV and EBV-BAC)

Name	**EBV strain/Backbone**	**Source of supply and description**
B110	M81	M81 strain wild type
B1341	M81/B110	M81 strain wild type miR-BHRF1 deletion
B1011	M81/B110	M81 strain wild type miR-BART deletion
B1901	M81/B110	M81 strain wild type All-miRNAs deletion
2089	B95-8/2089	B95-8 strain wild type
B414	B95-8/2089	B95-8 strain wild type miR-BHRF1 deletion

B400.30	B95-8/2089	B95-8 strain wild type miR-BHRF1-1 deletion
B391	B95-8/2089	B95-8 strain wild type miR-BHRF1-2 deletion
B392	B95-8/2089	B95-8 strain wild type miR-BHRF1-3 deletion
B555	B95-8/2089	B95-8 strain wild type miR-BHRF1-2-3p seed mutation
B741	B95-8/2089	B95-8 strain wild type miR-BHRF1-2-5p seed mutation

4.1.8 Plasmids used for making recombinant EBV

Name	**Purpose**	**Description**
pCP15	Template of kanamycin	It carries a region of homology with the EBV genome in which the kanamycin resistance cassette is inserted.
pCP16	Template of tetracycline	It carries a region of homology with the EBV genome in which the tetracycline resistance cassette is inserted.
pKD46	Red recombinase expression	It contains an Arabinose-inducible recombinase that is used for homologous recombination in *E. coli*. And this plasmid also contains an Ampicillin resistance gene and a lacZ gene.
pCP20	Encoding the FLP recombinase	It shows the temperature sensitive replication and the thermal induction of FLP synthesis.

4.1.9 Antibodies

Name	Clone	Usage	Origin	Source of supply
Anti-α-actin	C2	WB	Mouse	Santa Cruz
Anti-mouse IgG (HRP, secondary antibody)		WB	Goat	Promega
EBNA2	PE2	IHC	Mouse	Hybridoma supernatant
LMP1	CS1-4	WB	Mouse	Hybridoma supernatant
NGFR		IF, cell isolation	Mouse	From Dr. med. Elisabeth Kremmer
RIG-I		WB	Mouse	Santa Cruz
IF: Immunofluorescence staining; IHC: Immunohistochemistry; WB: Western blot				

4.1.10 Enzymes

Name	Company	Usage
Phusion High-Fidelity DNA Polymerase	Thermo Scientific	PCR for cloning
Restriction Enzymes	Fermentas	Checking of the genomic integrity of EBV-BAC; plasmid construction
Alkaline Phosphatase	Roche	Regular cloning
Klenow Enzyme	Roche	Regular cloning
T4 DNA Polymerase	Fermentas	Regular cloning
RNase A	Roche	Mini-prep
Lysozyme	Serva	Mini-prep

DNase I	Fermentas	qPCR
Proteinase K	Roche	Viral titer measurement
AMV Reverse Transcriptase	Roche	qPCR
Taqman Universal Master Mixes	Life Technologies	qPCR

4.1.11 Commercial Kits

Name	**Company**	**Usage**
Dynabeads CD19 Pan B	Invitrogen	Isolation of human primary B cells
DETACHaBEAD CD19	Invitrogen	Isolation of human primary B cells
High Pure PCR product purification kit	Roche	Regular cloning
Human IFN-α ELISA development kit	Mabtech	IFN-α measurement
Human IL-6 ELISA development kit	Mabtech	IL-6 measurement
MicroRNA reverse transcript	Applied Biosystems	RT-PCR
NucleoBond BAC 100	Macherer-Nagel	EBV-BAC preparation
RNU48	Applied Biosystems	Internal controls for miRNA RT-qPCR
Western Lighting Plus ECL	PerkinElmer	Western Blot

4.1.12 Buffers and Media

Name	**Composition**
5x RIPA lysis buffer	750mM NaCl, 2.5% NP40, 5% Sodium Deoxycholat,

	0.5% SDS, 25mM EDTA, 100mM Tris-HCl (pH=7.5)
Sample loading buffer	100mM Tris-HCl (pH=6.8), 4% (w/v) SDS, 0.2% (w/v) Bromphenol blue, 20% (v/v) Glycerol, 200mM beta-Mercaptoethanol
10x SDS running buffer	250mM Tris, 1.92M Glycine, 1% SDS; pH=8.5-8.8
1x Blotting buffer	25mM Tris, 150mM Glycine, 20% Methanol
Ponceau S	0.1% Ponceau S, 5% Acetic acid
5% low-fat milk	5% Low-fat milk powder in 1x PBST
Mild stripping buffer	0.2M Glycine, 0.1% SDS, 1% Tween-20
PBS	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; pH=7.4
PBST	0.1% Tween-20 in PBS
TAE	40mM Tris, 1mM EDTA, 19mM Acetic acid
Circle prep lysis buffer	50mM NaCl, 40mM NaOH, 1% SDS, 2mM EDTA
DNA gel extraction buffer	300mM NaCl, 10mM Tris (pH=8.0), 1mM EDTA
DNA loading buffer	0.25% Bromphenol blue, 40% (w/v) Sucrose
TE	10mM Tris-HCl, 1mM EDTA (pH=8.0)
LB medium	(2:1:2) Tryptone, Yeast extract, NaCl in H_2O; pH=7.0
LB-Agar	15g Bacto-Agar in 1L LB medium
4% PFA	4% Paraformaldehyde in PBS; pH=7.4

4.1.13 Chemicals and Reagents

Name	**Company**	**Usage**
1-Butanol	Carl Roth	Circle prep
12-myristate 13-acetate (TPA)	Sigma-Aldrich	Lytic induction
Sodium butyrate	Sigma-Aldrich	Lytic induction
Protease inhibitor	Roche	Western blot

Acrylamide	Carl Roth	Western blot
Page Ruler Prestained Protein Ladder	Fermentas	Western blot
DNA ladder 1kb	Life Technologies	All DNA electrophoresis
Ethidium bromide	Life Technologies	Nuclei staining
TRIzol reagent	Life Technologies	RNA isolation
Chloroform	Sigma-Aldrich	RNA isolation
Roti-Phenol	Carl Roth	Circle prep
RNase free Water	Invitrogen	RT-PCR
Ficoll Plus	Amersham Bioscience	Buffy coat isolation
Heparin sodium salt	Sigma-Aldrich	Buffy coat isolation
Glycerin	VWR International	Various purposes
Hygromycin	Invitrogen	Antibiotics
Metafectene	Biontex Laboratories	Transfection for all eukaryotic cells
Trypsin-EDTA	Invitrogen	Cell culture

4.1.14 Consumables, equipment and software

Category	**Name**	**Company**
Consumable	0.5, 1.5, 2, 5 ml reaction tubes	Eppendorf
Consumable	15, 50 ml Falcon tubes	TPP
Consumable	Cell culture plates and flasks	TPP
Consumable	Syringe-driven sterile filter unit	Millipore
Equipment	Nanodrop	Thermo Scientific
Equipment	Amersham membrane Hybond^{TM} ECL	GE Healthcare
Equipment	Various centrifuges	Haraeus

Equipment	CO2 cell incubator	Thermo Scientific
Equipment	PCR Thermocycler (PTC-200)	MJ Research
Sequence analyzer	Macvector 12.0.6	Macvector

4.2 Methods

4.2.1 Culture conditions

E. coli strains were cultured in a sterile LB-medium by shaking or alternatively on LB-agar plates at favorable temperatures (32°C or 37°C) to obtain individual colonies. Different antibiotics, such as ampicillin (100 µg/ml), chloramphenicol (15 µg/ml) or kanamycin (50 µg/ml) were used to culture cells in LB-medium or LB-agar plates. The type of antibiotics to be used depends on the antibiotic resistance genes cloned into the plasmids. For long-term storage, 10% glycerol was added to bacteria culturing to freeze and store cells at -80°C.

4.2.2 Transformation of bacteria

Heat-shock: Plasmids or the ligation productions were mixed with chemically competent cells (e.g., DH5α) on ice for 10 mins. Then the mixture was incubated at 42°C for 90 sec and put on ice again for 1 min. The mixture was added to 2 ml of LB-medium (without antibiotics) and left in culture at 37°C for 45 mins for recovery. Bacteria were spun down by centrifugation at 3000 rpm for 5 mins and the pellet was suspended and cultured on LB-agar plates (with antibiotics) overnight.

Electroporation: In general, 25 µl of electroporation-competent cells DH10B pre-

prepared in 10% glycerol were thawed on ice from -80°C. Bacteria was made by the technician in our lab. The thawed bacteria were then mixed with DNA and incubated on ice for 5 mins. The mixture was transferred into cuvettes and subjected to electroporation at 1.2 KV, 200 Ω, and 25 μFd and then immediately resuspended in 1 ml of LB-medium and cultured at 37°C for 1 h. Single colony was obtained in the same way as the heat shock method.

4.2.3 Eukaryotic cells culture and transfection

Culture conditions: In general, all eukaryotic cells were culture at 37 °C in an incubator stably provided with 100% humidity and 5% CO_2.

Cell lines that grow in suspension, such as LCLs, were cultured in RPMI 1640 with 10% FBS. The cells were split 1 to 10 regularly, but depending on the growth rate. EBV-infected primary B cells were kept in RPMI 1640 with 20% FBS in the first week after infection. Adherent cell lines, such as 293 cells, were cultured in RPMI 1640 with 10% FBS. The cells were incubated with 0.05% Trypsin in 37 °C for 1 min and then resuspended in RPMI 1640 with 10% FBS to neutralize the proteolytic activity of Trypsin and split 1 to 10 regularly, but depending on the growth rate.

Transfection: The cells were seed at a concentration of 5x 10^5 cells per well on a 6-well-plate in 2 ml RPMI-10% FBS one day before the transfection. The transfection procedure is described as following: 1 μg DNA was resuspended in 100 μl of RPMI 1640 and 3 μl of Metafectene, the liposomal-based transfection reagent, was also resuspended in 100 μl of RPMI 1640. Then two mixtures were mixed slowly, and kept

for 20 mins at room temperature. Finally, the DNA-Metafectene mixture was added dropwise into the cells gently. After 6 hours, the medium of the transfected cells was carefully replaced with 2 ml fresh medium plus 10% FBS. The transfected cells or cell supernatants can be used 3 days post transfection.

Electroporation: In our lab, I use Neon Transfection System to introduce plasmids into LCLs. Sufficient cells were washed with PBS without Ca^{2+} and Mg^{2+} for three times and then resuspended with abundant T buffer, at a final density of 2x 10^7 cells/ml in a 1.5 ml tube. At the same time, an appropriate amount of DNA in deionized water at a concentration of 3-5 μg/μl was transferred into the 1.5 ml tube. The mixture was mixed gently by pipetting several times. And the electroporation was then performed following the manufacturer's instructions (pulse voltage varies according to types of cell lines). The transfected cells can be used 3 days post transfection.

4.2.4 Construction of recombinant EBVs and related techniques

All recombinant EBV clones used in my thesis were shown in the table of 4.1.6 Recombinant EBVs (EBV and EBV-BAC). The details of some constructs and all these techniques have already been described precisely in published articles by our group (Feederle et al. 2011a; Lin et al. 2015).

4.2.5 Construction of RIG-I 3'UTR reporter plasmid and luciferase reporter assays

RNA was isolated from PBMCs and cDNA was obtained through RT-PCR. cDNA was used as PCR template to amplify wild type 3'UTR of RIG-I sequence.

Forward primer: GCGCCTCGAGTATCAGGTCCTCAATCTTCAGCTACAGGG, containing an XhoI cutting site.

Reverse primer: CGCGTCTAGATCATTTATAAAGAAAAGAGGCTTAATAGATTC ACAGTTCC, containing an XbaI cutting site.

RIG-I 3'UTR sequence was then introduced in a firefly luciferase reporter plasmid pGL4.5 (Promega). This construct was confirmed by sequencing.

Luciferase reporter assays: 293 cells were seeded at a density of 7x 10^4 cells per well in a 24-well plate. At day 2, 210 ng of the RIG-I 3'UTR firefly luciferase fusion plasmid, 840 ng of miR-BHRF1-2 in pRK5 or of the empty vector control pRK5 and 210 ng of a pRL-SV40 plasmid (Promega) encoding the renilla luciferase to control for differences in cell numbers and transfection efficiency were co-transfected using 3 µl metafectene per µg of plasmid DNA. At day 4, the activity of the firefly and renilla luciferase were determined using the dual-luciferase reporter assay system (Promega) according to the manufactures.

4.2.6 Stable transfection of EBV into 293 cells and circle prep

Stable transfection of EBV into 293 cells: EBV genome was amplifed by using NucleoBond BAC kit and subsequently transfected into 293 cells using the transfection reagent (Metafectene). At day 2 or day 3, cells were transferred into 150 mm culture plates in RPMI-10% FBS plus hygromycin (100 µg/ml). The antibiotics-resistant and GFP-positive cell colonies were expanded and selected.

Circle prep: It was used to confirm the integrity of the EBV genome in stably

transfected 293 cells. 2x 10^7 cells were initially washed by 1x PBS twice and lysed with 10 ml lysis buffer at room temperature for 5 mins. The lysates were then neutralized by adding 0.5 ml 1M Tris-HCl, pH=7.1. Proteins in the lysates were adjusted with 70 µl Proteinase K at 37 °C overnight. At day 2, the phenol extraction was performed in order to extract high-quality circular DNA. DNA was precipitated by adding 2.5 volumes of 100% non-water ethanol at -20 °C for 1 h. The pellet was collected and washed with 80% ethanol twice. Abundant TE buffer was added to dissolve the DNA pellet. The extracted EBV DNA was transformed into *E. coli* DH10B by electroporation. Individual colonies were cleaved with the BamHI or other restriction enzyme.

4.2.7 Virus production and viral titers measurement

Virus production: HEK293 producer cell lines can be lytically induced by transfection of a BZLF1 expression plasmid, a BALF4 expression plasmid together with a gp110 expression plasmid. Six hours later, the medium of the transfected cells was replaced with RPMI-10% FBS. Cell supernatants were collected at 5 days post transfection and centrifuged at 1200 rpm for 10 mins to get rid of the cell debris before being filtered through a 0.45 µm filter.

Viral titers measurement by qPCR: 50 µl of filtered supernatants were treated with 5 U DNAseI at 37 °C for 1 h and then inactivated at 70 °C for 10 mins. 5 µl of DNaseI-treated viral supernatants were treated with 5 µl Proteinase K at 50 °C for 1 h and then inactivated by at 75 °C for 20 mins. The qPCR master mixed with primers and probe targeted for BALF5 gene was prepared and then mixed with viral supernatants. Finally,

the mixture was amplified by real time PCR using a Step One Plus™ qPCR machine.

Components for a qPCR reaction (25 µl in total)

12.5 µl	Taqman 2x Universal Mastermix
2.5 µl	EBV Pol fwd primer (10 µM)
2.5 µl	EBV Pol rev primer (10 µM)
1.0 µl	FAM-labeled EBV Pol probe (20 µM)
1.5 µl	H_2O
+5.0 µl	Proteinase K treated samples

Thermocycle for a qPCR reaction

Step	Temperature	Time
1	75 °C	2 mins
2	95 °C	10 mins
3	95 °C	15 sec
4	60 °C	1 min
Repeat step 3 and step 4 for 40 cycles		

4.2.8 Virus infections

Human primary B cells isolated from peripheral blood were exposed to viral supernatants with various multiplicities of infection (MOI) for 2 h, then washed once with RPMI and cultured with RPMI-20% FBS in the first week. To perform transformation assays, the percentages of EBNA2-positive B cells in the whole EBV-infected B cells were evaluated by immunofluorescence staining at 3 days post infection.

