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The EBV M81 BGLF4 protein modulates infectious events

by regulating gp350 protein expression

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2. Summary

We used knockout recombinant viruses to examine the functions of the M81 tegument proteins. It has recently been shown that functions of EBV proteins and of some EBV non-coding RNAs differ between the EBV M81 and B95.8 strains. EBV M81 strain has the unique feature to show a spontaneous lytic replication within B cells. In particular, this allows for an easy investigation without addition of chemical agents or artificial induction of the virus replication to the cells. The first part of our work underscored the importance of tegument proteins for an efficient virus production and for the infectivity of the produced viruses. We then found that the deletion of BGLF4 does not block production of virus and transmission across B cells but only reduces it on average three times. This suggests that the effects of the deletion are less pronounced than in 293 producer cells in which the absence of BGLF4 reduces the efficiency of transmission by a factor 30. Altogether, the effect of BGLF4 deletion affected lytic replication and propagation only mildly, suggesting that the multiple effects in the literature have little influence in the context of the whole virus. We could confirm viral targets of BGLF4 such as BMRF1 and the role of this kinase in late protein expression. However, our work has also evidenced the role of BGLF4 in B cell infection, a function that was not identified clearly before. Here the impact of BGLF4 on late gene expression through reduced binding and fusion is probably also crucial. Finally, although the BGLF4 deletion mutant virus could transform B cells normally, we found that BGLF4 surprisingly regulates the expression of latent proteins, in particular of LMP1. How a protein expressed only in lytically replicating cells can influence the expression of proteins in latently infected cells remains to be elucidated.

Zusammenfasung

Wir verwendeten rekombinante Knockout-Viren, um die Funktionen der M81-Tegument-Proteine zu untersuchen. Es wurde gezeigt, dass sich die Funktionen von EBV-Proteinen und einigen nicht-kodierenden EBV-RNAs zwischen den Stämmen EBV M81 und B95.8 unterscheiden. Der EBV M81-Stamm besitzt die einzigartige Eigenschaft in B Zellen spontan replizieren zu können. Dies ermöglicht eine einfache Untersuchung des Vorganges der Virusreplikation ohne Zugabe chemischer Mittel oder künstlicher Induktoren. Der erste Teil unserer Arbeit unterstreicht die Bedeutung von Tegument-Proteinen für eine effiziente Virusproduktion und für die Infektiösität der produzierten Viren. Wir fanden heraus, dass die Deletion von BGLF4 die Produktion von Viren und die Übertragung auf B-Zellen nicht komplett blockiert, aber im Durchschnitt dreifach reduziert. Dies legt nahe, dass die Auswirkungen der Deletion weniger ausgeprägt sind als in 293 Producerzellen, in denen das Fehlen von BGLF4 die Übertragungseffizienz um den Faktor 30 verringert. Insgesamt beeinflusste der Effekt der Deletion von BGLF4 die lytische Replikation und Vermehrung nur gering, was darauf hindeutet, dass die in der Literatur beschrieben Effekte im Zusammenhang mit dem gesamten Virus wenig Einfluss hat. Wir konnten virale Zielproteine von BGLF4, wie BMRF1, und die Rolle dieser Kinase bei der späten Proteinexpression bestätigen. Jedoch zeigt unsere Arbeit auch die Bedeutung von BGLF4 für die B-Zell-Infektion, eine Auswirkung, die zuvor nicht eindeutig identifiziert war. Hierbei ist höchstwahrscheinlich auch der Einfluss von BGLF4 auf die Genexpression später Proteine durch die reduzierte Bindung- und Fusionsfähigkeit entscheidend. Schließlich konnten wir auch zeigen, dass obwohl das mutierte BGLF4-Deletionsvirus B-Zellen normal transformiert, die Expression latenter Proteine, insbesondere von LMP1, reguliert ist. Wie ein Protein, das nur in lytisch replizierenden Zellen exprimiert wird, die Expression von Proteinen in latent infizierten Zellen beeinflussen kann, bedarf weiterer Untersuchungen.

3. List of Abbreviations

Δ	Knockout ; deletion
Ab	Antibody
AKT	Protein kinase B
Amp	Ampicillin
BAC	Bacterial Artificial Chromosome
BART	BamHI A Rightward Transcripts
BCL2	B cell lymphoma 2
BCR	B cell receptor
BL	Burkitt's lymphoma
Cam	Chloramphenicol
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic Acid
EBER	Epstein Barr small encoded RNA
EBV	Epstein Barr Virus
EBNA	Epstein Barr Nuclear Antigen
EBNA-LP	EBNA Leader Protein
E.coli	*Escherichia coli*
EtBr	Ethidium Bromidium
FCS	Fetal Calf Serum
g	Gravity force
GC	Gastric Carcinoma
GFP	Green Fluorescent Protein
gp	Glycoprotein
HEK	Human Embryonic Kidney
HRP	Horseradish Peroxidase
IF	Immunofluorescence
IM	Infectious Mononucleosis
Kan	Kanamycin
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
LCL	Lymphoblastoid Cell Line
LMP	Latent Membrane Protein
miRNA	microRNA
NFκB	Nuclear Factor κ B
MOI	Multiplicity of infection

NPC	Nasopharyngeal carcinoma
$p16^{INK4a}$	cycline-dependent kinase inhibitor
PAGE	Polyacrylamide gel
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3 kinase
PK	Protein Kinase
pol	Polymerase
PTLD	Post-transplant lymphoma disease
PTEN	Phosphatase and Tensin Homolog
qPCR	Quantitative Polymerase Chain Reaction
rEBV	Recombinant EBV
RNA	Ribonucleic Acid
ΔBGLF4 rev	Revertant of ΔBGLF4
RIPA	Radio-Immunoprecipitation Assay
rM81	Recombinant M81
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SB	Southern Blot
SDS	Sodium Dodecyl Sulfate
TBE	Tris Borate EDTA
TK	Thymidine Kinase
UV	Ultraviolet
WB	Western Blot

4. Introduction

The introduction will first describe the general properties of the Epstein-Barr virus. It will then focus on the viral tegument and the proteins it contains, notably the BGLF4 viral kinase.

4.1. The Epstein Barr virus history

Epstein Barr virus (EBV), also known as Human Herpesvirus 4, is a *lympocryptovirus* and belongs to the *Herpesviridae* family. EBV and the Kaposi-Sarcoma Herpes virus are included in the *gammaherpesviridae* subfamily due to their ability to establish proliferation and long-term latency of infected human B cells (Fields et al., 1996).

Denis Burkitt was the catalyst of Epstein Barr virus's discovery. In 1962, he described a new malignant lymphoma that was prevalent in children from some African areas. Due to the distribution of the tumors in Africa, he hypothesized that a mosquito would be the main agent of its induction (Burkitt, 1962). Using the newly established electron microscopy technique, D. Burkitt and Michael Anthony Epstein observed virus particles in these tumors. Then M.A. Epstein and Yvonne Barr established Burkitt's lymphoma (BL) derived cell lines and together with Bert Geoffery Achong, they observed herpesvirus like particles in a minority of cells (Epstein et al., 1964). It was the first indication of a tumor-associated virus. Later on, Gertrude and Werner Henle found evidence that EBV is the cause of the mononucleosis syndrome (Henle et al., 1968). By performing indirect immunofluorescent tests on sera from African patients with BL or with other malignant and non-malignant diseases as well as on various control groups, they observed antibodies against EBV in African patients with BL at high frequency. However, control groups also show low antibody, demonstrating that EBV is widespread around the world (Henle et al., 1969).

It was then proved that viruses could transform cells even in absence of cytopathogenic agent. Continuous cell lines were previously obtained from leucocytes infected with herpesvirus like particles from BL cells *in vitro* (Henle et al., 1967; Pope et al., 1968). Nilsson and colleagues were the first authors to describe the growth-promoting role of EBV in lymphoblastoid cell lines (LCL) *in vitro* (Nilsson et al., 1971). In 1970, Hausen and colleagues used the new nucleic acid hybridization method to detect the EBV genome in tumors cells (Hausen et al., 1970). They

observed EBV's presence in BL, but also in epithelial cells from nasopharyngeal carcinoma (NPC). Finally, Gerber et al. showed that the virus could be rescued from throats washings obtained from patients that carried EBV antibodies (Gerber et al., 1972). The persistence and ubiquity features of EBV in humans are characteristic for herpesviruses. However, its strong association with BL and its growth-promoting role distinguish EBV from the other human herpesviruses.

In more recent years, EBV has been found to be associated with 1 to 2% of all cancers worldwide including gastric cancers, Hodgkin's lymphomas, T cell lymphomas, immunoblastic lymphomas (Münz, 2015). Understanding the processes through which EBV causes cancers could facilitate their prevention or cure. Developing effective vaccines against this virus would also contribute to a reduction in cancer rate.

4.2. The Epstein Barr viral architecture

The structure of Herpesviridae consists of a DNA core in a nucleocapsid, a viral envelope with glycoprotein spikes on its surface and material that surrounds the capsid and called tegument. The size of a mature EBV virion is about 122-180nm.

4.2.1. The Epstein Barr viral structure

4.2.1.1. The Epstein Barr viral capsid

EBV mature virions contain a toroid-shaped DNA core enclosed within an icosadeltahedral nucleocapsid which presents 162 regularly arranged capsomeres, a feature common to all Herpesviruses (Fields et al., 1996). Mocarski has reviewed the proteins that form the capsid and that are conserved in Herpesviruses (Mocarski Jr., 2007). These proteins are involved in different mechanisms ranging from structural molecular activity, viral genome packaging and viral release from the host cells (Table 1).

Table 1: Identity, function and nomenclature of known Epstein Barr virus capsid gene products.