4.2.9 Immunofluorescence staining

Cell pellets were washed twice in 1x PBS and air-dried on glass slides at room temperature. Cells were then fixed with 4% paraformaldehyde for 20 mins at room temperature. After that, fixed cells were permeabilized in 1x PBS with 0.5% Triton X-100 for 2 mins, besides samples stained for viral membrane proteins. Cells were then incubated with the primary antibody for 30 mins at room temperature, washed in 1x PBS three times, and subsequently incubated with the secondary antibody derived from goat conjugated to Cy-3 for 30 mins at room temperature, and washed in 1x PBS three times. Finally, the stained slides were embedded in 90% glycerol and pictures were taken with a camera attached to a fluorescence microscope (Leica). If pictures could not be taken, samples were stored at 4 °C.

4.2.10 RNA extraction and Real-time qPCR (RT-qPCR)

RNA extraction: RNA was extracted from different types of cells according to experimental purposes. TRIzol reagent was used to isolate RNA, cell pellets were washed with 1x PBS once, then lyzed with 1 ml TRIzol and extracted with 200 µl CHCl3, then shaked violently for at least 30 sec and then incubated at room temperature for 5 mins. All samples were centrifuged at 4 °C, at 13600 rpm, for 15 mins. The upper part was immediately transferred into a new tube and 500 µl isopropanol was added into each tube. After mixing carefully by inverting tubes for several times, RNA was precipated at -20 °C for 30 mins. RNA was pelleted at 4 °C, at 13600 rpm, for 15 mins. Pellets were washed in 1 ml 80% ethanol and centrifuged at 4 °C, at 13600 rpm, for 5

mins. RNA pellets were finally resuspended in 40 µl of pre-heated nuclease-free water. RNA concentration was measured at OD260 nm in a Nanodrop photospectrometer. RNA was kept at -80 °C for a long-time storage.

Real-time qPCR: Total RNA was reverse transcripted with AMV-reverse transcriptase by using a mixture of random hexamers.

Components for a RT reaction

1.5 µl	10x RT buffer
3.0 µl	RT stem-loop primer mix
1.5 µl	RNU48 RT primer as endogenous control
0.15 µl	100 mM dNTPs
1.0 µl	MultiScribe MuLV-RT polymerase
0.2 µl	RNA inhibitor
2.65 µl	H_2O

10 µl in total

5 µl (110 ng) of RNA sample was added to the mix and incubated on ice for 5 mins.

Thermocycle for RT reaction

Step	Temperature	Time
1	16 °C	30 mins
2	42 °C	30 mins
3	85 °C	5 mins
4	4 °C	Hold
Add 40 µl water and use 5 µl per sample for RT-qPCR reaction		

Components for a qPCR reaction

10 µl	Taqman 2x Universal Mastermix
2 µl	Primer/Probe mix
3 µl	H_2O

15 µl in total

Add 5 µl cDNA to obtain 20 µl final volume.

Thermocycle for qPCR reaction

Step	Temperature	Time
1	50 °C	2 mins
2	95 °C	10 mins
3	95 °C	15 sec
4	56 °C	1 min
Repeat step 3 and step 4 for 40 cycles		

4.2.11 Western blots

Cell pellets were washed with 1x PBS once and then resuspended with a standard RIPA buffer. To break the genome DNA, cell pellets were sonicated. Lysates were then centrifuged at 4 °C, at 13600 rpm for 20 mins, and supernatants were collected. The concentration of protein samples was detected. Proteins were denatured in protein loading buffer at 100 °C for 15 mins. Boiled samples (20 µg of proteins) were separated in a SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred into a nitrocellulose membrane. After blocking in 5% milk powder in 1X PBST, primary antibodies against targeted proteins were added and membranes were incubated at 4 °C, overnight. At day 2, Membranes were washed with 1X PBST three times, 10 mins for

once. HRP-labeled secondary antibodies were added and membranes were incubated at room temperature for 1 h. Membranes were washed with 1X PBST three times, 10 mins for once. Revelation was performed by using Western Lightening Plus ECL kit.

To quantify the immunoblots, graphs were analyzed with the ImageJ software (NIH).

4.2.12 ELISA (measurement of IFN-α and IL-6 productions)

1x 10^6 cells per well were seeded in 24-well plates. After 2 days, cells were harvested and cell numbers were counted again. Cell culture supernatants were analyzed for the production of cytokines by using relevant ELISA development kits according to the manufacturer's protocol.

4.2.13 Statistical analysis

Paired student's *t*-test was used between two groups and ANOVA was used for more than two groups. All data are expressed as mean ± SEM (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

5. Results

5.1 EBV miRNAs were involved in the regulation of innate immune responses

5.1.1 Construction of EBV (ΔmiR-ALL) recombinant virus strain and production of EBV/ΔmiR-ALL viral producer cells

In an attampt to explore the role of EBV miRNAs in innate immune responses, an EBV recombinant virus (M81/ΔmiR-ALL), in which viral miRNAs were deleted, was first constructed. I used the wild type (WT) EBV strain M81 that was previously cloned onto a prokaryotic F-plasmid, which carried a chloramphenicol resistance gene, a gene for GFP and a hygromicin resistance gene. The EBV recombinant viruses were based on this wild type M81 EBV strain and obtained by replacing the different viral miRNAs with a kanamycin resistance cassette in *E. coli*. (Figure 5.1).

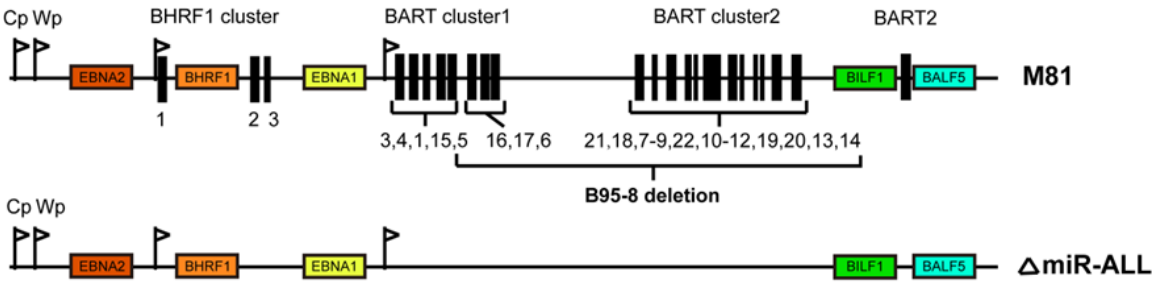


Fig. 5.1 Schematic map of M81/WT and M81/ΔmiR-ALL EBV strains.

M81 recombinants DNA was then stably introduced into HEK293 cells to generate virus producing cell lines. The integrity of the EBV genome in the stably expressed

HEK293 cell clones was confirmed by transporting back the viral episomes that they harbored into *E. coli* cells. Subsequently, enzyme restriction analysis could be performed (Figure 5.2). In order to induce virus production, BZLF1 plasmid was transfected into the producer cell lines to initiate the lytic replication. Additionally, gp110 plasmid was also co-transfected into the producer cell lines to improve the infection efficiency in primary B cells.

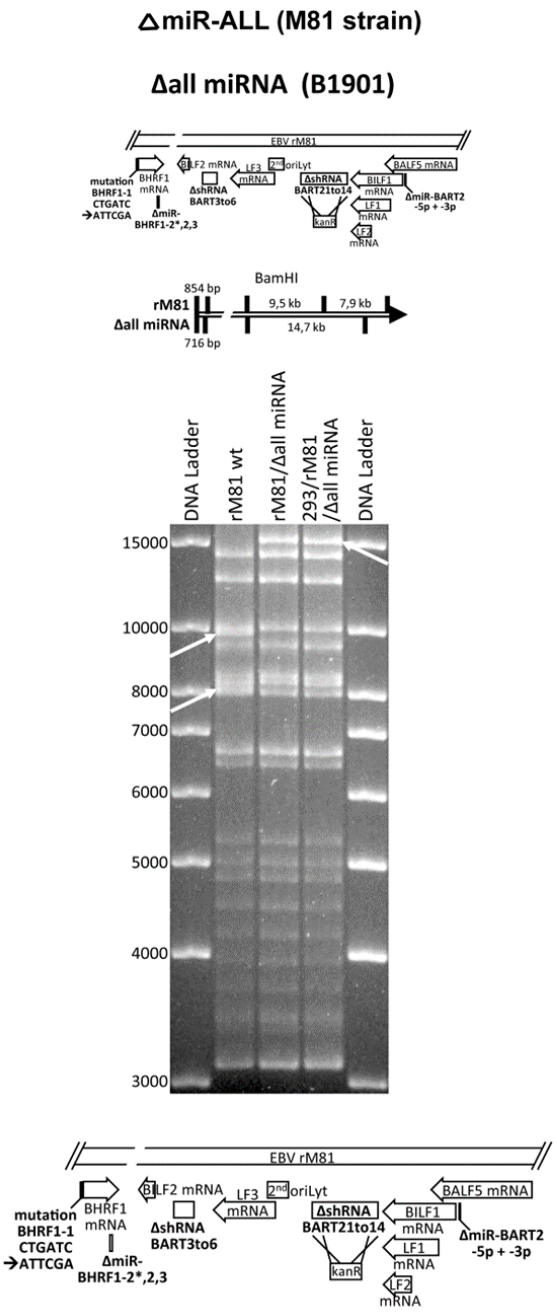


Fig. 5.2 Restriction enzyme analysis of the BAC miniprep shows restriction patterns of M81/ΔmiR-ALL mutant. Viral genome was extracted from virus producer cells and cleaved with restriction enzyme BamH I, then separated on an 0.8% agarose gel. The parental M81/WT genome was loaded as a control and arrows indicate the viral DNA fragments whose sizes differ between the M81/WT and this mutant.

5.1.2 EBV miRNAs could inhibit the production of IFN-α and IL-6 in EBV-infected human primary B cells

I subsequently wanted to determine the effects of the EBV miRNAs on the secretion of type I IFNs and proinflammatory cytokines released from EBV-infected human primary B cells. Above all, primary B cells from five independent blood samples were isolated from peripheral blood mononuclear cells (PBMCs). Resting B cells were exposed to M81/WT or M81/ΔmiR-ALL viruses at a multiplicity of infection (MOI) of 0.3 infectious particles per B cell. Upon infection with different virus strians for 2 hours, infected B cells were seeded at equal densities in fresh medium with 20% fetal bovine serum. Two days or five days later, cell supernatants were collected and cell numbers were calculated again. The concentration of cytokine secretions was measured by performing enzyme-linked immunosorbent assay (ELISA). The final value of cytokine concentration was quantified to the same cell number of 1.5 million cells per well for each sample.

As shown in Figure 5.3A and B, both IFN-α and IL-6 were secreted at a higher concentration when human primary B cells were infected with M81/ΔmiR-ALL mutant compared to that of M81/WT group, indicating that EBV miRNAs contribute to inhibit innate immune responses in EBV-infected primary B cells. This finding prompted me to further explore the functions of EBV miRNAs in EBV-induced innate immune responses.

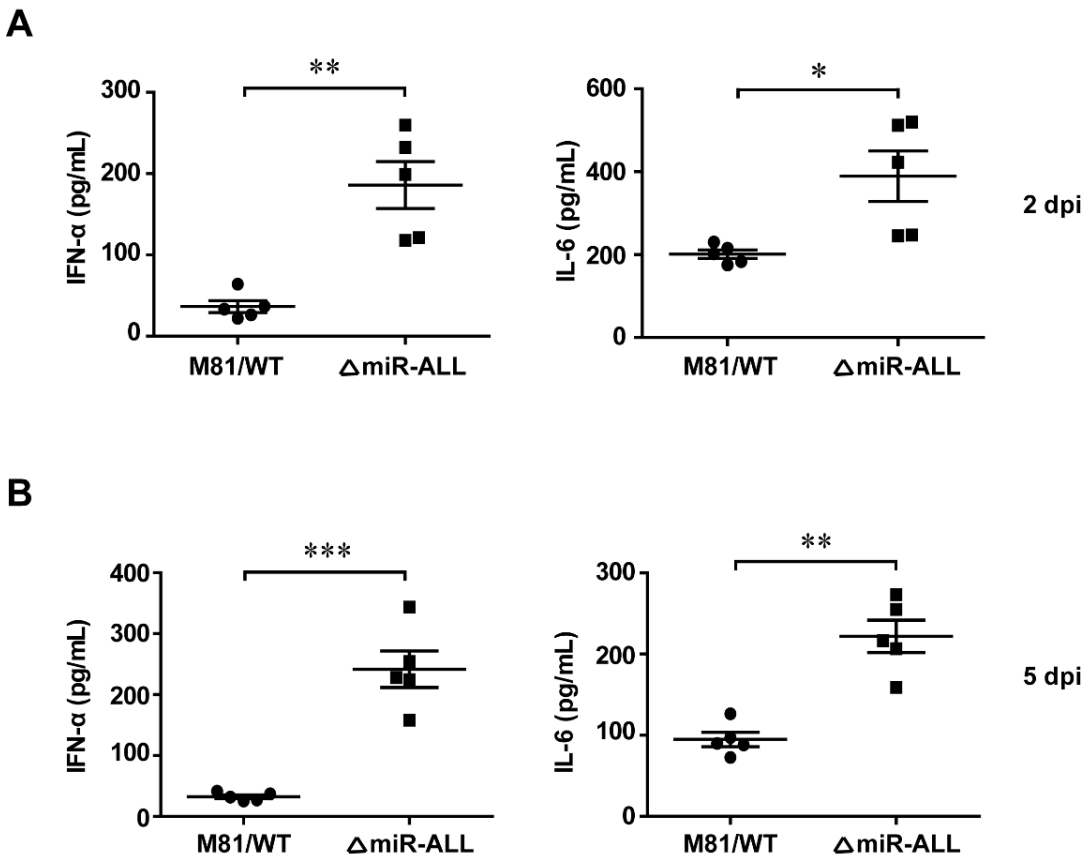


Fig. 5.3 Primary B cells were infected with M81/WT or M81/ΔmiR-ALL mutant viruses at two days (A) or five days (B) post infection, cell supernatants were collected and cell numbers were counted. The level of cytokine secretions was measured by ELISA. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

Interferons are named for their capability to interfere with viral replication by protecting host cells from virus infections. Interferons activate the JAK-STAT signaling pathway to induce the secretion of IFN-stimulated genes (ISGs). In addition to activate the JAK-STAT signaling pathway, interferons also activate other non-STAT signaling pathways, such as MAPKs signaling pathway, to be involved in the antiviral immune process

(Gonzalez-Navajas et al. 2012). Due to the powerful controlling ability of interferons for defensing the viral infection, it seems that EBV miRNAs participate in manipulating the activation of EBV-induced signaling pathways to hamper IFNs release and creating a suitable cellular enviroment for the establiment of EBV infection.

Importantly, EBV expresses all viral miRNAs at two days post infection and the expression level of viral miRNAs will gradually increase in the first week (Bernhardt et al. 2016). For this reason, the secretion of IFN-α at the EBV late infection phase was then detected in order to verify that each viral miRNA was expressed with comparatively high expression levels. Therefore, I extended my observations from the EBV early infection phase (< 10 days) to the EBV late infection phase (> 10 days). Thus, ELISA was performed at 15 days, 21 days or more than 28 days (LCLs: EBV-transformed lymphoblastoid cell lines) post infecion to assess the role of EBV miRNAs in the production of IFN-α at the late infection phase. It was found that the absence of EBV miRNAs led to the increased production of IFN-α in M81/ΔmiR-ALL-infected B cells compared to M81/WT-infected B cells at all studied time points (Figure 5.4A-C). Taken together, these previous results showed that EBV miRNAs can modulate innate immune responses by inhibiting the production of IFN-α and IL-6 in EBV-infected primary B cells. Moreover, EBV miRNAs curb the IFN-α production early after infection, but this effect persists in established EBV-transformed cell lines.

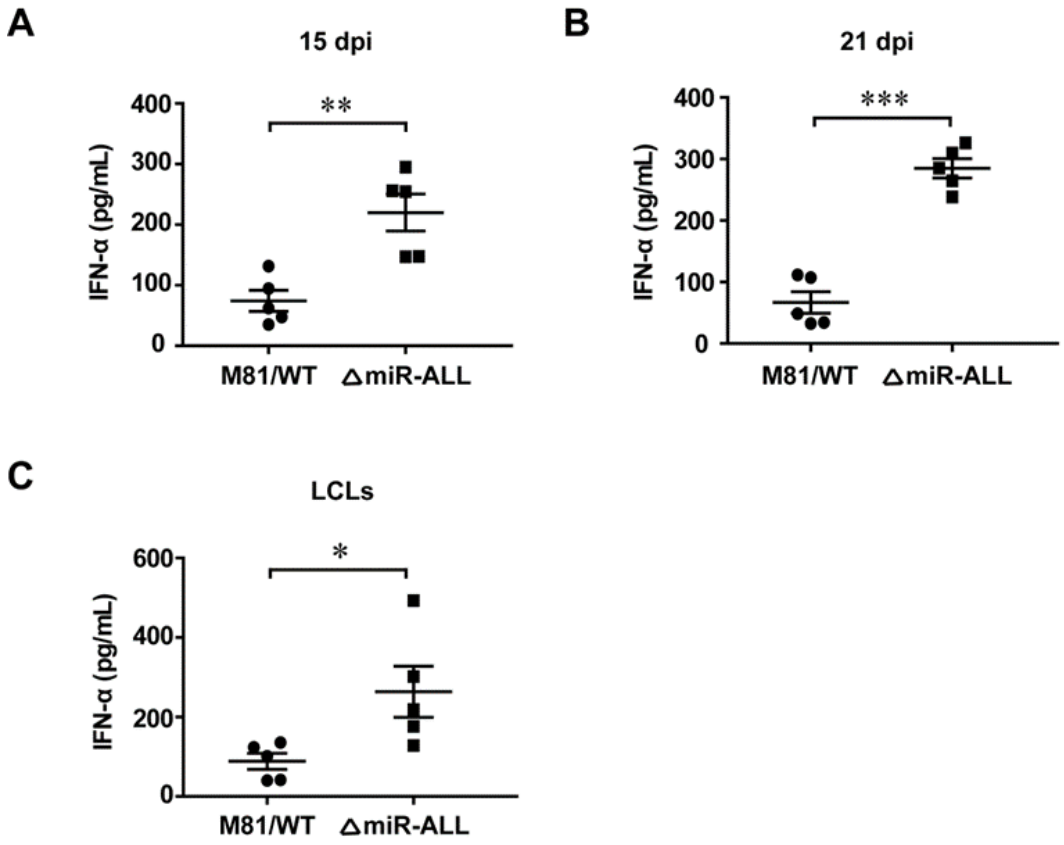


Fig. 5.4A-C Primary B cells were infected with M81/WT or M81/ΔmiR-ALL mutant viruses at different time points. Cell supernatants were collected and cell numbers were counted again. The level of IFN-α secretions was measured by ELISA. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

5.2 The EBV miR-BHRF1 locus played an important role in the downregulation of IFN-α secretion

5.2.1 Constructions of EBV (ΔmiR-BHRF1 and ΔmiR-BART) recombinant virus strains

To investigate which of both EBV miRNA clusters was responsible for the downregulation of type I interferon-alpha secretion. Another two EBV recombinant viruses (M81/ΔmiR-BHRF1 and M81/ΔmiR-BART), in which relative clusters of viral miRNAs were deleted, were subsequently constructed (Figure 5.5).

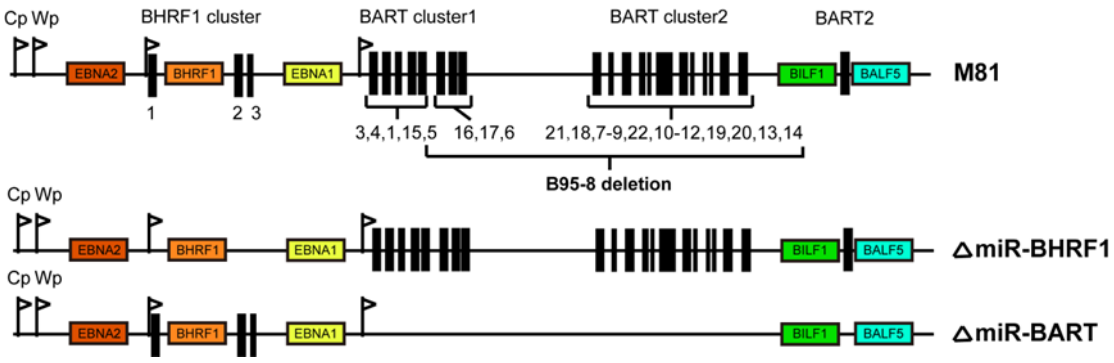


Fig. 5.5 Schematic map of M81/ΔmiR-BHRF1 and M81/ΔmiR-BART EBV strains.

M81 recombinants DNA was then stably introduced into HEK293 cells to generate virus producer cells. The integrity of the EBV genome in the stably expressed HEK293 cell clones was confirmed by transporting the viral episomes that they harbored into *E. coli* cells and enzyme restriction analysis was then performed (Figure 5.6).

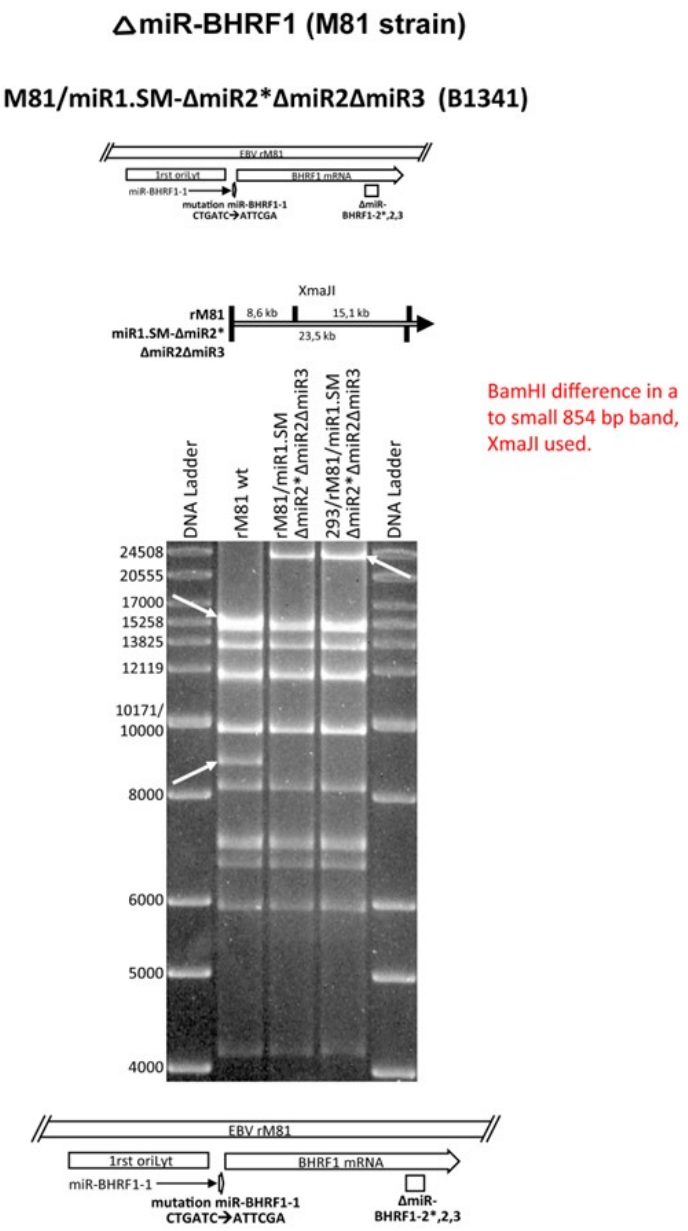


Fig. 5.6 Restriction enzyme analysis of the BAC miniprep shows restriction patterns of M81/ΔmiR-BHRF1 mutant. Viral genome DNAs were cleaved with XmaJ I and then separated on an 0.8% agarose gel. The parental M81 recombinant EBV genome was loaded as a control. The arrows indicate the viral DNA fragments whose sizes differ between the M81/WT and this mutant. The detailed information of the M81/ΔmiR-BART mutant construction was shown in Dr. Lin's paper (Lin et al. 2015).

5.2.2 EBV miR-BHRF1 locus suppressed IFN-α secretion in EBV-infected primary B cells

Previous work on the study of the BHRF1 miRNA cluster has confirmed its contribution to B-cell proliferation and transformation (Feederle et al. 2011a). Primary B cells infected with EBV virus that lacks miR-BHRF1 undergo more frequently cell apoptosis. A virus that lacks the BHRF1 miRNA gene cluster and a virus that lacks the BART miRNA gene cluster were then used to analyze the concentration levels of IFN-α secreted by primary B cells infected by M81/WT, M81/ΔmiR-BHRF1 mutant or M81/ΔmiR-BART mutant. It was found that at 15 days, 21 days or more than 28 days post infection, the expression level of IFN-α secreted by EBV-infected primary B cells in M81/miR-BHRF1 deletion mutant group was significantly higher than that in other groups, as indicated in Figure 5.7A-C. The miR-BART deletion mutation showed a slight increase relative to the wild type control at 15 days post infection, but this difference vanished overtime.

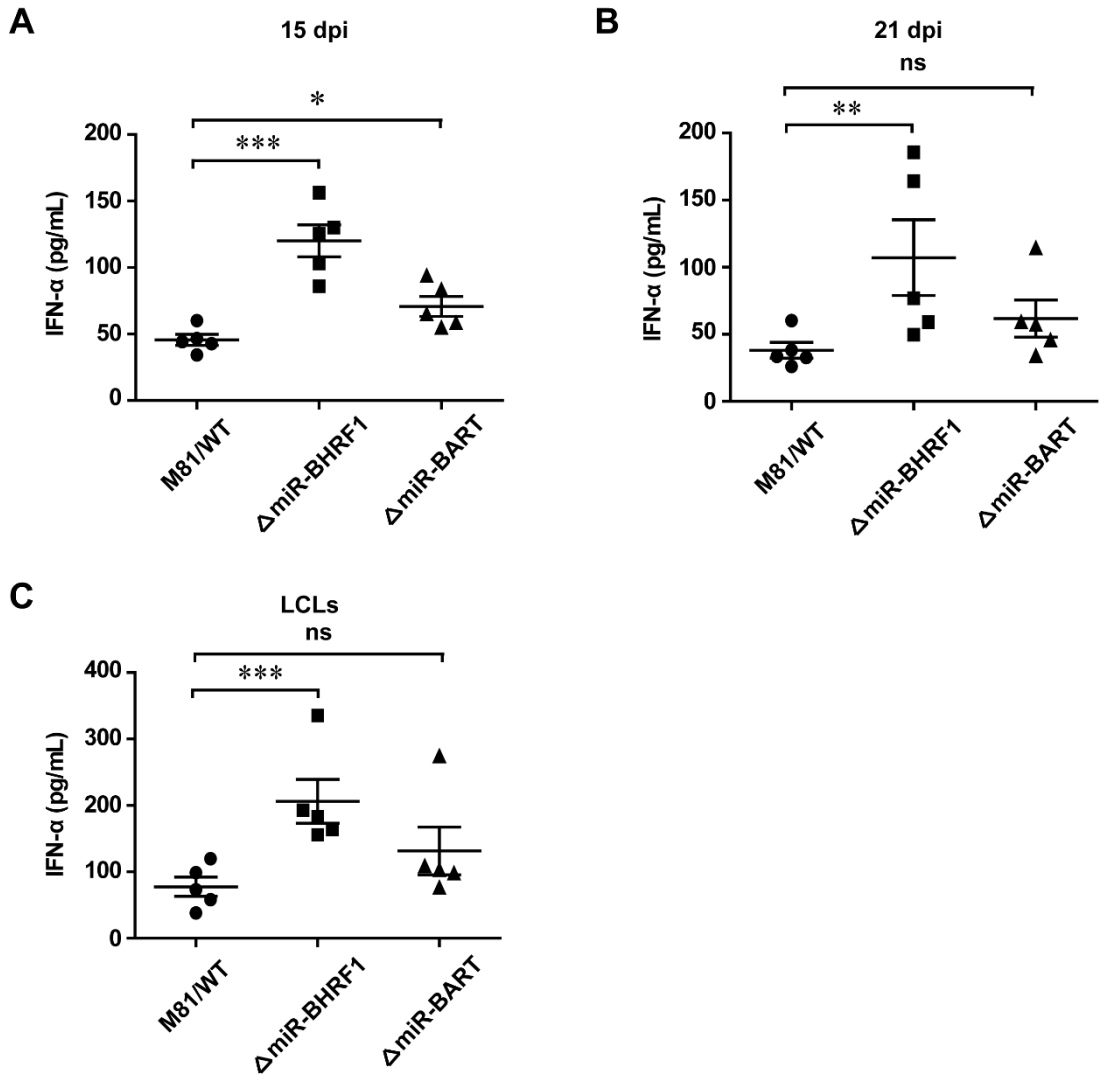


Fig. 5.7A-C Primary B cells isolated from PBMCs were infected with M81/WT, M81/ΔmiR-BHRF1 or M81/ΔmiR-BART mutants respectively. At 15 days , 21 days or more than 28 days post infection, culture supernatants were collected and cell numbers were counted again. The release of IFN-α was analyzed by using ELISA. Data are representative of three independent experiments and ANOVA was used for more than two groups (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

5.2.3 The miR-BHRF1 homolog on the B95-8 EBV genome could also influence the IFN-α release in primary B cells

Both M81 and B95-8 are distinct EBV strains. To our knowledge, M81's target cells are epithelial cells and B95-8 has a stronger tropism for B cells (Lin et al. 2015). In our lab, it has been demonstrated that B95-8 induced cell growth more efficiently than M81 strain, which was positively correlated with the expression levels of miR-BHRF1 (Tsai et al. 2017). To verify these previous results, I moved forward a single step to explore whether the miR-BHRF1 homolog on the B95-8 EBV genome could also influence the IFN-α release or not.

B95-8/WT and B95-8/ΔmiR-BHRF1 viruses were used (Figure 5.8) and the expression levels of IFN-α secreted by primary B cells infected with B95-8/WT or B95-8/ΔmiR-BHRF1 were assessed. Primary B cells were exposed to B95-8/WT or B95-8/ΔmiR-BHRF1 viruses at a multiplicity of infection (MOI) of 0.1 infectious particles per B cell. Similar results were obtained, the IFN-α secretion was increased after EBV miR-BHRF1 deletion (Figure 5.9A-C), suggesting that B95-8 and M81 behave similarly. Interestingly, the B95-8 genome carries a large deletion of the BART miRNA locus but this deletion had no impact on IFN-α release. This confirms that mainly the BHRF1 locus and not the BART locus modulates the IFN response.