Common name	Abbreviation name	EBV gene name	Function
Portal protein	PORT	BBRF1	Viral genome packaging,

			viral release from the host cells (Pavlova et al., 2013; Visalli et al., 2019)
Major Capsid Protein	MCP	BcLF1	Structural molecule activity (Henson et al., 2009)
Triplex capsid protein 2	TRX2	BDLF1	Structural molecule activity (Henson et al., 2009; Wang et al., 2011, 2015b)
Scaffold protein		BdRF1	Structural molecule activity (Henson et al., 2009)
		BDRF1	Capsid core component (Visalli et al., 2019)
		BFLF1	Capsid core component (Visalli et al., 2019)
Small capsomere-interacting protein	SCP	BFRF3	Structural molecule activity (Wang et al., 2015b)
		BGLF1	Viral DNA packaging (Sugimoto et al., 2019)
Triplex capsid protein 1	TRX1	BORF1	DNA binding (Huang et al., 2020; Wang et al., 2015b)
Capsid vertex component 2	CVC2	BVRF1	Viral genome packaging (Sugimoto et al., 2019)
		BVRF2	

4.2.1.2. The Epstein Barr viral tegument

An asymmetrically fibrous material, called tegument surrounds the capsid (Fields et al., 1996). Tegument proteins are essential for the virus life cycle as they display multiple enzymatic activities and are involved in various mechanisms ranging from virion maturation to virus infection (Diefenbach, 2015; Full et al., 2017; Guo et al., 2010; Kalejta, 2008; Sathish et al.,

2012; Zhang and van Drunen Littel-van den Hurk, 2017). Although Herpes Simplex Virus tegument proteins are well studied (Batterson et al., 1983; Chadha et al., 2012; Chouljenko et al., 2016; Metrick et al., 2020; Owen et al., 2015), little is known in EBV. In 2004, Eric Johannsen and colleagues purified and determined EBV mature virions composition (Johannsen et al., 2004). He showed that EBV tegument contains several viral proteins, among which some proteins are already described such as the major tegument protein BNRF1 (Adhikary et al., 2020; Feederle et al., 2006; Tsai et al., 2011) and the large tegument protein BPLF1. These were found as the more abundantly represented tegument proteins. Surprisingly, host cells proteins involved in the cell cytoskeleton like β-actin, cofilin or tubulin and some heat shock proteins, Hsp70 and Hsp90, were also found (Table 2)(Alberts et al., 2002; Young et al., 2007). This suggests that the proteins derived from the cell play a role as "mediators of the morphogenesis".

Table 2: Identity, functions and nomenclature of known Epstein Barr virus tegument gene products.

Common name	Abbreviation name	EBV gene name	Function
Major DNA binding protein	ssDNABP	BALF2	Viral DNA replication (Tsurumi et al., 1996)
Cytoplasmic envelopment protein 3	MyrP	BBLF1	Virion maturation (Chiu et al., 2012; Hung et al., 2019)
Cytoplasmic envelopment protein 1	BBRF2	BBRF2	Cytoplasmic virus egress (Watanabe et al., 2017)
BDLF2		BDLF2	Virus cell to cell spreading (Gill et al., 2008; Loesing et al., 2009)
Capsid vertex component 1	CVC1	BGLF1	DNA packaging, viral release from host cell (Sugimoto et al., 2019)
Cytoplasmic envelopment protein 2	MyrPBP	BGLF2	Virus infectivity (Hung et al., 2019; Konishi et al., 2018; Liu and

			Cohen, 2015; Paladino et al., 2014)
Protein BGLF3		BGLF3	Component of the viral pre-initiation complex (Li et al., 2019a; McKenzie et al., 2016)(Li et al., 2019a; McKenzie et al., 2016)
Virion protein kinase	vPK	BGLF4	Serine-threonine kinase (Asai et al., 2009; Chang et al., 2012c; Feederle et al., 2009; Kato et al., 2001; Lee et al., 2007; Paladino et al., 2014; Wang et al., 2005, 2009)
Tegument protein BKRF4		BKRF4	Progeny production, inhibition of the cellular DNA Damage signaling pathway in latent and lytic EBV infection (Ho et al., 2018; Masud et al., 2017)
Tegument protein BLRF2		BLRF2	Contains an important motif for viral replication (Duarte et al., 2013)
DNA polymerase processivity factor EA-D	dsDNABP	BMRF1	Polymerase processivity factor that inhibits the DNA damage response (Neuhierl and Delecluse, 2006; Salamun et al., 2019)
Major tegument	MTP	BNRF1	Centrosome

protein			amplification, viral transport from endosomes to the nucleus, chromatin assembly modulator (Adhikary et al., 2020, 2020; Feederle et al., 2006; López et al., 2005; Lu et al., 2016)
Inner tegument protein	LTPbp	BOLF1	Viral infectivity (Kaushik and Kukreti, 2020; Masud et al., 2019)
Ribonucleoside-diphosphate reductase large subunit	RNR-L	BORF2	Viral genome integrity (Cheng et al., 2019, 2019; Paladino et al., 2014)
Large tegument protein deneddylase	LTP	BPLF1	Virus entry, transport, and assembly, deubiquitynylase activity… (van Gent et al., 2014; McKenzie et al., 2016)
Tegument protein BRRF2		BRRF2	Virus production (Watanabe et al., 2017)
BSRF1		BSRF1	Virion cytoplasmic egress (Serrano-Solis et al., 2019; Yanagi et al., 2019)
BTRF1 protein		BTRF1	Viral self-regulation network (Serrano-Solis et al., 2019)
Thymidine Kinase	TK	BXLF1	Viral thymidine kinase

		localized within the centrioles (Gill et al., 2007; Meng et al., 2010)
BXRF1	BXRF1	unknown
	Actin	Host cell cytoskeleton component (Alberts et al., 2002)
	β-tubulin	Host cell cytoskeleton component (Alberts et al., 2002)
	Cofilin	Host cell cytoskeleton component (Alberts et al., 2002)
	Enolase	Host cell cytoskeleton component (Alberts et al., 2002)
	Hsp70	Host cell heat shock protein (Cheung and Dosch, 1993)
	Hsp90	Host cell heat shock protein (Cheung and Dosch, 1993)

4.2.1.3. The Epstein Barr viral envelope

The EBV capsid is enveloped by a trilaminar lipid membrane with glycoprotein spikes on its surface (Fields et al., 1996). This property explains EBV's sensitivity to detergents. EBV encodes more than 12 glycoproteins, which interact with surface molecules expressed by host cells and thus determine the tropism for its host cells. EBV glycoproteins serve critical functions during the virus life cycle that are involved in multiple processes. EBV glycoproteins belong to three groups (i) those involved in virus entry and spread (Nemerow et al., 1987); (ii) others are involved in virus assembly (Murata, 2018). (iii) The remaining proteins are involved in

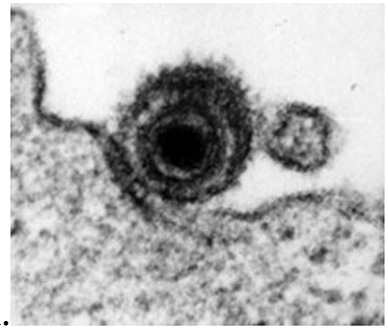
manipulating the host cell. Most glycoproteins have multiple functions (Table 3) (Figure 1) (Hutt-Fletcher, 2015).

Table 3: Identity, function and nomenclature of known Epstein Barr virus envelope gene products.

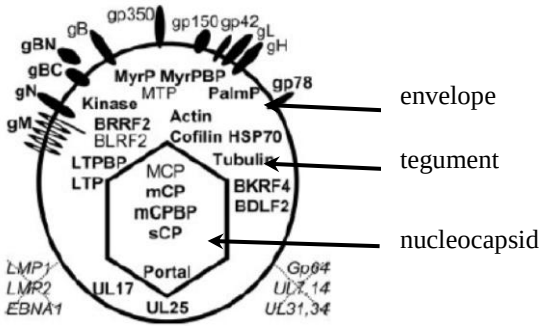
Common name	Abbreviation name	EBV gene name	Function
BARF1		BARF1	CSF1 receptor/immune evasion/ oncogene (Hoebe et al., 2013; Wei and Ooka, 1989; Wei et al., 1994)
BDLF2		BDLF2	Epithelial spread? (Loesing et al., 2009)
Membrane glycoprotein	Gp150	BDLF3	Membrane glycoprotein/ immune modulator (Chesnokova et al., 2016; Quinn et al., 2016)
G-protein coupled receptor BILF1	BILF1	BILF1	G-protein-coupled receptor/immune evasion (Zuo et al., 2011)
Glycoprotein BILF2	gp78	gp78	Single pass type 1 membrane (Mackett et al., 1990)
Protein BMRF2	Epi ligand	BMRF2	Epithelial cell attachment and spread (Loesing et al., 2009)
Glycoprotein B		gB	Fusion (Hutt-Fletcher, 2007)
Envelope glycoprotein H	gH	gH	Regulation and triggering of fusion

			(Hutt-Fletcher, 2007)
Envelope glycoprotein L	gL	gL	Regulation and triggering of fusion (Hutt-Fletcher, 2007)
Envelope glycoprotein M	gM	gM	Assembly and release (Changotra et al., 2016)
Envelope glycoprotein N	gN	gN	Assembly and release (Lake and Hutt-Fletcher, 2000)
Glycoprotein 42	gp42	gp42	Triggering of fusion/immune evasion (Li et al., 1995)
Membrane antigen	MA	gp350/220	Attachment (Tanner et al., 1988; Thorley-Lawson and Poodry, 1982)

a.



b.



<u>Figure 1</u>: a. Epstein Barr Virus particle electron micrograph (Delecluse & al.). The picture represents an electron micrograph of EBV. **b. Epstein Barr virion proteins structure.** The schema depicts the EBV virion composition. Modified from (Johannsen et al., 2004). Mature EBV virions were purified in order to perform a mass spectometry analysis. This analysis allowed to know which proteins are present in the capsid, tegument and in the envelope as well as its relative amount.

4.2.2. The Epstein Barr virus genome

The EBV genome was the first herpesvirus to be sequenced (Baer et al., 1984). The EBV genes were named according to (i) the BamHI restriction fragment in which they start, (ii) the orientation of their transcription (rightward/ leftward) and (iii) the number of Open Reading Frames that carry the same configuration. For example, the BGLF4 gene encodes a viral protein kinase and is located within the BamHI G fragment at the fourth leftward open reading frame. Today, the sequence of EBV wildtype genome, used as a reference in the laboratory, was created from the B95.8 and Raji sequence assembling. The introduction of EBV B95.8 genome into Bacterial Artificial Chromosome (BAC) allowed later to create recombinant EBV (rEBV) mutant and therefore to study single EBV gene function (Delecluse et al., 1998; Feederle et al., 2010). The rEBV mutants are obtained from homologuous recombination and the "en passant mutagenesis" is a method particularly used when the modification of a single gene will disturb the expression of the neighboring EBV genes (Tischer et al., 2010).