Taken together, all these results suggest that the EBV miR-BHRF1 locus is involved in the downregulation of IFN-α production in EBV-infected primary B cells, confirming that the EBV miRNAs control innate immune evasion in EBV-infected primary B cells.

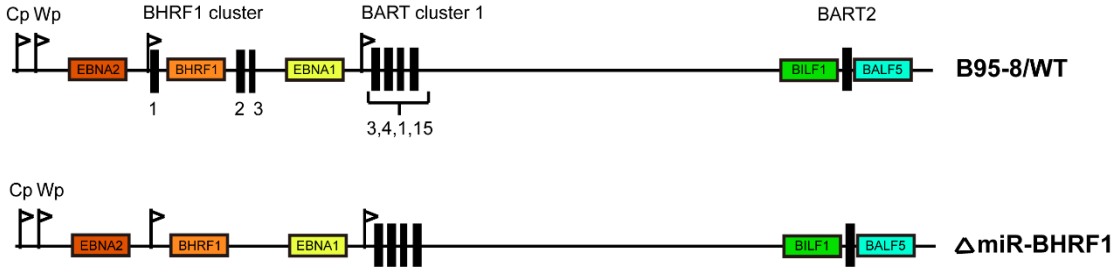


Fig. 5.8 Schematic map of B95-8/WT and B95-8/ΔmiR-BHRF1 EBV strains. More information about EBV mutants construction could be obtained in (Feederle et al. 2011a).

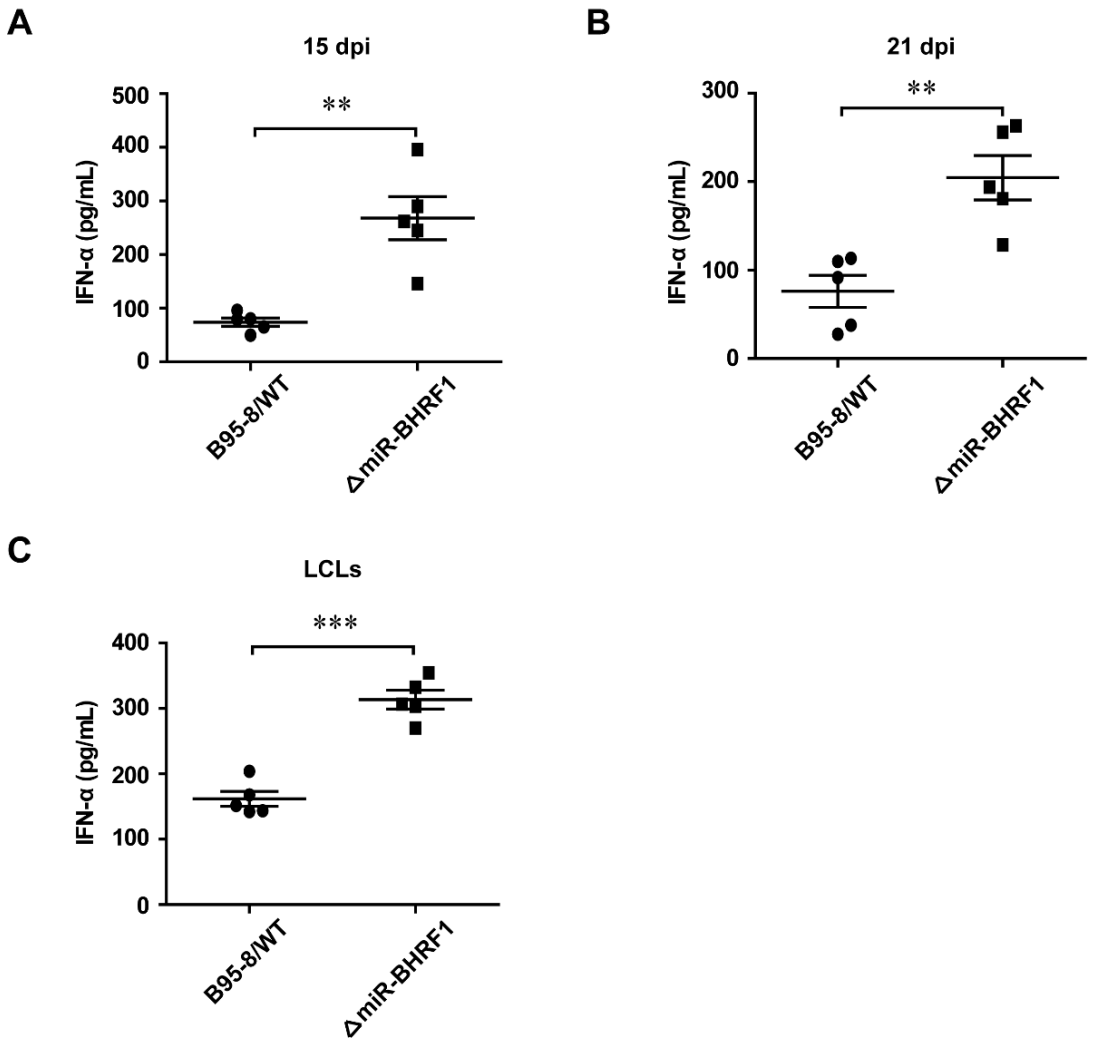


Fig. 5.9A-C Primary B cells isolated from PBMCs were infected with B95-8/WT or B95-8/ΔmiR-BHRF1 mutant respectively. At 15 days, 21 days or more than 28 days post infection, culture

supernatants were collected and cell numbers were counted again. The release of IFN-α was analyzed by using ELISA. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

5.3 EBV miR-BHRF1-2 was mainly responsible for the inhibition of IFN-α production in EBV-infected primary B cells

5.3.1 The production of IFN-α was increased after the deletion of miR-BHRF1-2 in EBV-infected primary B cells

It has been reported that all three BHRF1 pre-miRNAs are present and highly expressed in host cells with a latency III pattern of the viral antigen expression (Bernhardt et al. 2016). There are mainly four members of EBV mature BHRF1 miRNAs, including that miR-BHRF1-1, miR-BHRF1-2 that contains miR2 (miR-BHRF1-2-3p) and miR2* (miR-BHRF1-2-5p), and miR-BHRF1-3. Details of construction of these single miRNA-deletion mutants were already described in previously published paper (Feederle et al. 2011a).

I aimed to figure out which of these three pre-miRNAs contributes to the downregulation of type I interferons release in EBV-infected primary B cells. Therefore, the concentration levels of IFN-α secreted by primary B cells infected with B95-8/WT or different single miR-BHRF1 deletion mutants were analyzed. It was observed that there was no significant change in the production of IFN-α secreted by EBV-infected

primary B cells infected with a virus that lacks miR-BHRF1-1 or miR-BHRF1-3. However, EBV miR-BHRF1-2 exhibited a powerful role in the downregulation of IFN-α secretion in EBV-infected primary B cells (Figure 5.10A-C).

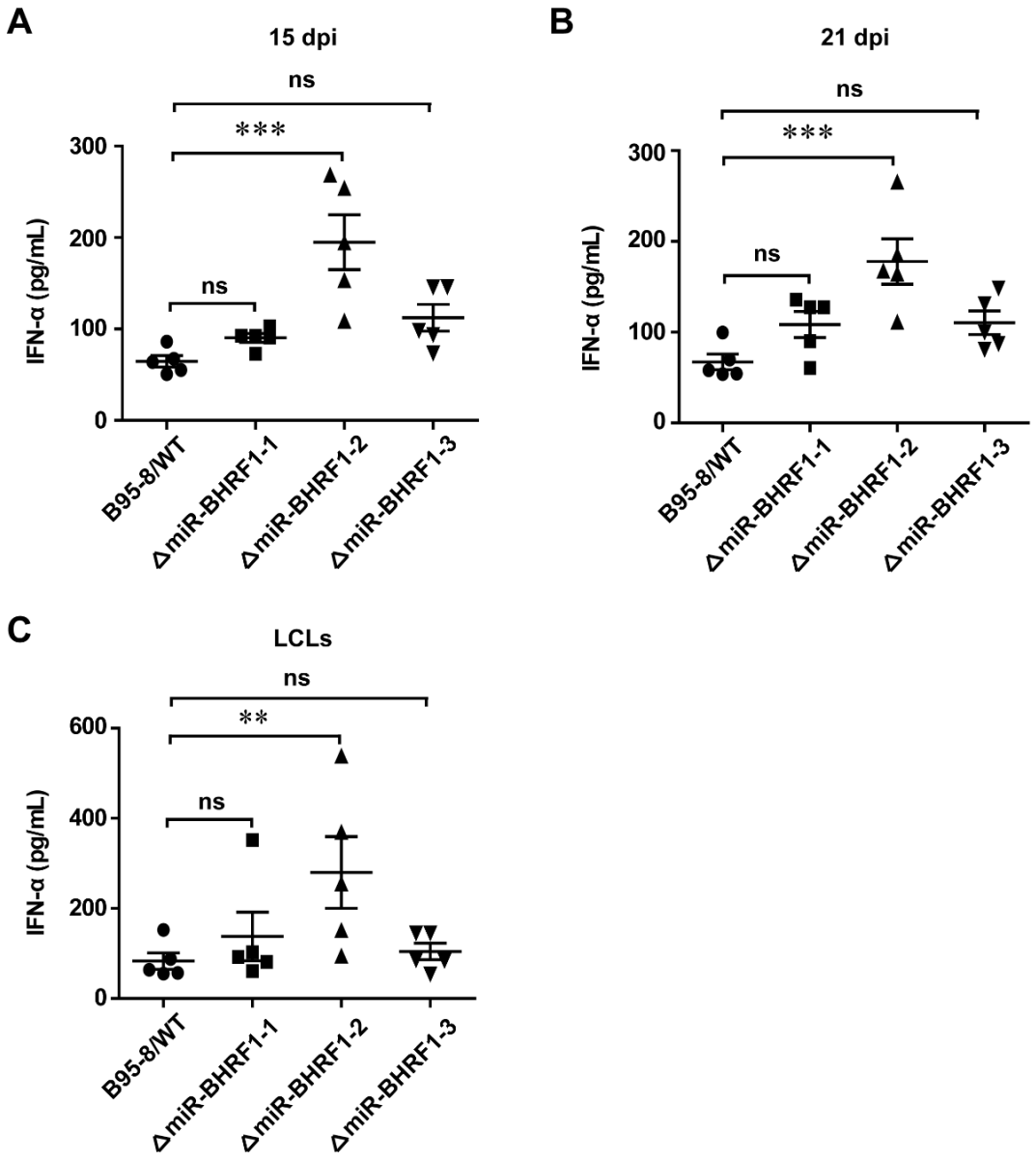


Fig. 5.10A-C Primary B cells were infected with B95-8/WT or different single ΔmiR-BHRF1 mutants respectively. At 15 days, 21 days or more than 28 days post infection, the culture supernatants were collected and cell numbers were counted again. The release of IFN-α was

analyzed by using ELISA. Data are representative of three independent experiments and ANOVA was used for more than two groups (*P-value < 0.05; **P-value < 0.01; ***P-value < 0.001; ns, non-significant).

5.3.2 Rescue experiments by reintroducing miR-BHRF1-2 into LCLs

I then attampted to complement the phenotype of LCLs transformed by B95-8/ ΔmiR-BHRF1-2 by transfecting them with a plasmid that encodes miR-BHRF1-2, as well as a truncated nerve growth factor receptor (NGFR) gene which is expressed at the cell surface of transfected cells, or with a control vector plasmid. After purifying transfected cells with NGFR antibody, qPCR was first used to confirm the miR-BHRF1-2 expression (Figure 5.11A), and ELISA was subsequently performed with the same cells to evaluate the secretion of IFN-α. The existence of miR-BHRF1-2 could dramatically decrease the expression of IFN-α (Figure 5.11B).

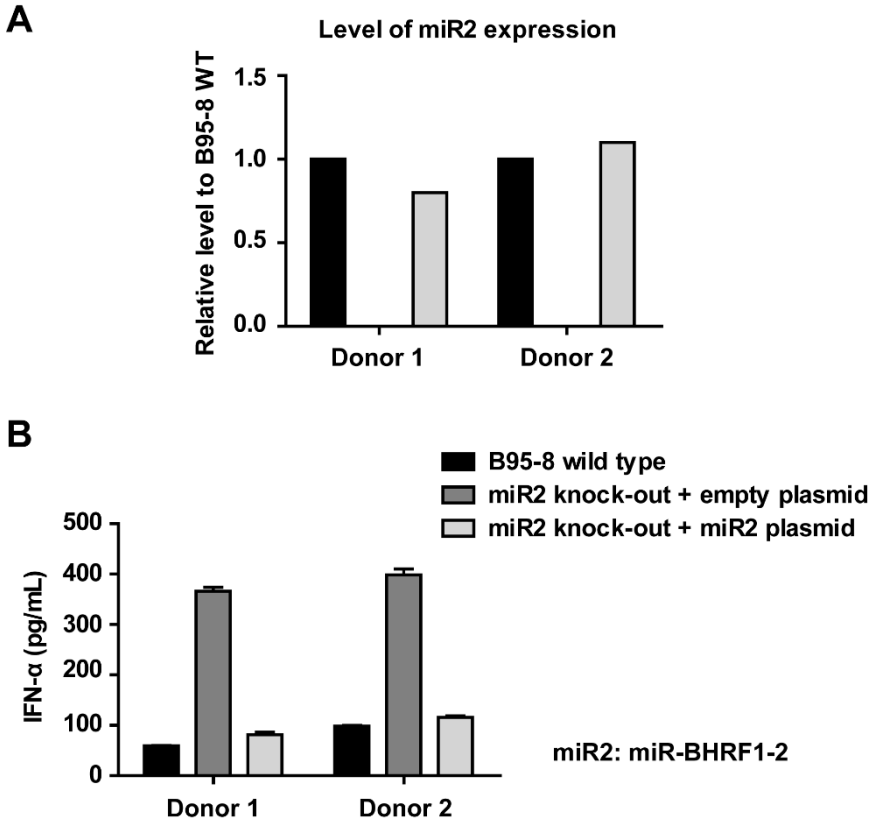


Fig. 5.11 LCLs transformed by B95-8/ΔmiR-BHRF1-2 EBV viruses were stably transfected with a plasmid that encodes a truncated form of NGFR and miR-BHRF1-2 or with an empty plasmid. The NGFR-positive cells were then purified by using the NGFR antibody. We determined the miR-BHRF1-2 expression in these cells relative to B95-8/WT-transformed LCLs (A) and their IFN-α expression (B). Data are representative of three independent experiments.

Until now, I have determined the identifications and characterizations of EBV miRNAs in EBV-induced innate immune responses. EBV miR-BHRF1-2 contributes to help establishing the EBV latent infection by suppressing the release of IFN-α in EBV-infected primary B cells. However, to reveal the molecular mechanism, viral and cellular targets of EBV miR-BHRF1-2 need to be examined.

5.4 LMP1 was not involved in the inhibition of IFN-α secretion by EBV miR-BHRF1-2 in EBV-infected B cells

Scientists have found that the EBV oncoprotein, latent membrane protein 1 (LMP1) is indispensable for B-cell transformation. LMP1 is highly expressed in a large majority of EBV-associated malignancies (Kieser and Sterz 2015). It has been found that LMP1 was strongly upregulated upon the induction of viral lytic replication (Ahsan et al. 2005). During latency, LMP1 has been reported to induce the expression of IRF7, which is the main transcriptional factor of IFN-α promoter and promote IRF7 phosphorylation and its nuclear translocation (Ning et al. 2011). Meanwhile, previous work has shown that EBV miRNAs could dramatically decrease both mRNA and protein expression levels of LMP1 in EBV-infected B cells (Albanese et al. 2016; Skalsky et al. 2014).

I hypothesized that there might be a potential regulatory circuit between miR-BHRF1-2 and LMP1. To reveal the unclear mechanism that IFN-α was downregulated by miR-BHRF1-2, the protein expression level of LMP1 between B95-8/WT- and B95-8/ΔmiR-BHRF1-2-infected B cells at different infection time points was detected. Immunoblots were performed at 15 days, 21 days or more than 28 days post infection. However, there was no significant difference in LMP1 protein expression levels between B95-8/WT- and B95-8/ΔmiR-BHRF1-2-infected B cells at all investigated infection time points, indicating that LMP1 has no influence on the inhibition of IFN-α secretion by miR-BHRF1-2, as shown in Figure 5.12A-C (left part), even though there was still a striking difference of IFN-α secretion between these two groups (right part).

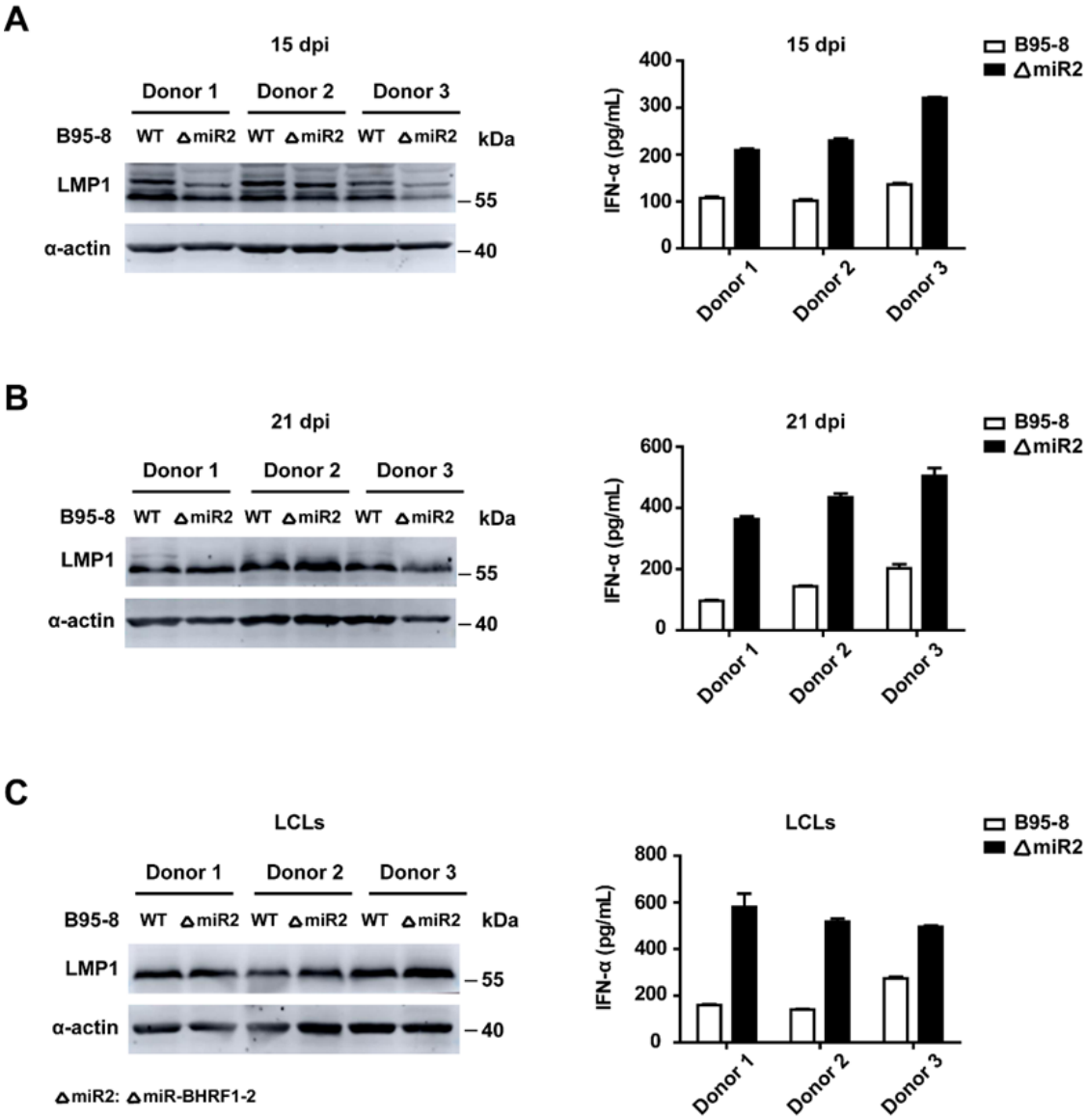


Fig. 5.12A-C Primary B cells isolated from PBMCs of three donors were stimulated with B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant viruses for the indicated times. The cell lysates were subjected to immunoblot analysis with indicated antibodies. The culture supernatants were collected and cell numbers were counted again. The release of IFN-α was analyzed by using ELISA. Data are representative of three independent experiments.

5.5 EBV miR-BHRF1-2 downregulated the production of IFN-α by targeting RIG-I, a dsRNA virus sensor

5.5.1 Proteomic analysis

All results gathered so far led me to conclude that EBV miR-BHRF1-2 plays an important role in repressing IFN-α secretion in EBV-infected primary B cells. I aimed to reveal the underlying mechanistic link between miR-BHRF1-2 expression and IFN-α inhibition in EBV-infected B cells. To this end, three panels of LCLs infected with B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant viruses were generated and subjected to a proteomic analysis.

As a result of statistical analysis, all cellular potential targets of EBV miR-BHRF1-2 were summarized in Table 7 (upregulated proteins) and in Table 8 (downregulated proteins). RIG-I (DHX58), a dsRNA virus-cytosolic receptor, was upregulated in B95-8/ΔmiR-BHRF1-2 mutant-transformed LCLs.

Table 7: Proteins that are upregulated

Gene name	Protein name
BIN2	Bridging integrator 2
LAMP3	Lysosome-associated membrane glycoprotein 3
CR1	Complement receptor type 1
SLC25A22	Mitochondrial glutamate carrier 1
DPM1	Dolichol-phosphate mannosyltransferase subunit 1
DHX58	Probable ATP-dependent RNA helicase DHX58 (RIG-I)
ZFYVE19	Abscission/NoCut checkpoint regulator
CC2D1B	Coiled-coil and C2 domain-containing protein 1B
FCER2	Low affinity immunoglobulin epsilon Fc receptor
SLC35A4	Probable UDP-sugar transporter protein SLC35A4
CLIC2	Chloride intracellular channel protein 2
TOM1	Target of Myb protein 1
HIP1R	Huntingtin-interacting protein 1-related protein
GRN	Granulins; Acrogranin
ARFIP1	Arfaptin-1
TANGO6	Transport and Golgi organization protein 6 homolog
PHPT1	14 kDa phosphohistidine phosphatase
STK17A	Serine/threonine-protein kinase 17A
MYH4; MYH1	Myosin-4; Myosin-1
SNAPIN	SNARE-associated protein Snapin
ITGAM	Integrin alpha-M
CHTOP	Chromatin target of PRMT1 protein
EMC3	ER membrane protein complex subunit 3

Table 8: Proteins that are downregulated

Gene name	Protein name
KIAA1524	Protein CIP2A
CHD8	Chromodomain-helicase-DNA-binding protein 8
PCCB	Propionyl-CoA carboxylase beta chain, mitochondrial
NOCR1	Nuclear receptor corepressor 1
SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3
CAPN2	Calpain-2catalytic subunit
GNPDA1	Glucosamine-6-phosphate isomerase 1
CASP4	Caspase-4
METAP1	Methionine aminopeptidase 1
GLE1	Nucleoporin GLE1
FCRLA	Fc receptor-like A
GHDC	GH3 domain-containing protein
SIGMAR1	Sigma non-opioid intracellular receptor 1
CIT	Citron Rho-interacting kinase
NSUN4	5-methylcytosine rRNA methyltransferase NSUN4
STK38	Serine/threonine-protein kinase 38
OXSM	3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial

5.5.2 Protein level of RIG-I was increased by the deletion of EBV miR-BHRF1-2 in EBV-infected B cells

EBV infection in primary B cells induces the activation of multiple cellular signaling pathways that lead to the production of type I IFNs and the transcription of ISGs. It has been reported that EBV-encoded non-coding RNAs, such as EBERs, can be recognized by RIG-I, TLR3 or TLR7, finally resulting in the production of type I IFNs and proinflammatory cytokines (Iwakiri and Takada 2010; Li et al. 2019; Samanta et al. 2006). Retinoic acid-inducible gene I (RIG-I) is encoded by the DDX58 gene in human beings and it is one of family members of the RIG-I like receptors (RLRs) that also comprise Melanoma Differentiation-Associated protein 5 (MDA5) and Laboratory of genetics physiology 2 (LGP2) (Kell and Gale 2015). As a cytosolic protein, RIG-I recognizes viral double-stranded RNAs (dsRNAs) in the cytosolic and initiates IRF3/7 signaling pathways leading to the induction of type I IFNs.

I hypothesized that EBV miR-BHRF1-2 might target RIG-I and inhibit RIG-I-induced signaling pathways in EBV-infected cells. Hence, the protein expression level of RIG-I in B95-8/WT- or B95-8/ΔmiR-BHRF1-2 mutant-transformed LCLs was analyzed by performing a western blot. It was found that RIG-I protein level was increased by miR-BHRF1-2 deletion in EBV-transformed LCLs, as shown in Figure 5.13.

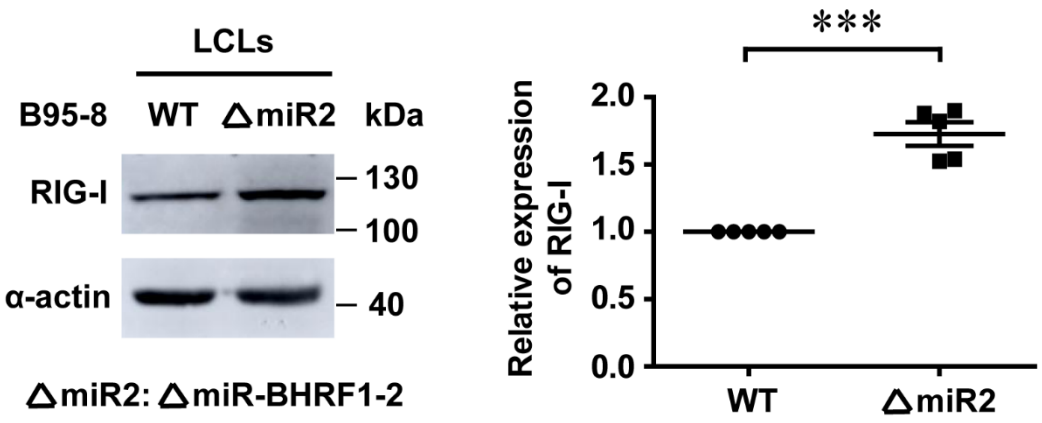


Fig. 5.13 Immunoblot analysis was performed on LCLs transformed with B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant viruses with antibodies specific for RIG-I and α-actin. The graph shows the relative intensity of the signals quantified by the ImageJ software. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

To confirm this observation, an immunoblot analysis of EBV-infected primary B cells at the early infection phase was further performed. Primary B cells were isolated from PBMCs and subsequently exposed to B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant viruses respectively for 5 days. It was found that the protein level of RIG-I was correspondingly increased in B95-8/ΔmiR-BHRF1-2 mutant-infected B cell at 5 days post infection, as indicated in Figure 5.14.

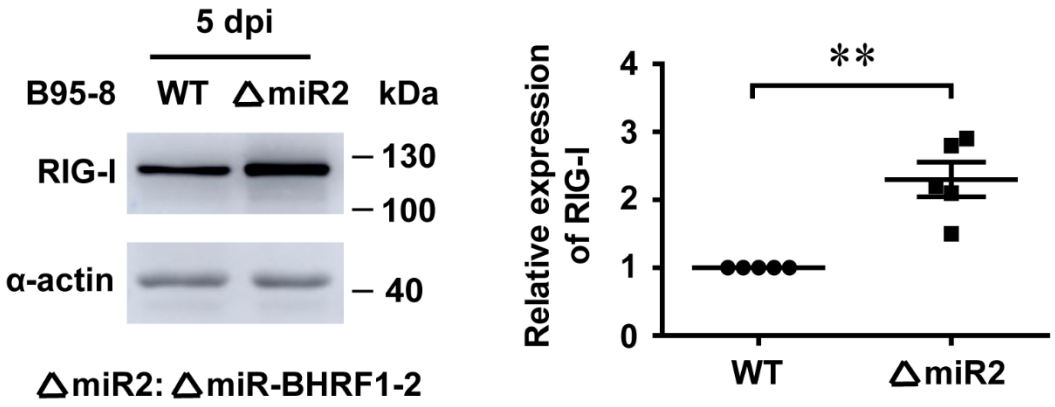


Fig. 5.14 Immunoblot analysis was performed on primary B cells infected with B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant viruses with antibodies specific for RIG-I and α-actin. The graph shows the relative intensity of the signals quantified by the ImageJ software. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

5.5.3 Protein level of RIG-I was decreased after the reintroduction of EBV miR-BHRF1-2 in LCLs

To confirm the previous observation that EBV miR-BHRF1-2 could downregulate the protein level of RIG-I, the expression of RIG-I protein was studied by using B95-8/ΔmiR-BHRF1-2 mutant-transformed LCLs that were then transfected with a plasmid that encodes EBV miR-BHRF1-2. LCLs were analyzed for RIG-I expression by performing a western blot. As expected, the protein level of RIG-I was substantially decreased after the reintroduction of miR-BHRF1-2 in B95-8/ΔmiR-BHRF1-2-transformed LCLs, as shown in Figure 5.15.

In this chapter, it can be concluded that EBV miR-BHRF1-2 contributes to inhibit IFN-

α production in EBV-infected primary B cells by targeting RIG-I and downregulating its protein expression.