Since 2010, many EBV strains from patients have been isolated, sequenced and cloned into BAC such as the M81 strain or the YCCEL1 strain. Although the YCCEL1 strain was isolated from Gastric Carcinoma (Kanda et al., 2016), the M81 strain was isolated from a nasopharyngeal carcinoma in a Chinese patient (Tsai et al., 2013). Even if the EBV strains show a similar genome structure, there are few genes polymorphisms that might be responsible of different functions. The impact of these polymorphisms is currently under study (Tsai et al., 2013).

Like other herpesviruses, the EBV genome is a linear and double-stranded DNA packaged in viral particles. Its molecular weight is around 172kbp and sequencing revealed a 60% guanine-plus-cytosine content (Fields et al., 1996). The EBV genome encodes more than 85 proteins and 46 functional small-untranslated RNAs. The presence of repeated DNA sequence called Terminal Repeats (TR) flanking the EBV genome at both ends allows its circularization once the cells are latently infected (Zimmermann and Hammerschmidt, 1995). Therefore, the latently infected cells contain the genome as a nuclear episome which is attached to the human chromosomes by binding to AT-rich tracts of DNA via the EBV Nuclear Antigen 1 (EBNA1) protein (Lieberman, 2013). One TR is constituted of 2 to 5 tandems of same sequence and is 0,5kbp in size (Kieff et al., 1982) . During EBV lytic replication, random recombinations occur between the TR. Therefore, the number of TR is variable among the EBV virions (Münz, 2015). However, when the cells are infected, the episome copy number in daughter cells will have noticeably a similar number of TR in case they derive from a single cell. This characteristic feature allows to easily

determine whether an EBV-infected cell population grew from a single infected cell or instead from different cells (Bánáti et al., 2017). The number of TR can be determined by Southern blot that represents a useful experimental tool to distinguish between monoclonal and polyclonal proliferations (Bánáti et al., 2017; Serquiña and Ziegelbauer, 2017). The viral genome is separated into the two domains U_S and U_L by a DNA sequence that consists of reiterated 3kbp internal direct repeats. The latter sequence is called IR1or major internal repeat. The IR1 is significant for EBV because of the Wp promoter presence; this promoter regulates the EBNA genes, which are immediately activated when B-lymphocytes are infected. Although the U_S domain encodes only LMP2 and the EBER non-coding RNA, the U_L domain encodes most of the EBV genes. U_L is further subdivided into four segments (U_{L2-5}) by the internal repeat domains IR2-4 (Alfieri, 2006) (Figure 2).

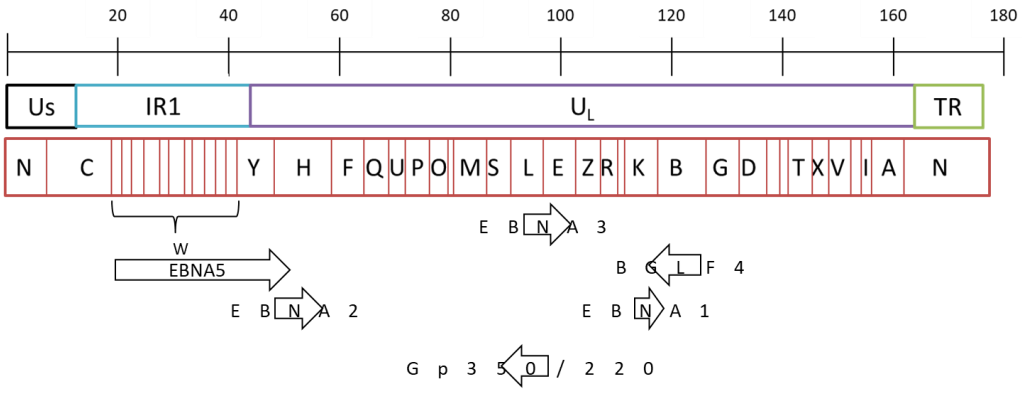


Figure 2: Genomic map of the Epstein Barr virus genome. Modified from (Dillner et al., 1991). The schema depicts the genomic map of the EBV genome. The four main regions: US, IR1, UL and TR are represented on the top of the figure. Below are represented the BamHI restriction fragments. Arrows indicate some EBV proteins encoded by the genome. The arrow's tip indicates the direction of the translation from the coding sequence.

4.3. The Epstein Barr virus life cycle

4.3.1. The Epstein Barr virus B95.8 and M81 strains

EBV has a preferential tropism for B cells but can also infect epithelial, T and NK cells, even if with a much lower efficacy (Alfieri, 2006; Cai et al., 2015). The tropism of EBV for some

cellular types is also dependent of the EBV strain. For example, the EBV B95.8 and M81 strains differ in their (i) cellular tropism, (ii) cellular transformation ability and (iii) sequence. The EBV B95.8 strain was isolated from an American patient with an infectious mononucleosis (IM)(Baer et al., 1984). In contrast to the B95.8 strain, the EBV M81 strain was isolated from a Chinese patient with a NPC (Tsai et al., 2013). While EBV B95.8 strain infects well human B cells, the infection of epithelial cells is less efficient. On the contrary, EBV M81 strain is less efficient for infecting human B cells but it shows a higher infectivity towards epithelial cells. Furthermore, a spontaneous lytic replication is observed *in vitro* and in vivo in lymphoblastoid cell lines (LCL) obtained from M81 infected B cells. Unlike the M81 strain, the replication of EBV B95.8 strain needs to be chemically induced in B cells. These few differences make the EBV M81 strain a novel and more interesting model for studying the viral replication.

4.3.2. The Epstein Barr viral entry within the cells

Epstein Barr virus is transmitted in saliva as cell-free virus. Although the molecular mechanisms that govern EBV epithelial cell infection are poorly understood (Wang et al., 2015a), those that control B cell infection are well characterized (Schäfer et al., 2015). First, EBV particles bind their cellular host through interaction of the glycoprotein gp350/gp220 with the cellular receptor CD21 also known as CR2 (Birkenbach et al., 1992). Recently, it has been found that CD35 might be an alternative receptor for the EBV attachment in CD21 negative cells (Ogembo et al., 2013). Moreover, the EBV BMRF2 glycoprotein was shown to interact with β1 and α5 integrins only in oral epithelial cells (Xiao et al., 2008, 2009). Then, cell membranes are fusioned to the EBV envelope. In B cells, the fusion follows the endocytosis between the endocytic vesicle membrane and the EBV envelope. This fusion requires the viral glycoproteins comprising the core fusion machinery and called the gH-gL heterodimer and gB glycoproteins but also the binding of the viral glycoprotein gp42 to the cell surface human leukocyte antigen class II (HLA class II) (Kirschner et al., 2006; Molesworth et al., 2000; Wang and Hutt-Fletcher, 1998; Wang et al., 1998). This allows the tegumented capsid entry into the cytoplasm. Unlike B cells, EBV entry is independent of gp350/220 and gp42 in epithelial cells. Chesnekova and colleagues showed that the virus entry within the cells occurs through direct fusion with the host cell plasma membrane at the cell surface and involved the interaction of the neuropilin1 protein to the glycoprotein gB and a binding of gH-gL and αVβ6 and αVβ8 integrins (Chesnokova et al., 2009). In 2018, it was

shown that EphA2 (erythropoietin-producing hepatoma) receptor was required for the entry of EBV within epithelial cells (Chen et al., 2018; Zhang et al., 2018). Eph receptors are tyrosine kinase receptors and consist in three part: an extracellular domain, a transmembrane domain and an intrecellular domain (Schlessinger, 2000). Chen and colleagues demonstrated that EBV can enter within the cells when gH/gL and gB interacts with Ephrin A2 receptor which leads to the internalization of EBV (Chen et al., 2018). However, they also demonstrate that the extracellular domain of EphA4 did not interact with gH/gL unlike EphA2. Finally, the EphA2 receptor is required for the infection of epithelial cells as it was not detectable in B lymphomas cell lines. Indeed, an up-regulation of this receptor was found in nasopharyngeal carcinomas and EBV-associated gastric cancers.

4.3.3. The Epstein Barr viral life cycle

During its life, EBV can be found into two forms within its cellular host: latent form and lytic form (Fields et al., 1996). During latency, the EBV DNA is maintained as a covalently closed circular episome in the cell nuclei and only few proteins and miRNAs are expressed (Laux et al., 1988). During the lytic stage, the EBV DNA is replicated and the viral particles are produced within the cells. EBV remains in a latent form in most of the infected B cells (Decker et al., 1996) and it is rare to detect lytic replication-associated proteins in healthy people or in people with EBV-associated malignancies. A major feature of EBV is its ability to shut down latent gene transcription in some B cells that are used by the virus as a reservoir in order to counteract the host immune response but also to express back the EBV latent genes in immune deficient host (Babcock et al., 1998; Miller, 1989). Only few infected cells show a switch from the latency to lytic replication. However, the understanding of the molecular mechanisms initiating the switch from the latency to lytic replication remains limited.

4.3.4. The Epstein Barr viral latent infection

Depending of the infected cell type, as well as immunological environment, EBV will establish diverse latency types that are called latency 0, I, II or III (Cohen, 2000). Only few EBV proteins (Laux et al., 1988), as well as EBV RNA are expressed to maintain the viral genome in EBV infected cells by helping the proliferation and survival of these cells (Table 4) (Takada and Ono,

1989). The main proteins and RNA involved in this process belong to the EBNA (Epstein Barr Nuclear Antigen)(Weigel et al., 1985) and LMP (Latent Membrane Protein)(Fennewald et al., 1984; Hennessy et al., 1984) family and EBER (Epstein Barr small Encoded RNA)(Howe and Steitz, 1986), BART-miRNA (BamHI-A rightward transcripts micro-RNA)(Chen et al., 1999) and BHRF1-miRNA family (Table 4) (Farrell, 2019). BHRF1 is an anti-apoptotic viral homolog of the Bcl2 protein.

Table 4: Summary of the Epstein Barr virus genes and their function expressed during the pattern of latent cycle in EBV infected B cells. Proteins are in blue.

Protein/RNA family	EBV gene name	Function	Latency pattern			
			0	I	II	III
EBNA	EBNA1	Viral genome persistence (Rawlins et al., 1985)		✔	✔	✔
	EBNA2	Cell growth proliferation (Kempkes et al., 1995)				✔
	EBNA3A/3C	Differentiation into plasma cells down-regulation (Allday et al., 2015)				✔
	EBNA-LP	Innate response inhibitor (Echendu and Ling, 2008)				✔
LMP	LMP1	Apoptosis down-regulation (El-Sharkawy et al., 2018)			✔	✔
	LMP2A	Apoptosis down-regulation (El-Sharkawy et al., 2018)			✔	✔
BART miRNAs		T and NK cells evasion (Ramakrishnan et al., 2011)	✔	✔	✔	✔
BHRF1		B cell transformation				✔

miRNAs		(Poling et al., 2017)				
EBER	EBER 1/2	Immune evasion (Li et al, 2019b)	✔	✔	✔	✔

The EBV latency established in human B cells is well understood (Figure 4) (Fields et al., 1996). Immediately after the infection of human B-lymphocytes, cells proliferate and grow as lymphoblastoid cell line (LCL) (Hurley and Thorley-Lawson, 1988). The EBV genome is transported in the nucleus of the host cells and circularized into an episome, with approximatively 10 copies of EBV genome per cell (Alfieri, 2006). This number of EBV copies is maintained through the viral DNA replication using the host cell polymerase. It has been shown that EBNA1, EBNA2, EBNA3C and LMP1 are essential for the conversion of the infected B cells into LCL (Gordadze et al., 2004; Jiménez-Ramírez et al., 2006; Jones et al., 1989). Permanent expression of EBNA1 allows the protein to bind to the EBV latent origin of replication and thus allows efficient EBV genome partitioning and persistence after cell division. EBNA2 is a transcription factor that is responsible of the activation of the EBV LMP genes, as well as cellular genes (Rawlins et al., 1985). EBNA2 activates LMP1 protein expression, one protein with transformation ability (Wang et al., 1990). Besides, EBNA-LP (EBNA Leader Protein) cooperates with EBNA2 to overcome a cellular innate response to the EBV genome transcription (Kempkes and Ling, 2015). In order to help LMP1, EBNA 3A and EBNA3C will contribute to the B cell transformation as well as being involved in the regulation of the cellular genes (Jiang et al., 2017). Finally, the three BHRF1 region miRNA play a role in B cell transformation as well as targeting PTEN and p27 (Bernhardt et al., 2016). LMP1 expression leads to the NFκB activation which induces the Bcl2 protein; this will prevent apoptosis (Voigt et al., 2020). Like LMP1, LMP2A plays a role in the prevention of the apoptosis due to its signal transduction from the BCR (B Cell Receptor). LMP2 induces PI3K (Phosphoinositide 3 kinase) to activate the AKT (Protein kinase B) pathway. EBNA3A and EBNA3C proteins repress the pro-apoptotic BIM protein and the cycline-dependent kinase inhibitor $p16^{INK4a}$, which in turn will facilitate the proliferation of LCL (Skalska et al., 2013). During latency I, the EBV gene expression is reduced to a low level of EBNA1 and the B activated lymphoblasts become memory B cells. Finally, EBNA1 expression is shut down and the virus persists in these memory cells, counteracting the

detection from immune system. This is the latency 0. Intermittently, EBNA 1 is reactivated in order to lead cell division and the EBV genome persistence during latency I (Figure 3)(Kerr et al., 1992).

EBV can be reactivated when memory B cells differentiate into plasma cells. This leads to the lytic replication and thus production of EBV virions.

Interestingly, the specific latency programs are associated with some types of EBV-positive malignancies. The latent I stage genes encoding EBNA1 protein, EBER and BART-miRNA are the only latent genes expressed in Burkitt´s lymphoma. In latency II, cells express the EBNA1, LMP1 and LMP2A proteins: this is observed in nasopharyngeal carcinoma, Gastric cancers (GC), Hodgkin and T cell lymphomas. Finally, latency III is observed in LCL *in vitro* (Farrell, 2019).

4.3.5. The Epstein Barr viral lytic replication

Lytic replication leads to EBV virions production. During lytic replication, EBV increases the number of viral DNA genome copies. The EBV genome is linearized and replicated (Fields et al., 1996).

To initiate the lytic replication cycle, the EBV proteins encoded by the BZLF1 (Zta/ZEBRA) and BRLF1 (Rta) immediate early genes (or transactivators) need to be expressed to regulate their own promoter but also the expression of the other lytic viral genes (Münz, 2015). A sequential expression of viral genes follows the expression of the transactivators (Sample et al., 1984). These latter represent the genes encoding the proteins responsible of the viral DNA replication as well as the genes encoding the proteins involved in late gene expression, which in turn will induce the expression of the genes encoding the EBV structural proteins (Alfieri, 2006). BZLF1 and BRLF1 alter the host cell cycle process to promote the transcription of the early and late lytic viral genes by binding on the promoter of the other lytic genes (Huang et al., 2020; McKenzie et al., 2016). During lytic replication, EBV is replicated as a linearized concatemer, which will undergo random excision within the TR region (Zimmermann and Hammerschmidt, 1995). After DNA replication, EBV genome is encapsidated, gathered in viral particle and then released outside of the cell through Golgi apparatus budding and transport (Figure 3).

The lytic replication cannot happen if the cell host expresses a protective mechanism against the virus. Accordingly, many EBV tegument proteins induce harmful events in the cells. Notably, the

EBV protein kinase BGLF4 is an example of tegument protein, which induces alterations in the host cells to favor EBV replication. The functions of BGLF4 will be described later.

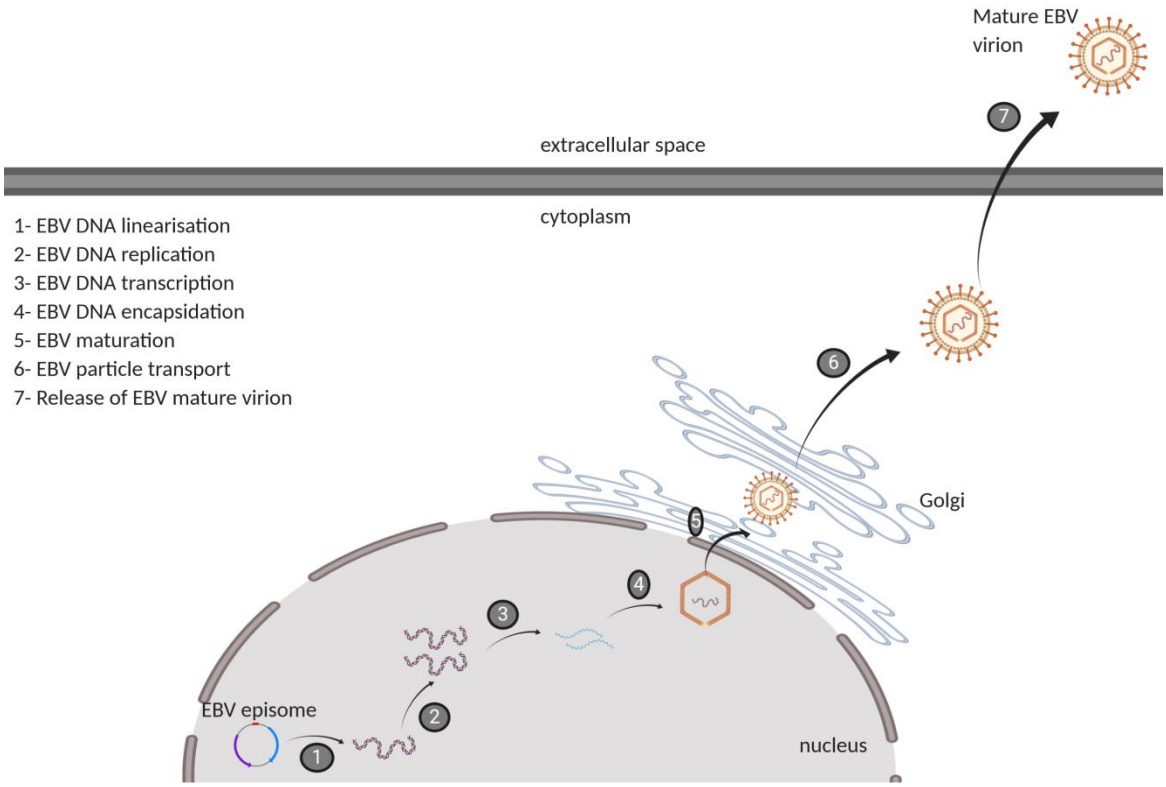


Figure 3: Schematic representation of the Epstein Barr viral lytic replication in human B cells. Figure created on BioRender.

4.3.6. The Epstein Barr viral reactivation: from latency to lytic replication

It is rare to observe replicating cells after the primary infection of the B cells *in vitro* as well as in EBV associated tumors and in healthy individuals. Since many years, the molecular mechanism that allows the switch from latency to lytic replication in EBV has been extensively studied *in vitro*(Miller, 1989). Notably, studies performed with many EBV strain showed that the expression of BZLF1 induces the EBV lytic replication in permissive cells (Countryman et al., 1987). Therefore, BZLF1 has been admitted to be the main key activator of the EBV switch from latency to lytic replication. In particular, the studies performed on the EBV B95.8 strain use chemical agents such as phorbol esters, n-butyrate and N-nitrosamines as inducers of the lytic replication. The activation of some signaling pathways is directly linked to the reactivation of the

lytic cycle of EBV. This has been observed in BL cell lines where the crosslinking of antibodies on the B cell receptor (BCR) with antibodies or the supplementation to the cells with TGF- β strongly suggest a link between the activation of the BCR/TGF- β pathways and lytic replication (Daibata et al., 1991).

The activation of the Protein Kinase C (PKC) pathway or the Ataxia telangiectasia mutated (ATM) pathways seems to show some other involved pathways in the reactivation of EBV (Nikitin et al., 2010). On the contrary, some B cells factors inhibit the lytic replication: this is the case of Pax5 transcription factor (Arvey et al., 2012).