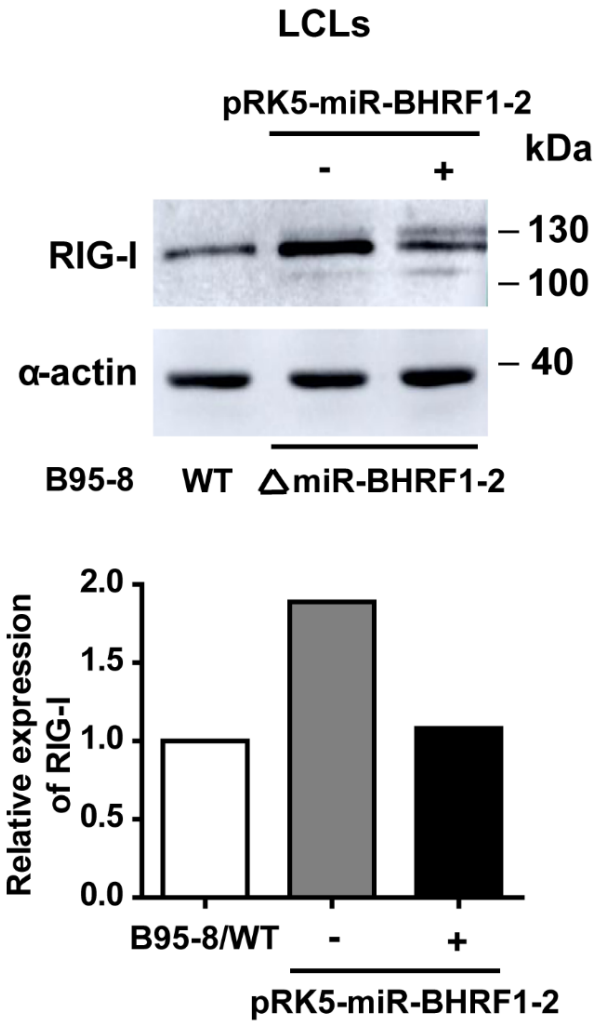


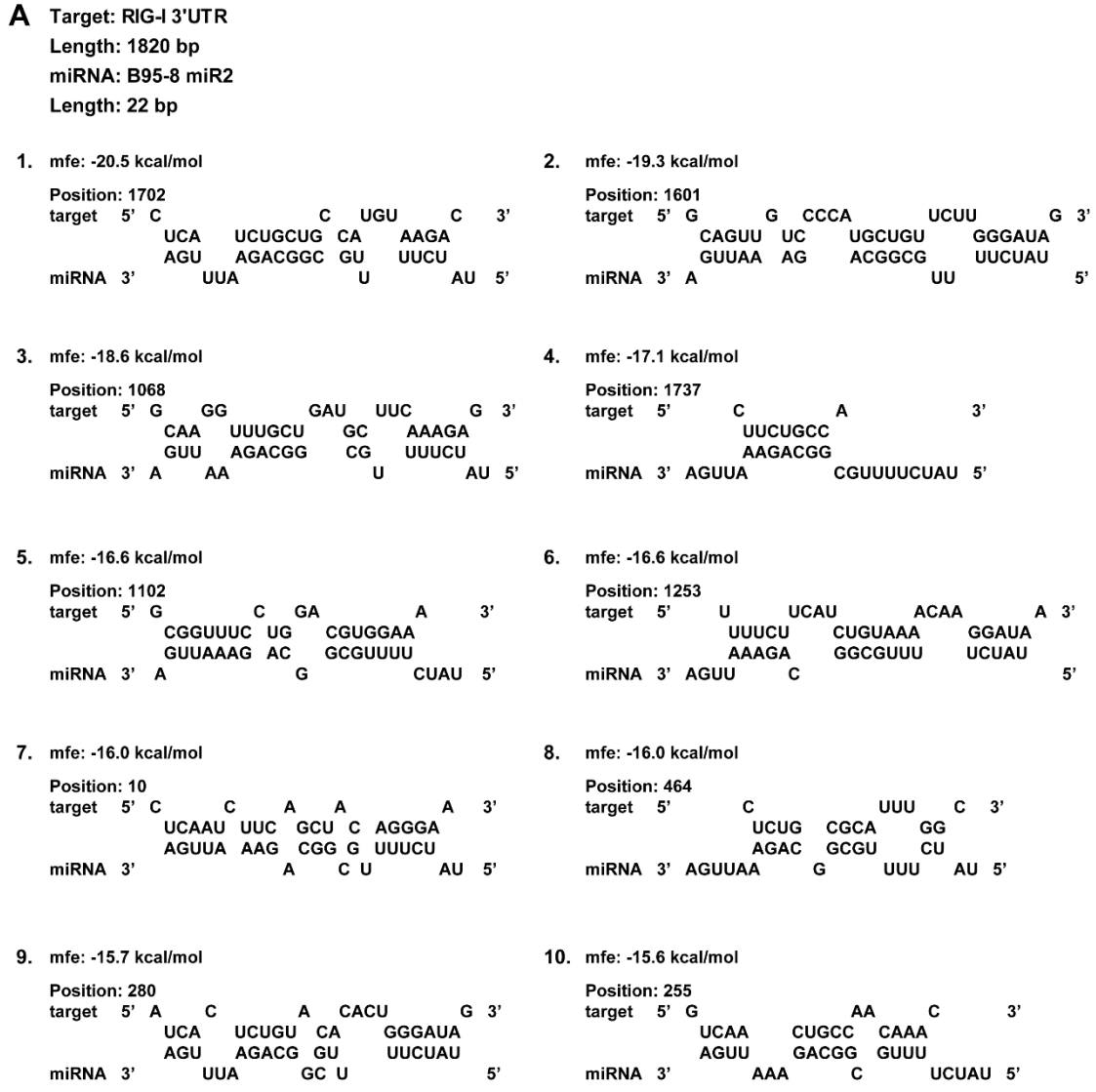
Fig. 5.15 Immunoblot analysis was performed on LCLs transformed with B95-8/WT or B95-8/ΔmiR-BHRF1-2 mutant that were then transfected with control vector or miR-BHFR1-2 plasmid later with antibodies specific for RIG-I and α-actin. The graph of bars shows the relative intensity of the signals quantified by the ImageJ software.

5.5.4 EBV miR-BHRF1-2 downregulated RIG-I protein level by targeting its 3'UTR for degradation

The predicted RNA-RNA interaction sites between miR-BHRF1-2 and the RIG-I 3'UTR were analyzed with the online software RNAhybrid "Fast and effective prediction of microRNA/target duplexes" (2004). The best canonical binding sites usually have a perfect match of the seed nucleotides 2 to 8 and also have a free energy of <-27 kcal/mol. However, for the non-canonical binding sites, there are different possibilities with less conserved binding of the seed sequence but then more binding in the 3' terminal of the miRNA. In this situation, the free energy of the duplex miRNA/mRNA can be less than -20 kcal/mol. It was confirmed that there were multiple possible non-canonical binding sites for miR2 (miR-BHRF1-2-3p) and the RIG-I 3'UTR, and for miR2* (miR-BHRF1-2-5p) and the RIG-I 3'UTR. Here I listed all predicted interaction regions of the RIG-I 3'UTR with the EBV miR2 (miR-BHRF1-2-3p) or with the EBV miR2* (miR-BHRF1-2-5p), as indicated in Figure 5.16A and B.

To further confirm the interaction between miR-BHRF1-2 and the RIG-I 3'UTR. The 3'UTR of RIG-I was cloned into a firefly luciferase reporter plasmid (pGL4.5), and the RIG-I 3'UTR reporter plasmid was co-transfected into HEK293 cells with a negative control or a miR-BHRF1-2 expression plasmid. It was observed that miR-BHRF1-2 could mediate an inhibition of the luciferase expression compared to the control group, as shown in Figure 5.17. Subsequently, the miR2 or the miR2* seed match sites were mutated to disrupt the miRNA binding ability (Figure 5.18A). It was found that miR2 (miR-BHRF1-2-3p) but not miR2* (miR-BHRF1-2-5p) could considerably reduce the

RIG-I 3'UTR activity, while mutation of its binding site abolished this inhibitory activity, as shown in Figure 5.18B.



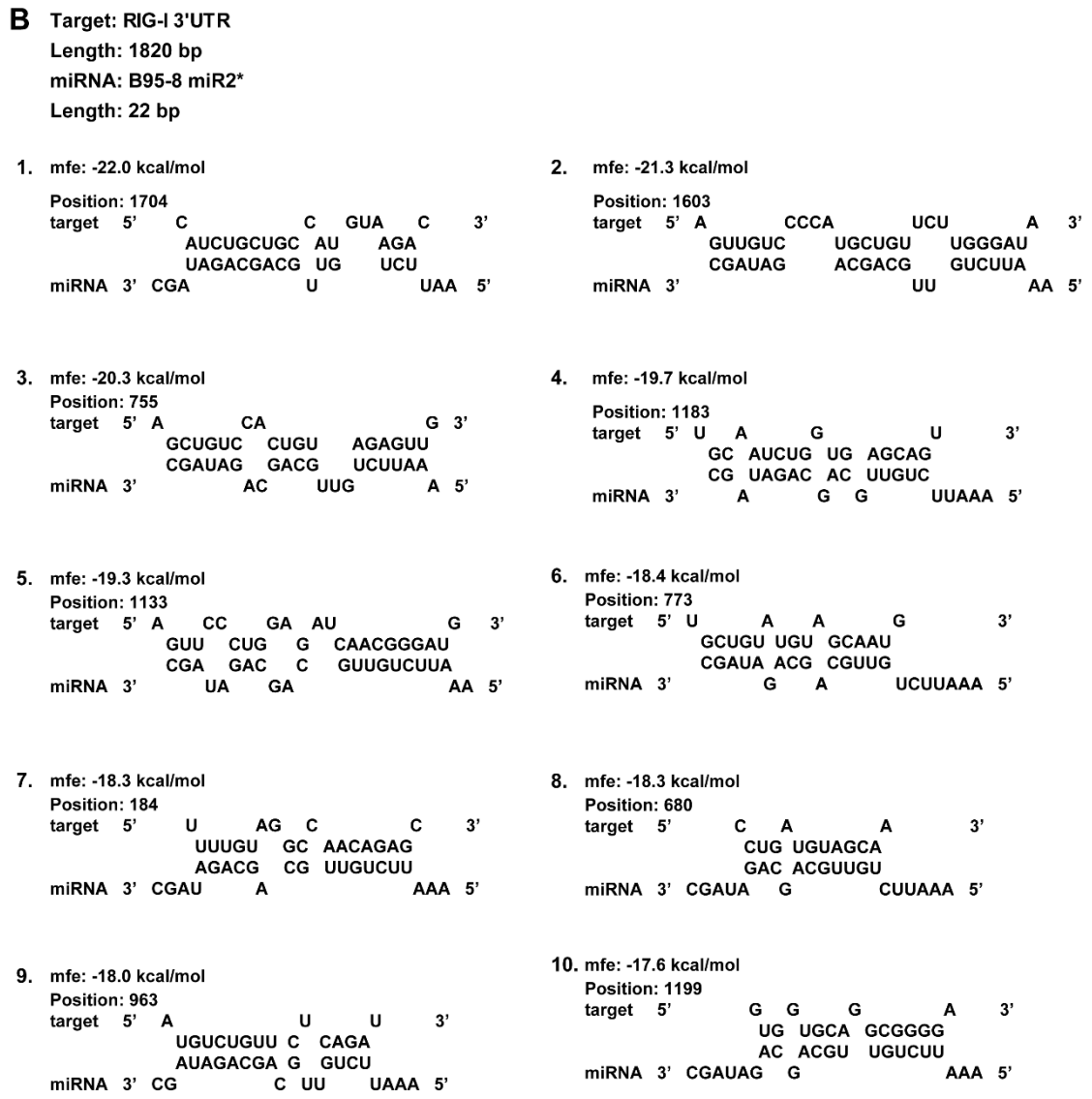


Fig. 5.16 Alignment of miR2 (miR-BHRF1-2-3p) (A) or miR2* (miR-BHRF1-2-5p) (B) with their predicted target sites in the 3'UTR of RIG-I.

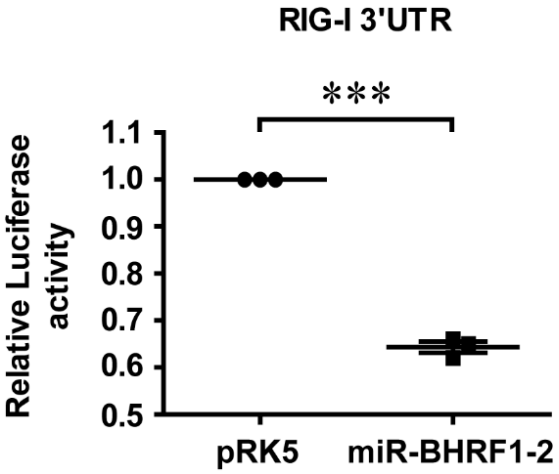


Fig. 5.17 Luciferase reporter assays were performed. The RIG-I 3'UTR plasmid was co-transfected into HEK293 cells with miR-BHRF1-2 expression plasmid or an empty pRK5 negative control. After 48 h, cell lysate was analyzed for the luciferase activity. Data are representative of three independent experiments and all the *p* values are obtained from paired student's *t*-tests (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

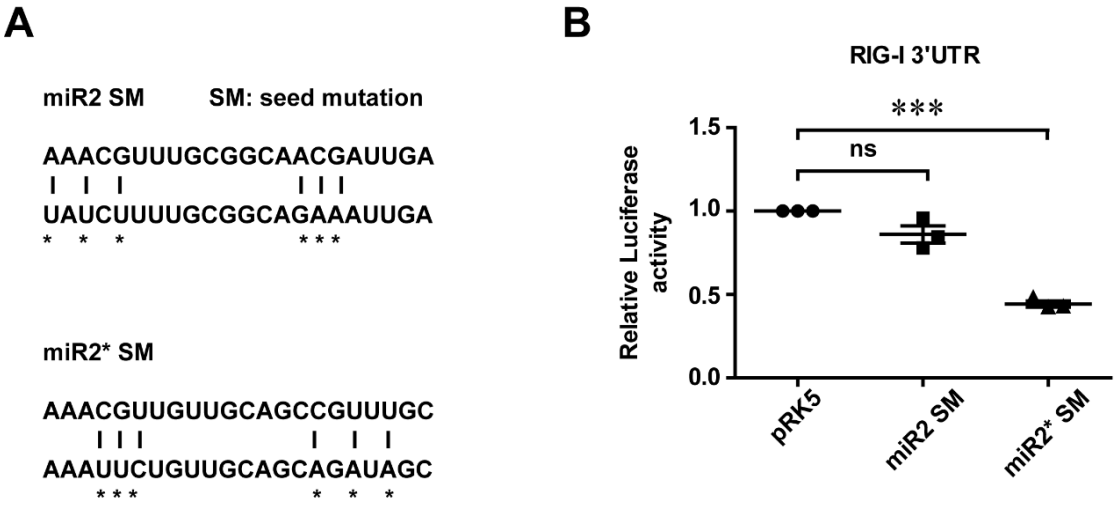


Fig. 5.18 (A) Schematic maps of miR2 seed mutant and miR2* seed mutant. (B) The RIG-I 3'UTR plasmid was co-transfected into HEK293 cells with miR2 SM, miR2* SM or an empty pRK5

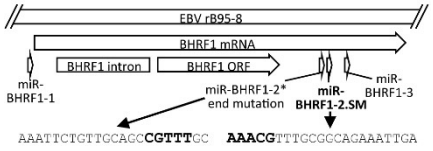
negative control. After 48 h, cell lysate was analyzed for the luciferase activity. Data are representative of three independent experiments and ANOVA was used for more than two groups (*P-value < 0.05; **P-value < 0.01; ***P-value < 0.001; ns, non-significant).

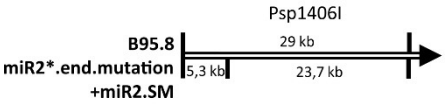
I also constructed another two EBV recombinant viruses that carry a seed mutation in miR2 or miR2*. These recombinants DNA was then stably introduced into HEK293 cells to generate virus producer cells. The integrity of the EBV genome in the stably expressed HEK293 cell clones was confirmed by transporting the viral episome that they harbored into *E. coli* cells and enzyme restriction analysis was then performed (Figure 5.19A, B).

B95-8/WT, B95-8/miR2 SM and B95-8/miR2* SM viruses were subsequently used to infect primary B cells and the expression levels of IFN-α secreted by primary B cells infected with B95-8/WT, B95-8/miR2 SM or B95-8/miR2* SM viruses were assessed. At 15 days, 21 days or more than 28 days post infection, the expression level of IFN-α secreted by EBV-infected primary B cells in B95-8/miR2 SM group was higher than that in other groups, as indicated in Figure 5.20A-C, confirming my previous findings that miR2 was responsible for the inhibition of the luciferase activity of RIG-I 3'UTR.