In humans, the EBV reactivation occurs spontaneously. Few years ago, our lab described spontaneous lytic replication in LCL established from EBV M81 strain infected B cells (Tsai et al., 2013). We therefore used the M81 strain to deeper characterize the EBV proteins functions that are still poorly studied, e.g. the tegument proteins.

4.4. Epstein Barr Virus related diseases

Most humans are infected by EBV and do not develop disease. However, EBV is linked to 1.5% of all human cancer cases worldwide (Farrell, 2019). EBV was the first identified virus associated with cancers (Burkitt, 1962). It infects both B cells and epithelial cells. EBV-associated cancers are mainly lymphomas and carcinomas that are respectively derived from B cells and epithelial cells (Farrell, 2019). Some EBV-associated diseases are described below.

4.4.1. Epstein Barr virus associated diseases

4.4.1.1. Infection Mononucleosis (IM)

Primary EBV infection usually occurs during the childhood and is asymptomatic. However, if the infection occurs during adolescence or in adults, an Infectious Mononucleosis (IM) syndrome develops. EBV is linked to 90% of IM syndromes (Fugl and Andersen, 2019). The main symptoms of this disease are fever, lymphadenopathy, and pharyngitis. During the EBV infection and up to 180 days after the first symptoms appear, individuals produce high amounts of EBV. IM is characterized by the activation of cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells during EBV dissemination throughout the body. However EBV restricts its gene expression,

which avoids the recognition by the CTLs, and thus results in latent infection in the infected B-cells through an unknown mechanism.

4.4.1.2. Post-Transplant Lymphoma Disease (PTLD)

After organ transplantation, the immunosuppressive treatment leads to EBV reactivation in some patients. Reactivation is characterized by an increased virus replication and/or B cell proliferation of EBV-infected B cells. This can lead to an uncontrolled EBV-driven B-cell proliferation, called EBV post-transplant lymphoma disease (PTLD). PTLD can develop immediately or several years after transplantation, although the peak frequency is observed after one year (Hamed et al., 2020). The latency found in PTLD is usually but not always latency III. While most of the PTLD are B cells tumors, some are T cells lymphoma. Treatment includes withdrawal of immunosuppression, conventional chemotherapy and T cell therapy in specialized medical centers. Acyclovir and ganciclovir are also given in patients with PTLD (Funch et al., 2005). Indeed, anti-viral drug administration has been shown by some authors to be associated with a reduction in the risk of PTLD in renal transplant patients with a better gancyclovir curative power compared to the acyclovir.

In cells infected by Herpes Simplex Virus and Human Cytomegalovirus, these drugs are phosphorylated by a viral kinase homolog to the EBV BGLF4 protein and by cellular kinases. The fully phosphorylated form of the drug is toxic to infected cells. The benefit of acyclovir and ganciclovir in patients with PTLD has been noted in some but not all clinical studies (Rasche et al., 2014).

4.4.2. Epstein Barr virus associated cancers

4.4.2.1. Epstein Barr Virus associated Gastric Carcinoma (EBVaGC)

Approximately 10% of all gastric cancers are associated with EBV infection (Naseem et al., 2018). Gastric cancers are heterogeneous cancers and an accurate classification would lead to personalized treatment. For many years, gastric cancers have been classified into intestinal and diffuse cancer types according to the Lauren classification, or by another system that was proposed by the World Health Organization. In 2014, the Cancer Genome Atlas (TCGA)

distinguished gastric carcinomas on a novel molecular and genomic classification (Wang et al., 2019; Zhang, 2014). The TCGA network used the results of genome, exome and methylome DNA sequencing, RNA sequencing and protein arrays as well as other tools on 295 gastric cancer tumors samples to distinguish four subtypes of gastric cancers: Epstein-Barr virus (EBV)-positive tumors, microsatellite instable (MSI) tumors, genomically stable (GS) tumors, and tumors with chromosomal instability (CIN).

Multiple factors contribute to EBV-associated gastric carcinoma (EBVaGC) such as (i) hypermethylation of tumor suppressor genes driven by EBV, (ii) inflammatory changes in gastric mucosa, (iii) host immune evasion by EBV and (iv) changes in cell cycle pathways. The unique molecular feature of these cancers consists in programmed death ligand 1 (PD-L1) overexpression. A latency type I characterizes EBVaGC: the expression of EBNA-1 leads to an impaired DNA damage response and reduces apoptosis. The EBV lytic protein BARF1 is the only lytic protein detected in these cancers. EBVaGC are separated from other gastric carcinomas due to the lower expression of p16, p27, p73 and E-cadherin. Finally, the Cyclin D1 and the transcription factor NF-κB are produced at higher levels in these cells.

4.4.2.2. Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinomas (NPC) are endemic in Southeast China. The first observation of an association between EBV and NPC goes back to 1966. The regular presence of EBV DNA fingerprints and the high antibodies titers against EBV VCA, EA and EA-D, EBNA and ZEBRA antigens characterize this association. The presence of EBER and BARTs RNAs, as well as EBNA-1, LMP2, and variable expression of LMP1 proteins classified this cancer in latency II class disease.

The development of EBV associated nasopharyngeal carcinomas is strongly associated with (i) carcinogenic agents commonly found in the Chinese diet. These agents induce DNA damage and somatic genetic alterations in epithelial cells. One explanation to the somatic genetic alterations may be due to the major histocompatibility complex MHC class I (MHC class I): indeed the expression of variants from MHC class I is strongly associated with the expression of EBV genes. It decreases the host cell's immune response and facilitates the growth of cancer cells. This mechanism yet remains unclear and needs to be elucidated. (ii) Genetic alterations such as

accumulation of driver events (activation of telomerase activity and inactivation of tumor suppressor genes *RASSF1A* and *p16).* The EBV gene expression also affects the cellular signaling pathways. For example, the expression of LMP1 plays a role in NF-κB activation in NPC. LMP1 plays also a role at the epigenetic level. It enhances the global hypermethylation which affects later the role of the tumor suppressor genes. Altogether, it drives an abnormal PI3K/MAPK signaling pathway and chromatin remodeling which will support the EBV infection in cells (Tsao et al., 2017; Wu et al., 2018).

In 2013, Delecluse and his team showed a link between a specific EBV strain and NPC in Chinese restricted areas. After its isolation from Chinese NPC sample and its cloning in BAC, they described a novel phenotype: the M81 strain induces a spontaneous lytic replication after 21 days compared to the B95.8 strain. Besides, EBV M81 presents an increased tropism for epithelial cells (Tsai et al., 2013). Altogether with the high levels of anti EBV VCA antibodies, which are expressed before NPC development, it implies that the replication of EBV is important for NPC's development.

4.4.3. Epstein Barr associated lymphomas

4.4.3.1. Burkitt's Bymphoma (BL)

In 1964, M.A. Epstein and Yvonne Barr observed herpesvirus like particles in few cells derived from a Burkitt lymphoma (BL) biopsy taken from an African child patient. Later, EBV has been demonstrated as the viral agent causing BL in association with malaria infection. It is now known that all BLs share one of these features: (i) a unusual chromosomal translocations leading to the constitutive activation of the c-*MYC* oncogene (Rowe et al., 2014); (ii) alteration of the *TP53* pathway; (iii) alteration of the retinoblastoma protein (pRb) pathway.

It has been shown that malaria infection affects germinal center B cells. Upregulation of the expression of Activation-Induced cytidine Deaminase (AID) is observed and AID is known for increasing the risk of the *MYC* gene translocation in EBV infected germinal center B cells. Recently, the EBNA3C protein was found to induce the AID mRNA and protein expression in EBV-infected B cells supporting a stronger association between EBV and development of BL. In endemic regions of malaria, 100% of BL cases are associated with EBV presence in cells. In fact, Cystein-rich interdomain region 1 α (CIDR1α) is expressed on the surface of plasmodium

infected cells and reactivates EBV lytic replication as it was shown in Akata BL cell line. Another argument that favors the association between EBV and BL is the very low INF-γ T cell response against EBV that was measured in children with BL. Whereas all of this evidence could convince of the EBV-BL association *in vitro, in vivo,* it has been demonstrated that only EBV-positive BL cell lines express a high level of the TCL-1 (T cell leukemia 1) protein, which is responsible of the B cell lymphoma formation in transgenic mice.

4.4.3.2. Hodgkin's Lymphoma

Hodgkin's lymphomas, are classified into two groups according the World Health Organization (WHO): classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL). However, classical HL is separate because of its virological features, regarding EBV infection and expresses a type II latency disease. cHL are characterized by (i) Reed-Sternberg cells (RSC) which are separated into four types of cells: lymphocyte-rich cHL (LRCHL), nodular sclerosis cHL (NS), mixed cellularity cHL (MC), and lymphocyte depletion cHL (LD) and (ii) a cellular distinct tumour microenvironment. Phenotypically, all RSC express $CD30^+$, $CD40^+$, $CD15^+$, IRF4/$MUM1^+$. EBV is found mostly in MC and LD cHL. Inflammatory cells such as activated T helper and immune cells like EBV-specific cytotoxic T lymphocytes surround the RSC. The LMP1 protein contributes to generate this environment by enhancing the production of immunosuppressive cytokines such as IL6, IL8, and IL10.

Due to the EBV replication long before the Hodgkin's lymphoma diagnostic, the RSC show elevated titers of antibodies directed to the EBV VCA, EBNA2 and EA-D antigen (Carbone and Gloghini, 2018).

The latent proteins LMP1 and LMP2A are involved in aberrant activation of key signaling pathways in cHL such as NF-κB, Janus activated kinase/signal transducers and activators of transcription (JAK/STAT), and phosphatidylinositol 3-kinase (PI3K)/AKT pathways. Indeed, these pathways are constitutively active in these cells. The high activation of NFκB in cHL EBV positive RSC can be induced by LMP1. However, EBV negative RSC also show an activation of NFκB. This suggests that other proteins activate the NFκB pathway. LMP1 is involved at the epigenetic level in cHL. The polycomb gene BMI1 belongs to the polycomb repressive complex (PRC1) and plays a role in gene silencing and development of lymphomas in transgenic mice. In

fact, LMP1 and BMI1 regulate the ATM tumor suppressor; among some other Hodgkin's lymphomas associated genes.

LMP2A interferes with normal B-cell development *in vivo* and contributes to cell survival by activating the RAS/PI3K/AKT pathway. LMP2A is highly expressed in cHL and is shown to help the EBV-infected GC B cells to transit through the germinal center then to help the differentiation into memory B cells (Kapatai and Murray, 2007; Vockerodt et al., 2014).

4.4. Functions of the EBV tegument

As all herpesviruses, mature Epstein Barr virions contain a unique proteinaceous layer between the virion envelope and the capsid, termed tegument.

Viral infection requires binding and fusion to the cell membrane, viral transfer to the nucleus, and then expression of the viral genome in the target cell's nucleus. This transfer from the host cell membrane to the nucleus is mediated at least in part by tegument proteins. These proteins display unusual enzymatic activities and are involved in mechanisms including virion maturation and immunity. Therefore, they are potential targets for antiviral therapies.

4.4.1. Epstein Barr tegument generalities

4.4.1.1. Epstein Barr tegument composition

The tegument is composed of lytic proteins that are expressed in the late phase of the EBV lytic cycle. In 1976, Myron Dolyniuk and colleagues performed a study on purified EBV enveloped viruses. They observed that the tegument from enveloped particles consisted predominantly of amorphous material. Furthermore, the EBV tegument proteins migrated quite similarly between Herpex Simplex Virus-1 (HSV-1) and Equine Herpesvirus (EHV) in western blot analysis (Dolyniuk et al., 1976). In 2004, Eric Johannsen determined the EBV mature virion composition and highlighted the presence of viral and derived host cell proteins within the tegument (Johannsen et al., 2004). He showed that the tegument contains several viral proteins among which the dominant major tegument protein BNRF1 and the large tegument protein BPLF1. Currently, only scanty information is available on EBV tegument proteins. Indeed, their respective functions are postulated to be more or less homologous to the HSV tegument proteins. However, there are tegument proteins, e.g. BLRF2, BRRF2 or BKRF4 that are unique to γ-

herpesviruses. This suggests that the herpesviruses followed different evolution routes and that the functions of the tegument proteins can differ between α/β-herpesviruses and EBV.

4.4.1.2. Regulation of the Epstein Barr viral tegument proteins expression

During its productive cycle, Epstein Barr virus exhibits a strictly regulated temporal cascade of gene expression and expression of viral lytic genes that are categorized in three groups (i) the immediate early genes (IE) BZLF1 and BRLF1 which are the transactivators of the EBV lytic cycle; (ii) the early lytic genes (E) which are implicated mostly in viral DNA synthesis; (iii) the late lytic genes encode structural proteins such as the tegument proteins. Promoter complexity differs strikingly between IE/E genes and L genes. IE and E promoters contain *cis*-regulating sequences upstream of a TATA box, whereas L promoters comprise a unique *cis* element. The IE BZLF1 and BRLF1 expression induce the activation of the viral early genes promoter in intact genomes. Then, the combination of both BZLF1 and BRLF1 is required for activating the transcription of BMRF1, BHLF1 and BHRF1 (Feederle et al., 2000). The EBV early gene products are involved in multiple processes like transcription regulation, RNA transport and stability, immune evasion and cellular apoptosis. Finally, the late lytic genes encode structural proteins. EBV encodes a complex of six proteins necessary for the activation of the late viral genes (Aubry et al., 2014). This complex is formed around a viral TBP-like protein and interacts with cellular RNA polymerase II. These genes are expressed after the onset of viral replication and are secreted in the cell cytoplasm. The late lytic genes are classified into two groups: (i) the late genes encoding the two immunoevasins, BCRF1 and BPLF1 which are transcribed independently of the viral pre-initiation complex and (ii) the viral structural proteins which are dependent on the viral pre-initiation complex. Among the late lytic genes, only the serine-threonine protein kinase BGLF4 is required for expression of both groups of late genes suggesting that this protein might be one of the first late genes to be transcribed (McKenzie et al., 2016).

4.4.2. Functional roles of Epstein Barr viral tegument proteins

4.4.2.1. Viral entry

When EBV infects its target cells, its particles are internalized into cytoplasmic vesicles where decapsidation takes place to allow transfer of the viral DNA to the cell nucleus. Within the nucleus, the linear genome is released through nuclear pores. In the cytoplasm, the viral genome is enclosed and protected by its capsid proteins. However, the roles of the tegument proteins in the cytoplasm are less well known. Interestingly, upon entry in the cells, the tegument proteins are released and can interact with the infected cell. In 2006, Regina Feederle gave the first evidence that BNRF1, the EBV major tegument protein, plays a crucial role in viral transfer from the endosome/lysosome compartment to the nucleus (Feederle et al., 2006). Performing electron microscopy analyses, she identified that a virus devoid of BNRF1 protein can reach vesicles of infected cells similarly to the EBV-wildtype infection. Because BNRF1 was not required for viral transport from the cellular membrane to the endosome/lysosome compartment, it was likely play a role at a later stage during infection.

4.4.2.2. Viral particles maturation and release

These last few years, the mechanisms behind EBV virion assembly and release have received much attention. For many years, research was based on studies performed with alpha and beta herpesviruses due to their similar particle structure. As the capsid proteins, the tegument proteins are also conserved. It has been speculated that EBV viral DNA is packaged within the capsid in the nucleus and that the capsids bud through the nucleus to the cytoplasm by a fusion of the envelope with the nuclear membrane. Finally, the tegument proteins are inserted between the nucleocapsid and the envelope in the cytoplasm. In 2012, Chiu and al., demonstrated that the tegument protein BBLF1 is involved in virion maturation as expected before. Although this protein shares only about 15% of amino acid sequence with its homologues in HSV and HCMV, the post-translational modification of BBLF1 is conserved among the herpesviruses. This phenomenon facilitates the anchoring of BBLF1 to the membrane of the trans-Golgi network and therefore stabilizes the proteins. This stabilization serves to promote BBLF1 as a docking site in order to recruit other tegument proteins. Knocking down BBLF1 showed a decrease in the

production of viral particles validating the role of BBLF1 in virion maturation (Chiu et al., 2012). In 2018, Nanbo and al., confirmed the importance of intracellular compartments with Golgi markers in the EBV final envelopment (Nanbo et al., 2018).

4.4.2.3. Tegument proteins and signal transduction

4.4.2.3.1. Enzymatic activities

Post-translational protein modifications play crucial roles in eukaryotes cells. Viruses make use of the cellular ubiquitin proteasome system to inactivate cellular proteins with antiviral properties. For example, the EBV large tegument protein BPLF1 possesses a deneddylase activity (Gastaldello et al., 2010) that regulates virus production through modulating the activity of cullin-RING ligases. This enzyme also modulates cellular proteins, which create a virus replication permissive S-phase-like cellular environment.

4.4.2.3.2. Immune evasion

EBV is known for counteracting the host's innate immune response using the deubiquitinylation of cellular proteins that are involved in the toll-like receptor (TLR) signaling cascade. This process later facilitates the lytic replication as mentioned previously for the neddylation. Here the deubiquitinylase activity of the BPLF1 N-terminal region plays an important role. This second enzymatic activity suppresses the TLR-mediated activation of NF-kB. It allows EBV to target TRAF6, NEMO and IκBα (van Gent et al., 2014). EBV can counteract the TLR signaling in its host, either by degrading TLR mRNA or influencing downstream signaling of TLRs. Notably, the suppression of TLR9 signaling during lytic EBV reactivation may help the virus because the sensing of unmethylated EBV double stranded DNA to be packed in new virion particles is reduced. The replicative cycle is completed and evades innate immune control. New EBV particles can then be released from the host cell.

4.4.2.3.3. The cellular DNA damages response

EBV lytic proteins manipulate some cellular processes like the DNA Damage Response (DDR) by activating or inactivating different steps in response to the infection. This signaling pathway is

essential for the cells since it helps to maintain cellular genome integrity. However, EBV must inactivate it to replicate and persist in the target cells. The DNA ends of EBV genome mimic double strand DNA breaks. This can elicit the DDR when the viral genome enters in the nucleus. The ATM kinase is activated but the activation of p53 and apoptosis does not occur. In 2018, Ting-Hin Ho found that the tegument protein BKRF4 is a DDR inhibitor, which interferes with the histone ubiquitination at the DNA breaks during lytic and latent EBV infection. This protein was already shown to be involved in the production of infectious virions and to localize in the nucleus of the cells (Masud et al., 2017). The BKRF4 acidic domain allows it to bind directly to histones. This binding blocks the recruitment of RNF168 recruitment. Therefore, DNA repair cannot happen (Ho et al., 2018). The localization of the tegument protein within the cells might indicate their role in targeting particular cellular proteins to help the virus counteract the antiviral cellular system.

4.4.2.3.4. Cellular signaling pathways

Interaction of the tegument proteins with cellular signaling pathways allows EBV to deregulate the immune system and to subvert cellular processes. Reciprocally, cellular signaling pathways are also used for reactivating the lytic cycle of EBV. In 2014, Patrick Paladino found that some tegument proteins were involved in G1/S cell cycle arrest. Particularly, the BGLF2 protein induces p21 protein expression independently of p53 (Paladino et al., 2014). Few years later, two teams described a mechanism in which BGLF2 reactivates the EBV lytic cycle through the activation of the p38 Mitogen-Activated Protein Kinase (MAPK) pathway (Konishi et al., 2018; Liu and Cohen, 2015). Indeed, the over-expression of BGLF2 in latently infected cells induces BZLF1 expression. Subsequently, p38 and c-Jun N-terminal kinases (JNK) are phosphorylated and AP1 is activated. The activation of the MAPK signaling pathway helps the reactivation of the lytic cycle and then the production of mature virions.

4.5. Epstein Barr virus BGLF4 protein kinase

Epstein Barr virus encodes two protein kinases: the Thymidine Kinase (TK) and BGLF4, which is a serine/threonine protein kinase. Both kinases are conserved in herpesviruses, which lead to

speculate on their importance for the viruses. First, we will describe the serine/threonine protein kinases as well as their classification and then we will focus on BGLF4.