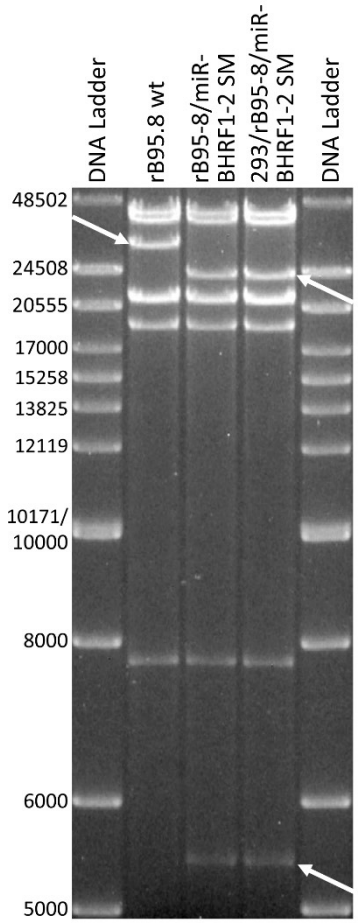
A **B95-8/miR2 SM**

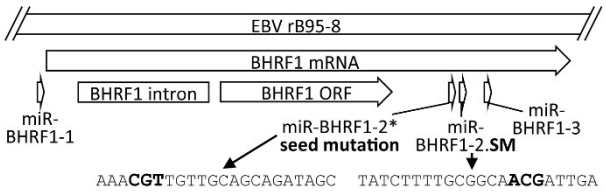
B95-8/miR-BHRF1-2 seed mutant (B555)





Identical BamHI pattern for wt and mutant, Psp1406I site created in the seed mutation.





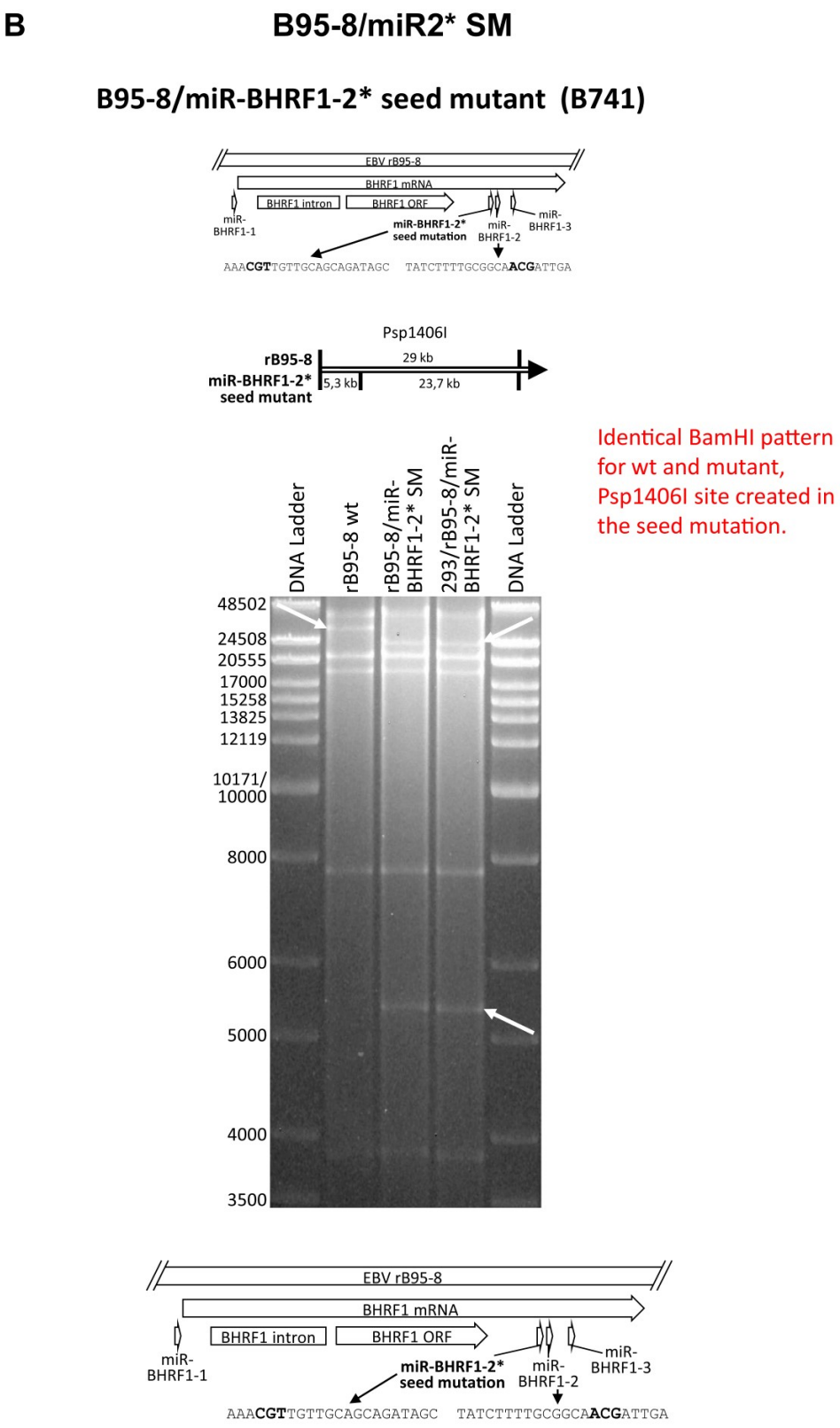


Fig. 5.19 Restriction enzyme analysis of the BAC miniprep shows restriction patterns of B95-8/miR2 seed mutant (A) and B95-8/miR2* seed mutant (B). Viral genome was extracted from virus

producer cells and cleaved with restriction enzyme Psp1406 I, then separated on an 0.8% agarose gel. The parental B95-8/WT genome was loaded as a control and arrows indicate the viral DNA fragments whose sizes differ between the B95-8/WT and this mutant.

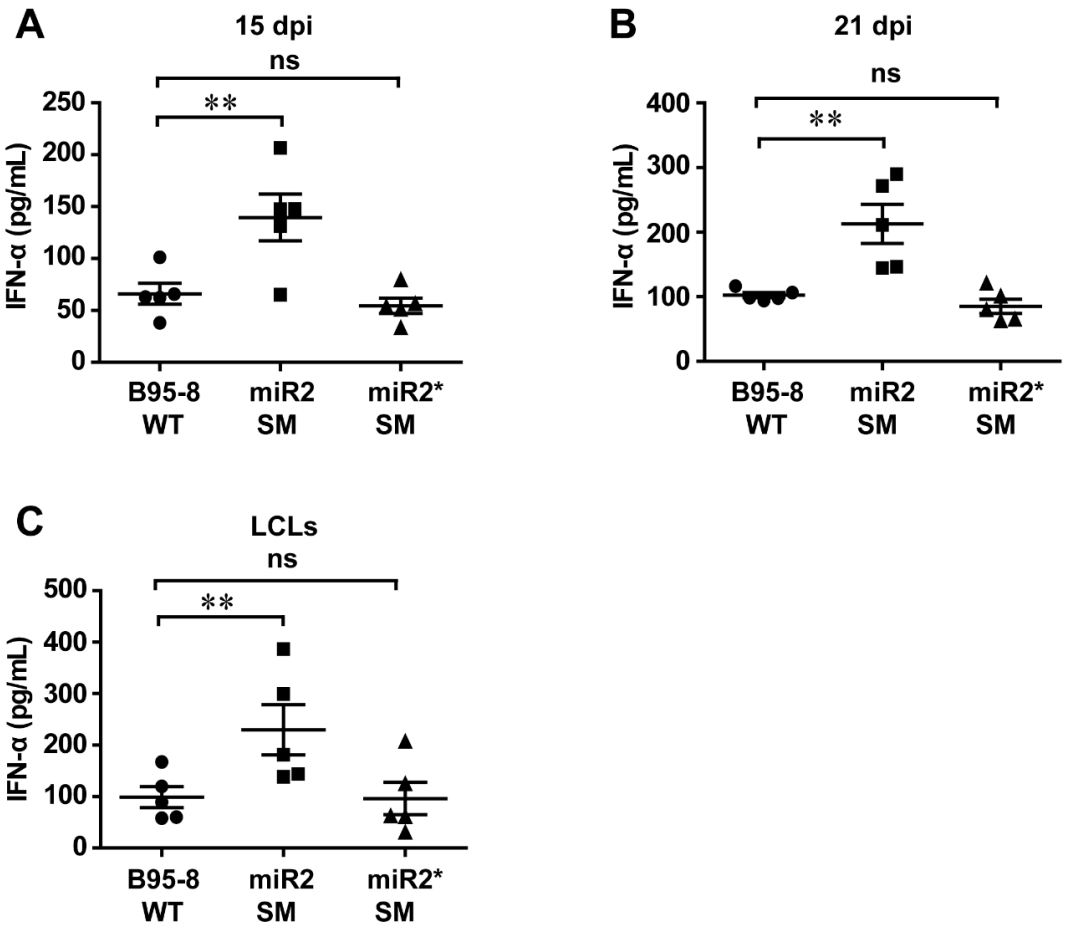


Fig. 5.20A-C Primary B cells isolated from PBMCs were infected with B95-8/WT, B95-8/miR2 SM or B95-8/miR2* SM respectively. At 15 days, 21 days or more than 28 days post infection, culture supernatants were collected and cell numbers were counted again. The release of IFN-α was analyzed by using ELISA. Data are representative of three independent experiments and ANOVA was used for more than two groups (**P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; ns, non-significant).

In an attempt to demonstrate that RIG-I is directly targeted by miR-BHRF1-2, I then constructed six different luciferase-RIG-I 3'UTR reporter mutants in which predicted RIG-I 3'UTR-miR2 binding sites were mutated. According to the statistical analysis, mutations of its binding sites 1024-1029 or 1087-1090 could slightly affect this inhibitory activity of RIG-I 3'UTR by miR-BHRF1-2, as shown in Figure 5.21. However, their effects were very limited, even though there was a statistical difference between RIG-I 3'UTR wild type and these two mutants. It means that the direct interaction between RIG-I 3'UTR and miR-BHRF1-2 remains to be demonstrated.

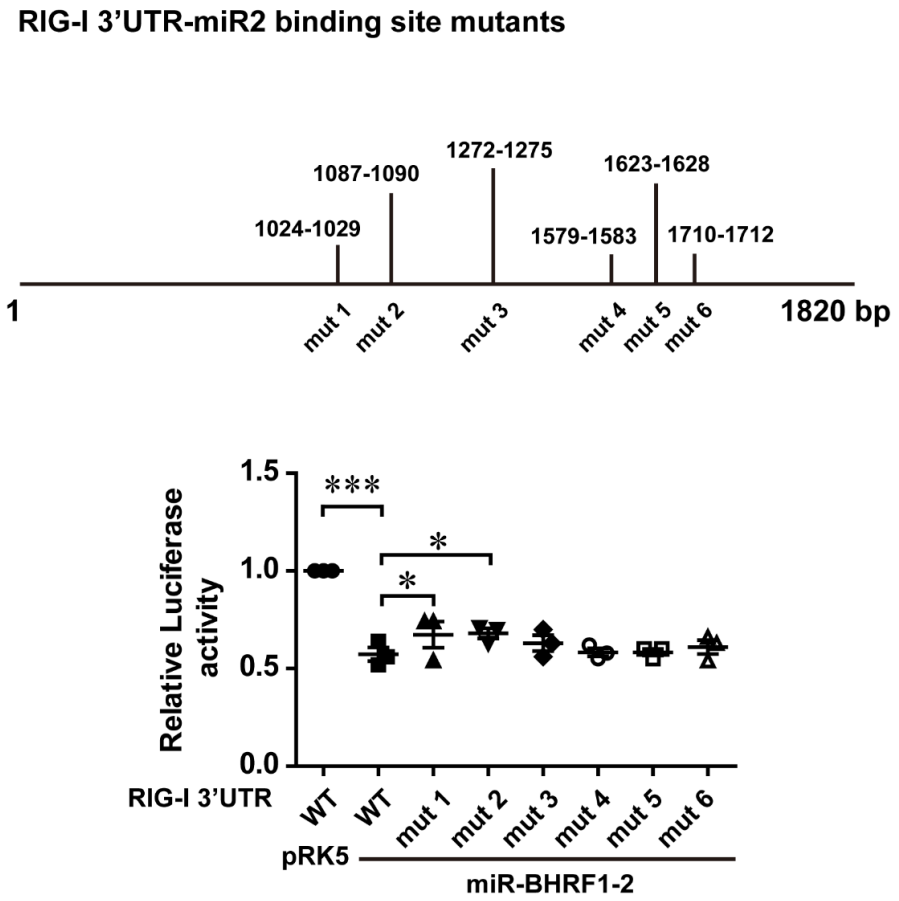


Fig. 5.21 Schematic map of luciferase-RIG-I 3'UTR reporter mutants (top graph). The RIG-I 3'UTR

wild type plasmid or different mutants were co-transfected into HEK293 cells with miR-BHRF1-2 expression plasmid or an empty pRK5 negative control. After 48 h, cell lysate was analyzed for the luciferase activity. Data are representative of three independent experiments and ANOVA was used for more than two groups (*P-value < 0.05; **P-value < 0.01; ***P-value < 0.001; ns, non-significant).

6. Discussion

It has been nearly twenty years since the first identification of EBV miRNAs. Initially, the expression of three BHRF1 miRNAs (miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3) and two BART miRNAs (miR-BART1 and miR-BART2) was identified by cloning the small RNAs from a Burkitt's lymphoma cell line (Pfeffer et al. 2004). Over the last ten years, additional thirty-nine EBV miRNAs have been identified (Skalsky 2017). However, our understanding of their functions is still incomplete. In my project, it was found that M81 EBV miRNAs were involved in the regulation of innate immune responses. I further confirmed it was the miR-BHRF1 cluster that participates in the antiviral immune evasion by downregulating the production of IFN-α. This property was shared by the B95-8 EBV strain, showing that it applies to different viral isolates. By infecting B cells with different single miR-BHRF1 deletion mutants, I could show that miR-BHRF1-2 played the most dominant role in the inhibition of IFN-α release. By performing a proteomic analysis, EBV miR-BHRF1-2 was found to modify the expression of multiple cellular genes including RIG-I. RIG-I is a dsRNA virus sensor that is able to induce the IFN-α production in innate immune responses and EBV-encoded non-coding RNAs could regulate the RIG-I signaling pathway. I confirmed miR-BHRF1-2 could target the 3'UTR of RIG-I and lead to its degradation. Therefore, these results indicate that EBV miRNAs contribute to antiviral immune evasion through inhibiting the release of type I IFNs.

6.1 The connection between EBV miRNAs and innate immunity

Since miRNA lin-4 was first reported to regulate the *lin-14* gene expression in 1993, multiple host genes, including genes that play important roles in mediating immune responses, have been reported to be regulated by host cellular or pathogen-encoded miRNAs (Lee et al. 1993). This property of miRNAs renders them potential candidates in the management of immunity.