4.5.1. The serine/threonine protein kinases

4.5.1.1. Generalities on serine/threonine protein kinases

The phosphorylation/dephosphorylation of proteins is crucial for their cellular activity and it is regulated respectively by protein kinases and phosphatases. The protein kinases display particular enzyme activities: they catalyze the transfer of phosphate from a donor source such as nucleotide triphosphate (ATP or GTP) to a substrate while phosphatases catalyze the transfer of phosphate from the phosphorylated proteins, also called phosphoproteins, to water molecule (Cheng et al., 2011). Protein kinases are involved in cellular regulation such as cellular metabolism, cell growth, cell motility, membrane transport, learning or memory (Choi et al., 2020; Hong et al., 2020; Loh et al., 2020). Their dysregulation leads to alterations in cell signaling and therefore to some diseases such as cancers (Bertrand, 2020; Gizak et al., 2020; Kohlmeyer et al., 2020). As an example of the importance of protein kinases, these proteins represent 2% of the genome in most eukaryotes but they phosphorylate more than 30% of the cellular proteins (Ubersax and Ferrell, 2007) and they are particularly studied for their potential usefulness in clinical applications such as in the treatment of pancreatic, lung or breast cancer (Sahin et al., 2020; Wu et al., 2020). Herpesviruses also encode viral kinases. Protein kinases play an important role in viral replication. In cells infected with Human Cytomegalovirus, indolocarbozoles decrease the activity of pUL97, a BGLF4 homologous protein. As a consequence, the viral replication is inhibited (Marschall et al., 2002). Sato et al. published that cyclin A- and E-associated Cyclin dependent Kinase 2 (CDK2) complexes phosphorylate the EBV BDLF4 protein in vitro, a member of the viral Pre-initiation Complex (vPIC) (McKenzie et al., 2016; Sato et al., 2019). This complex is required for the expression of the late viral genes (Aubry et al., 2014). The destabilization of BDLF4 by CDK2 inhibitors leads to the down-regulation of late viral genes. It suggests that targeting the cellular kinases with inhibitors may be a therapeutic choice against Herpesviruses and notably EBV lytic replication (Sato et al., 2019). Protein kinases are usually divided into two broad classes on the basis of whether the primary phosphate acceptor site is a serine/threonine or a tyrosine (Roskoski, 2007, 2014) and most of the protein kinases have 3D

similar structure. The work of Zheng et al., enabled a better understanding of the catalytic structure of protein kinases (Zheng et al., 1993). The crystal structure of the catalytic subunit of cAMP-dependent Protein showed a canonical catalytic domain, which consists of an active site in a cleft between two lobes (Zheng et al., 1993). These two lobes represent a small N-terminal lobe of β-sheets and a large C-terminal lobe of α-helices with helices E and F in the core especially conserved and a flexible hinge serves as a link between the two lobes. ATB binds into this cleft: the adenosine moiety is buried in a hydrophobic pocket and the phosphate backbone is orientated outwards of the slot. Protein's phosphorylation occurs when the protein bind alongside the cleft. Subsequently, a set of conserved residues within the kinase catalytic domain catalyzes the transfer of the terminal γ-phosphate of ATP to the hydroxyl oxygen of the substrate's amino acids. The cleft is conserved among the protein kinases, 80% of these proteins being serine/threonine kinases (Manning et al., 2002). However, it differs in terms of charge and hydrophobicity of surface residues and in terms of depth. This is particularly important for the kinase specificity and it is the cause of the binding preference at the substrate's phosphorylation site, also called P-site. For example, the tyrosine kinases have a deeper catalytic cleft compared to the serine/threonine kinases. Although a tyrosine residue spans the distance between the substrate and the γ-phosphate of ATP, the serine and threonine residues cannot achieve it. Furthermore, the amino acid sequence of the substrate that binds alongside the cleft is particularly important as it contributes to the binding energy. Indeed, the presence of phosphorylatable amino acid within the sequence leads to a decrease in the inhibition constant (Ki) and consequently a higher binding energy to the interaction (Ben-Shimon and Niv, 2011; Hubbard, 1997; Zheng et al., 1993) In addition to the phosphorylable substrate's residue, the amino acids sequence ranking the P-site plays a role in the recognition between the kinase and the substrate. Kinase and substrate interact via complementary sequence on the basis of charge, hydrogen bonding or hydrophobic interaction. These sequences are called the consensus phosphorylation site. Most of the time, the active site of the kinase interacts with a motif of four amino acids on either side of the P-site. The further sequences from the P-site can bind the kinase's portion outside of the active site (Zheng et al., 1993). Finally the substrate affinity is enhanced due to the interactions between kinase's interaction domains and docking motifs on the substrate. The distal docking sites are domains separated from the substrate's P site and the kinase's active site and increase the efficiency of phosphorylation (Goldsmith et al., 2007). The docking interactions are defined

as the recognition of a short peptide motif in substrate by a groove on the kinase's catalytic domain, which is separated from its active site (Reményi et al., 2006). It sometimes provides an allosteric regulation of the kinase (Biondi and Nebreda, 2003). The stimulus responsible of the activation of the serine/threonine kinases allows to subclassified them (Plattner and Bibb, 2012). Here are the main serine/threonine protein kinases.

4.5.1.1.1. Cyclic nucleotide dependent kinases

Cyclic nucleotides are important in signal transduction pathways as they act as second messengers within the cells (Beavo and Brunton, 2002). PKA (cAMP dependent protein kinase) and PKG (cGMP dependent protein kinase) are cyclic nucleotide dependent protein kinases and are regulated respectively by cAMP and cGMP (Seifert et al., 2015). These two kinases are involved in many cellular processes cAMP is a second messenger involved in the regulation of hormone-mediated events. It is generated by membranous adenylyl cyclases under the control of G-protein couplet receptors and by the bicarbonate-stimulated soluble adenylyl cyclases, which are a metabolic sensor while cGMP, is produced by the nitric oxide-stimulated soluble guanylyl cyclases and atrial natriuretic peptide-stimulated particulate guanylyl cyclases. As many protein kinases, PKA and PKG possesses a regulatory and a catalytic subunit. The catalytic subunit includes a core of approximately 250 amino acids, which contains the sequences responsible for the substrate binding but also the catalysis of the phosphate transfer. It has been shown that the complex formed by the interaction of the two subunits renders the kinases inactive. When the intracellular concentration of cAMP or cGMP increases, the complex formed by the association of the two subunits is dissociated and subsequently the kinases become activated (Scott, 1991). PKA is involved in the regulation of energy homeostasis (Liu et al., 2018) in skeletal muscle and adipose tissue. It has also a crucial role in synaptic plasticity and sympathetic stimulation of the heart (Gold, 2019) among some other functions. Like PKA, PKG is involved in neuronal plasticity and memory function (Argyrousi et al., 2020) .The role of PKA and PKG has been demonstrated in a diverse array of biochemical events through their substrate's phosphorylation demonstrating their importance in the signaling cascade regulation.

4.5.1.1.2. Ca^{2+}/calmoduline dependent protein kinases

While some protein kinases require the second messenger cAMP to be active, others are dependent of the intracellular concentration in calcium/calmoduline and are called CaMKs (Plattner and Bibb, 2012). These kinases play an important role in the metabolism of neurons. When the intracellular Ca^{2+} levels increase, the Ca^{2+}/calmoduline complexes interact with the kinases, which leads to their activation (Hamilton, 1998). Six CaMKs are known: CaMKI, CaMKII, CaMKIV and MLCK (Myosin Light Chain Kinase). They are particularly studied in neurons (Lisman et al., 2002; Wayman et al., 2008)

4.5.1.1.3. Phospholipids dependent protein kinases

The phospholipid-dependent protein kinases are sensitive to specific second messengers which are constituted by the phospholipid hydrolysis products (Kolczynska et al., 2020). Furthermore, these kinases can be dependent or independent of the Ca^{2+} levels. The Protein kinase C (PKC) family (Plattner and Bibb, 2012) represents one example of phospholipid dependent protein kinases. The PKC family comprises more than 14 isoforms that are subdivided into three groups on the basis of their cofactors requirement: the conventional PKC, the novel PKC and the atypical PKC. In fact, the atypical PKC ζ and λ are considered constitutively active, whereas the conventional PKC α, β and γ require Ca^{2+} and dyacylglycerols (DAG) to be active and the PKC novel isoforms δ, ε and η need only DAG to be active (Mérida et al., 2019; Plattner and Bibb, 2012). The PKC isoforms are specific to some cellular compartments and cellular specific within the brain (Artola et al., 2020; Hapak et al., 2019; Hirai, 2018). This spatial regulation is mainly controlled by the interaction between the PKC and some scaffold proteins (Pearce et al., 2010). PKC is involved in the regulation of memory (Hapak et al., 2019) through its role in glutamatergic, GABAergic and cholinergic neurotransmission systems.

4.5.1.1.4. MAPK signaling pathway

The mitogen-activated protein kinase (MAPK) signaling pathway is conserved from yeast to humans and it is involved in various cellular functions, such as inflammation, cell stress response, cell differentiation, cell division, cell proliferation, metabolism, motility and apoptosis (Plattner and Bibb, 2012). These pathways involve many proteins among which the MAPK are found. These originally named Extracellular signal-Regulated Kinases (ERK) signal through the

phosphorylation of neighboring proteins. This allows an "on" or "off" switch of the proteins activity (Widmann et al., 1999). The MAPK belong to the proline-directed protein kinase family. The kinases of the MAPK are called MAPKK for MAPK Kinase. These MAPKK phosphorylate MAPK on their serine and/or threonine residues. Furthermore, the enzymes that phosphorylate the MAPKK are called MAPKKK or MAP3K and so on until the latest kinases of this pathway (Lee et al., 2020). The kinases of the pathway, which are downstream the MAPK, are also called MAPK-APK. Multiple extracellular stimuli can activate the MAPK cascade (Haneda et al., 1999; Kyriakis, 1999). Finally, it has been shown multiple cross-talks within the cascade (Javadov et al., 2014). For instance, DNA viruses alter the MAPK signaling pathway to access DNA replication machineries, induce the cell proliferation, or prevent the mechanisms of cell death activated in response to the viral infection (DuShane and Maginnis, 2019). Therefore, this signaling pathway highlights its importance in regulating cellular processes that should be helpful for cells but which are counteracted by viruses to sometimes result in tumorigenesis.

4.5.2. The BGLF4 protein

BGLF4 is a serine/threonine protein kinase, which was discovered in 1989. It has been well studied for years and has been demonstrated to have roles in multiple processes. Here, we describe its role as a conserved protein kinase and then its specific functions.