Host cellular miRNAs are double-edged sword that has contradictory abilities. On the one hand, they exhibited anti-pathogen functions, such as, miRNA-29 that has been reported to inhibit HIV replication in T cells (Pilakka-Kanthikeel et al. 2011). On the other hand, they can promote the progression of viral infection, e.g., miRNA-122 binds to the 5' end of HCV genome, leading to the increase of viral replication (Schult et al. 2018). More generally, pathogen-encoded miRNAs influence the replication and survival of pathogens in the host body by interfering with multiple physiological processes. Scientists have found that viruses could use the host cellular machinery to produce their own miRNAs which conversely promote viral replication and establishment of latency (Chandan et al. 2019). Herpesviridae is a family of DNA viruses and at least 130 herpesviruses have been identified in animals, including humans (Brown and Newcomb 2011). In total, nine different types of herpesviruses, such as herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), EBV, human cytomegalovirus (HCMV), have been discovered to infect humans. Herpesvirus-encoded miRNAs have been demonstrated to participate in almost every aspect of immune responses, including development, maturation, differentiation and activation

of various immune cells. Particularly, more and more evidence has confirmed that a large fraction of these miRNAs plays important roles in innate immunity.

By infecting primary B cells with an EBV recombinant strain that lacks all miRNA clusters, EBV miRNAs were found to manipulate innate immune responses at both early and late infection phases. Hereafter, different EBV recombinants in which miR-BHRF1 or miR-BART clusters were knocked out, have been engineered and used to infect primary B cells. Altogether, my observations indicated that miR-BHRF1 originated from M81 strain or B95-8 strain might play an important role in the secretion of IFN-α in EBV-infected B cells.

6.2 Characteristics of EBV miR-BHRF1 and their roles in the IFN-α signaling pathway

EBV miR-BHRF1 cluster includes three precursor miRNAs, miR-BHRF1-1, miR-BHRF1-2 and miR-BHRF1-3, which will finally produce four mature miRNAs. A large majority of previous studies have demonstrated that one of the most important functions of miR-BHRF1 is to facilitate the establishment and maintenance of persistent infection by EBV. For example, our lab has indicated that miR-BHRF1-2 and miR-BHRF1-3 contributed to EBV-mediated B cell transformation and the growth of LCLs, they also found the mutation of the miR-BHRF1-2 encoding region affects processing of the synthetic miR-BHRF1-3, indicating a more important role of miR-BHRF1-2 in the survival of EBV-infected B cells (Haar et al. 2016).

A substantial number of cellular mRNAs targeted by EBV-encoded miR-BHRF1 have

been identified in immunity. EBV miR-BHRF1-3 targeted CXCL11, a ligand of CXCR3, resulting in a reduced T cells recruitment (Xia et al. 2008). And miR-BHRF1-2 targeted immune checkpoint ligands PD-L1 and PD-L2 (Cristino et al. 2019). The role of EBV miR-BHRF1 in the regulation of adaptive immunity has been well established. However, increasing evidence indicated that miR-BHRF1 of EBV targeted the host cellular mRNAs involved in innate immune responses, revealing novel parts of the miR-BHRF1 cluster (Zidovec Lepej et al. 2020). In innate immunity, miR-BHRF1-2 affected the inflammatory response by indirectly targeting the IL-1 receptor (Skinner et al. 2017), miR-BHRF1-3 targeted VAV2, a key activator of the Card 9 pathway and Card 9 engaged in the control of the NF-κB signaling pathway, regulating the proinflammatory cytokines secretion (Bouvet et al. 2021; Roth et al. 2016).

In my project, I used three single miRNA-deletion mutants and termed as B95-8/ΔmiR-BHRF1-1, B95-8/ΔmiR-BHRF1-2 and B95-8/ΔmiR-BHRF1-3 individually. Primary B cells were subsequently treated with these EBV recombinant mutants to analyze their impact on IFN-α release. I have noticed that miR-BHRF1-2 significantly decreased the production of IFN-α in EBV-infected B cells. It means that miR-BHRF1-2 might play the most important role to suppress the production of IFN-α.

Hereafter, the negative contribution of miR-BHRF1-2 to IFN-α secretion was confirmed by the complementation assay. Here reintroduction of the B95-8/miR-BHRF1-2 in B95-8/ΔmiR-BHRF1-2-transformed LCLs resulted in a decreased level of IFN-α that was expressed at almost the same level as B95-8 wild type. More recently, Mickael Bouvet et al. used a reporter luciferase system to assess the role of individual

EBV miRNAs in the exogenous type I IFN response. This luciferase reporter plasmid p6898 contains an improved firefly luciferase (luc2) gene under control of a chimeric ISG Mx2 promotor and five canonical ISREs. They identified miR-BHRF1-2 and other three miR-BARTs significantly attenuated the response to exogenous type I IFNs through this functional screen (Bouvet et al. 2021). This is the first time that scientists began to address a correlation between EBV miR-BHRF1-2 and the IFN-α signaling pathway in B cells. However, the precisely cellular target of miR-BHRF1-2 was still unclear. All these findings suggested a pivotal role for miR-BHRF1-2 in the IFN-α synthesis and IFN-α-induced ISGs processing.

6.3 Interplay between LMP1 and miR-BHRF1-2

Interferon regulatory factor 7 (IRF7), a key regulator of IFN-α, was first identified in the context of latent EBV infection in 1997 (Zhang and Pagano 1997). It has been reported to be associated with EBV latency process with a high expression level in type III latency (Ning et al. 2011). The EBV oncoprotein, latent membrane protein 1 (LMP1) could increase IRF7 expression and promote its phosphorylation. Moreover, IRF7 notably increased both LMP1 mRNA and protein levels, suggesting a positive circuit between IRF7 and LMP1 (Ning et al. 2003). Induction and activation of IRF7 by LMP1 during EBV latency might lead to large amounts of IFN-α secretion.

LMP1 has been demonstrated to be regulated by EBV miRNAs in B cells or epithelial cells. For instance, BART cluster 1 miRNAs directly targeted the 3'UTR of LMP1 and negatively regulated its protein expression in NPCs (Lo et al. 2007). In EBV-infected

B cells, the transcript level of LMP1 was downregulated after the deletion of EBV miRNAs (Albanese et al. 2016). All these observations prompted me to investigate whether LMP1 participated in the inhibition of IFN-α by miR-BHRF1-2 or not. I tested proteins level of LMP1 between B95-8/WT- and B95-8/ΔmiR-BHRF1-2-infected B cells at different infection time points and found that LMP1 was not affected by miR-BHRF1-2 in my system, suggestting that LMP1 might be preferentially modulated by EBV BART miRNA clusters as previous studies have shown. However, it needs to be further explored whether the transcript or protein levels of LMP1 are modulated by miR-BHRF1-2 at the early infection phase or not.

Therefore, it is interesting to note that there might be another mechanism how the IFN-α secretion was regulated by miR-BHRF1-2.

6.4 RIG-I plays an important role during EBV infection

By performing PAR-CLIP and Crosslinking, ligation And Sequencing of Hybrids (CLASH), scientists have identified an abundance of cellular targets of EBV miRNAs. For EBV miR-BHRF1-2, there are hundreds of cellular targets in which their mRNA 3'UTR interact with miR-BHRF1-2. EBV miR-BHRF1-2 targeted MALT4, GRB2 SOS1, RAC1 and PAG1, leading to a strong disruption of BCR signaling and the growth of latently infected B cells (Chen et al. 2019). And it also targets PD-L1/2, IL12B, CSTB and TAP2, making miR-BHRF1-2 as an important regulator in adaptive immunity (Cristino et al. 2019; Iizasa et al. 2020). To know which cellular targets are involved in the downregulation of IFN-α by miR-BHRF1-2, I performed a proteomic

analysis instead of performing a PAR-CLIP. I found that dozens of genes' protein levels were upregulated or downregulated by the deletion of miR-BHRF1-2 in EBV-transformed LCLs. Among these genes that I found, SLC25A22, ARFIP1 and STK38L have been identified as cellular targets of miR-BHRF1-2 before, but their functions have been unclear.

Intriguingly, the proteomic analysis results have shown that protein level of RIG-I is increased in the absence of miR-BHRF1-2 in the EBV-transformed LCLs. RIG-I is considered as an important pattern recognition receptor in antiviral innate immune responses, it can recognize double-stranded RNA that are generated during the viral life cycle. EBV-encoded RNAs, EBERs have been shown to be recognized by RIG-I. After binding to RIG-I, RIG-I is activated and type I IFNs (IFN-α/β) are induced through IRF3/7 signaling. Previous studies have found that multiple EBV components can directly or indirectly target RIG-I for its degradation. Meanwhile, miRNA-mediated degradation of RIG-I is an important mechanism of the virus that negatively regulates RIG-I-mediated innate immune responses. Specific examples are miR-BART6-3p targets RIG-I in NPCs, miR-BART3 and miR-BART19 are both able to regulate the RIG-I 3'UTR during the early phase of EBV infection in primary B cells, which suggests that EBV miRNAs are indispensable in the establishment of EBV infection both in B cells or epithelial cells (Bouvet et al. 2021; Lu et al. 2017). Here, it is demonstrated that the inhibitory effect of EBV miR-BHRF1-2 on IFN-α secretion is achieved via the degradation of RIG-I. This is the first time that miR-BHRF1 has been found to participate in the regulation of RIG-I, indicating that in latency III, miR-

BHRF1 might play more important role in the RIG-I-induced IFN-α signaling pathway in comparison to miR-BART. I reconfirmed that primary B cells infected with a virus lacking miR-BHRF1-2 or LCLs transformed by EBV/ΔmiR-BHRF1-2 exhibit an increased RIG-I protein expression by western blot. EBV miR-BHRF1-2 was able to significantly downregulate the luciferase-RIG-I 3'UTR reporter gene and the inhibition activity of the luciferase-RIG-I 3'UTR reporter gene was abolished by using the plasmid containing miR-BHRF1-2-3p seed mutant. Meanwhile, mutations of luciferase-RIG-I reporter genes could also slightly affect this inhibition activity by miR-BHRF1-2, indicating that miR-BHRF1-2 might target RIG-I, but we could not demonstrate a direct interaction between them. Thus, further work is needed to define the interaction between RIG-I and miR-BHRF1-2.

Interestingly, in my findings, I found that the BHRF1 miRNAs cluster but not the BART miRNAs cluster influenced the IFN-α secretion by targeting RIG-I, which is inconsistent with the previous study that has shown the miR-BART cluster was mainly responsible for the downregulation of IFN-α secretion (Bouvet et al. 2021). In this paper, the authors detected the secretion of IFN-α after EBV miRNAs deletion in EBV-infected primary B cells at nine days post infection and then identified RIG-I as one of potential targets of miR-BART using Ago PAR-CLIP analysis and luciferase reporter assay. The major difference between the present and published experimental systems is that they used a reconstituted B95-8 wild type that is complemented by a 11.8 kb fragment containing the coding sequences of the large part of BART miRNAs, and a B95-8/ΔmiR-All mutant, which might account for the differences between studies.

More work is needed to clarify the relationship between the different EBV miRNAs and the RIG-I-induced IFN-α signaling pathway.

In conclusion, my results demonstrate a novel role of miR-BHRF1-2 as a candidate viral component for immune evasion in the RIG-I-induced IFN-α signaling pathway.

7. Summary

Epstein-Barr virus (EBV) was discovered in 1964 and was the first identified human tumor-associated virus. EBV has been found to cause 2% of malignant tumors worldwide and it is etiologically associated with various malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma. MicroRNAs (miRNAs) are small noncoding RNAs of 19-22 nt in length that post-transcriptionally regulate gene expression. EBV is the first human virus that has been reported to encode miRNAs, including 4 mature BHRF1 miRNAs and 40 mature BART miRNAs. EBV miRNAs can be expressed in all phases of its complicated life cycle and are potentially involved in the regulation of hundreds of genes. However, their characteristics are just beginning to emerge. In my project, I found that EBV miRNAs were involved in the inhibition of innate immune responses. Among all 44 miRNAs, EBV-encoded miR-BHRF1-2 was identified as a novel viral immune-evasion factor that inhibited IFN-α release. EBV miR-BHRF1-2 targeted RIG-I, a viral double-stranded RNA (dsRNA) sensor that mediates IFN-α production, inducing the downregulation of RIG-I protein expression in EBV-infected B cells. My results further demonstrate the important role of miR-BHRF1-2 in the antiviral immune evasion and establishment of EBV infection, which may provide new targets for the treatment of EBV-associated malignancies.

8. Zusammenfassung

Das Epstein-Barr-Virus (EBV) wurde 1964 entdeckt und war das erste identifizierte humane tumorassoziierte Virus. Es wurde festgestellt, dass EBV weltweit 2 % der bösartigen Tumore verursacht und ätiologisch mit verschiedenen malignen Erkrankungen wie Burkitt-Lymphom, Hodgkin-Lymphom und Nasopharynxkarzinom assoziiert ist. MicroRNAs (miRNAs) sind kleine nichtkodierende RNAs mit einer Länge von 19–22 nt, die die Genexpression posttranskriptionell regulieren. EBV ist das erste menschliche Virus, von dem berichtet wurde, dass es miRNAs kodiert, darunter 4 reife BHRF1-miRNAs und 40 reife BART-miRNAs. EBV-miRNAs können in allen Phasen seines komplizierten Lebenszyklus exprimiert werden und sind möglicherweise an der Regulation von Hunderten von Genen beteiligt. Ihre Eigenschaften fangen jedoch gerade erst an, sich herauszubilden. In meinem Projekt fand ich heraus, dass EBV-miRNAs an der Hemmung der angeborenen Immunantwort beteiligt sind. Unter allen 44 miRNAs wurde EBV-kodiertes miR-BHRF1-2 als neuartiger viraler Immunevasionsfaktor identifiziert, der die Freisetzung von IFN-α hemmt. EBV miR-BHRF1-2 zielte auf RIG-I ab, einen viralen Sensor für doppelsträngige RNA (dsRNA), der die IFN-α-Produktion vermittelt und die Herunterregulierung der RIG-I-Proteinexpression in EBV-infizierten B-Zellen induziert. Meine Ergebnisse zeigen ferner die wichtige Rolle von miR-BHRF1-2 bei der antiviralen Immunevasion und Etablierung einer EBV-Infektion, was neue Angriffspunkte für die Behandlung von EBV-assoziierten Malignomen bieten könnte.

9. References

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10. Acknowledgements

Since 2018 when I first came to Heidelberg, I knew a new chapter of my life began. Now I am writing this part of my dissertation, I suddenly realised almost four years have past. It's time for me to say goodbye to this fantastic journey but I have to say studying PhD is a extremly memorable but tough.

First of all, I would like to thank my supervisor, Prof. Henri-Jacques Delecluse. Thanks a lot for giving me this facinating chance to study in DKFZ. Prof. Henri-Jacques Delecluse is really a good and professional virologist and his guidance helped me a lot during my 4 years PhD life. I also want to express my sincere thanks to him for helping revising my PhD dissertation.

I would also like to thank all my colleagues and all previous members of F100 research group for their patience, daily support and wonderful collaboration. I am grateful to all institute members of DKFZ for the comfortable atmosphere we enjoyed.

In addtion, I would like to thank my TAC members: Prof. Martin Müller and Dr. Florence Baudin for their helpful comments. I also want to thank Chinese Scholarship Council (CSC) for supporting my expenses in Germany.

Finally, I could not have completed this dissertation without the support of my family and friends, who provided stimulating discussions as well as happy distractions to rest my mind outside of my research.

11. Eidesstattliche Versicherung

1. Bei der eingereichten Dissertation zu dem Thema Epstein-Barr virus miR-BHRF1-2 targets retinoic acid-inducible gene I and inhibits interferon release in primary B cells handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.*

4. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.

5. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

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30. 06. 2022