4.5.2.1. BGLF4 is a Conserved Herpesvirus Protein Kinase

All viruses are cellular parasites that manipulate cellular processes such as transcription, translation, cell cycle regulation, protein degradation or apoptosis, to promote their own replication. Phosphorylation of cellular and viral proteins is observed during lytic replication of cells infected by all herpesviruses and it involves different protein kinases. The conservation of this process suggests that kinases are essential in the viral life cycles. For example, the Herpes Simplex Virus 1 (HSV-1), which is an alphaherpesviruses, encodes two protein kinases called UL13 and Us3 (Gershburg et al., 2015). These serine/threonine protein kinases play a critical role in the efficient assembly and release of infectious HSV-1 virions. While the Us3 protein is conserved within the alphaherpesviruses, UL13 homologous proteins are found in alpha-, beta- and gammaherpesviruses. Indeed, UL13 homologous viral kinases were found in EBV in 1989 (Smith

and Smith, 1989). The EBV BGLF4 protein is a serine/threonine protein kinase, which shares conserved domains with the HSV UL13 and HCMV UL97 (Chee et al., 1989). It was reported that all of these protein kinases shared a motif found in the host cellular kinases. Therefore, these proteins were called Conserved Herpesviruses Protein Kinases (CHPK) (Gershburg and Pagano, 2008). All CHPKs contain 11 conserved domains, which characterize the serine/threonine catalytic domain of the protein kinases (Hanks et al., 1988). Furthermore, when the lysine found in the second domain of the viral kinases is mutated, the kinases lost their enzymatic activity. This confirmed that these viral proteins might share functions with the cellular protein kinases (Kawaguchi et al., 2003). Analyses of the substrates of CHPKs showed a common substrate: the cellular translation elongation factor EF-1δ (Kato et al., 2001, 2003). EF-1δ is involved in translation of cellular proteins (Proud, 1994). All CHPK, and particularly the EBV BGLF4 protein kinase, have been reported to phosphorylate this factor in infected cells (Kato et al., 2001; Kuny et al., 2010). Thus, Herpesviruses manipulate the translation machinery to their own benefit. All CHPKs phosphorylate the same site in EF-1δ: the Serine 133 (Kawaguchi et al., 2003). Furthermore, the cellular protein kinase cdc2 also called CDK1 for Cyclin dependent Kinase 1 (Dorée and Hunt, 2002), is also able to phosphorylate this site which led to the hypothesis that there is a conserved function between cdc2 and CHPKs (Kawaguchi et al., 2003). Cdc2 is a proline-directed serine/threonine protein kinase, which plays a role in cellular mitosis (Santamaría et al., 2007), transcriptional termination (Bregman et al., 2000), nucleolar disassembly (Hernandez-Verdun, 2011), reorganization of the cytoskeleton (Chou et al., 1990; Karsenti, 1991) and condensation of the chromosomes (Roth et al., 1991). This kinase is active when it forms a complex with the cyclin B (Dorée and Galas, 1994; Yasuda et al., 1993). The CDKs are also known to be kinases that control the cell cycle progression. They phosphorylate many proteins such as the cellular retinoblastoma tumor suppressor protein Rb (Ewen, 1994; Zetterberg et al., 1995) or the components of the cellular nucleus: the lamin A/C proteins (Marschall et al., 2011). All beta- and gammaherpesviruses CHPKs share common functions such as Rb phosphorylation (Kuny et al., 2010; Marschall et al., 2011). They also phosphorylate the nuclear component lamin A and disrupt the nuclear lamina (Lee et al., 2008; Meng et al., 2010; Sharma et al., 2015). Interestingly, BGLF4 induces a hyper-phosphorylation of the Rb protein and delays the S-phase progression together with the formation of micronuclei and structural defects of chromosomes in an artificial model (Chang et al., 2012c). Finally, SAMHD1 is an anti-viral host restriction factor

that limits the infection of some viruses such as Human Immunodeficiency Virus 1 (HIV-1), Herpes Simplex Virus 1 (HSV-1), vaccinia virus, human T cell leukemia virus type 1 and hepatitis B virus (HBV) (Laguette and Benkirane, 2012; Mauney and Hollis, 2018). The cellular CDK 1 and 2 phosphorylate this factor to trigger the antiviral restriction function (Mauney and Hollis, 2018). BGLF4, as all beta- and gammaherpesviruses protein kinases, phosphorylates SAMHD1 to counteract the cellular host defense (Zhang et al., 2019). This confirms the existence of conserved functions between CHPKs from beta- and gammaherpesviruses.

The Conserved Herpesviruses Protein kinases have been demonstrated to be early late lytic proteins (Hamza et al., 2004; Michel et al., 1996; Wang et al., 2005). BGLF4 is a typical example of CHPK: it is an early-late lytic protein whose promoter activity is up-regulated by the two immediate-early transactivators (IE) BRLF1 and BZLF1 (Wang et al., 2010). However, different promoters of BGLF4 may be used in either a cell type-specific or lytic stage-dependent manner for expression. Furthermore, the presence of a non-conventional nuclear localization signal in the BGLF4 C-terminal region is a second argument to describe the CHPK membership of BGLF4 because this is a common feature of all CHPKs except of HSV UL13 (Gershburg et al., 2010). Some functions of the CHPKs were already demonstrated or speculated for the viral benefit. For example, they play a role in tegument assembly (Chevillotte et al., 2009; Leisenfelder et al., 2008; Tobler et al., 2019), modulation of the gene expression (McKenzie et al., 2016), capsid nuclear egress (Chang et al., 2015; Milbradt et al., 2018; Wild et al., 2019) or viral replication (Ambinder, 2018). As previously mentioned, like cdc2, BGLF4 phosphorylates EF-1δ and induces cellular DNA condensation; this takes place through the phosphorylation of condensin and the activation of Topoisomerase II (Lee et al., 2007). Besides, BGLF4 reorganizes the nuclear lamina to facilitate the nuclear egress of nucleocapsids (Lee et al., 2007). BGLF4 is a nuclear protein in infected cells (Gershburg et al., 2004). Its ability to translocate within the nucleus is due to direct interactions with nucleoporins. BGLF4 interferes with the usual functions of Nup62 and Nup153 and helps the nuclear import of viral proteins for viral DNA replication and assembly (Chang et al., 2012a, 2015).

Finally, BGLF4 betaherpesvirus homolog is required for the activation of drugs that inhibit the viral replication (Kim et al., 1991; Littler et al., 1992; Pulliam et al., 1986; Talarico et al., 1999) although it is the viral thymidine kinase encoded by alphaherpesviruses that is responsible of the activation of the drugs. These drugs are first phosphorylated by the viral kinases and then the

drugs inhibit the viral replication through subsequent drug phosphorylation steps by cellular kinases to become a triphosphorylated drug. Meng and colleagues demonstrated that BGLF4 plays a similar role and is required for the phosphorylation of ganciclovir and acyclovir (Meng et al., 2010) confirming some similar functions between the CHPK (Figure 4).

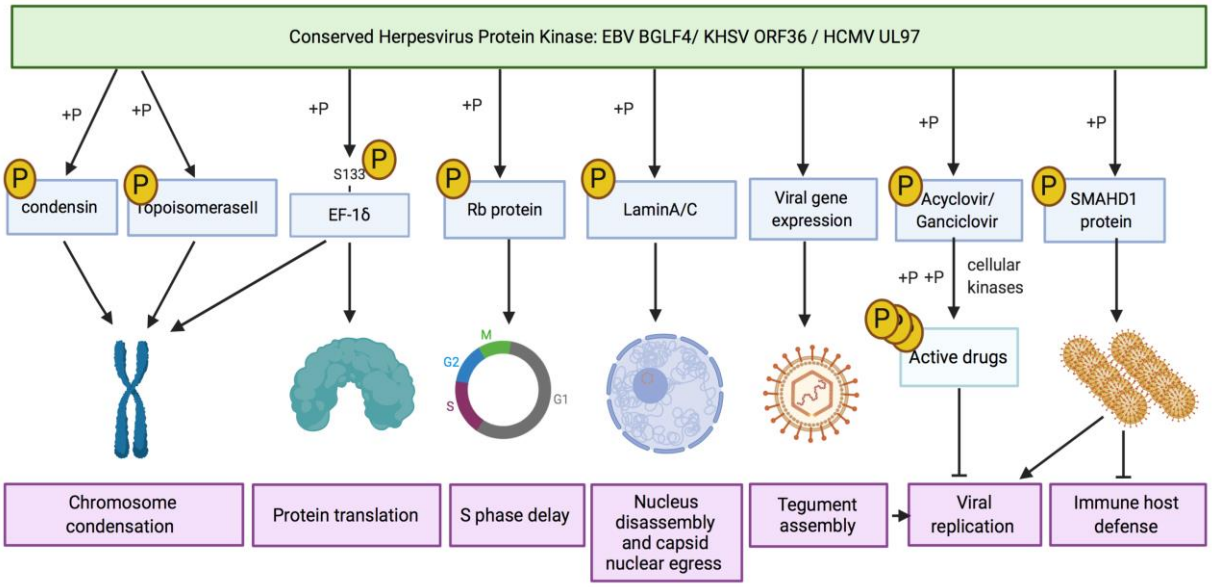


Figure 4: BGLF4 is a Conserved Herpesvirus Protein Kinase (CHPK). The figure summarized the roles in cellular and viral mechanisms in which all CHPK, and notably the EBV BGLF4 protein kinase, are involved. The process of phosphorylation is shown in the yellow circle. The arrows mean an activation process or an inhibition process. Figure created on BioRender.

4.5.2.2. Specific functions of BGLF4 in viral life and in cellular mechanisms

4.5.2.2.1. BGLF4 and the viral life

Although the various Herpesviruses protein kinases share many properties, differences have been noted. For example, UL13 is an insoluble protein whereas BGLF4 can be solubilized at physiological salt concentrations in *in vitro* tegument release assays (Asai et al., 2009). To demonstrate the role of BGLF4 in viral replication, (Gershburg et al., 2007) designed a knockdown of BGLF4 with RNA interference (siRNA). They showed that BGLF4 was involved in virions production and particularly in the nuclear egress as BGLF4 knockdown cells retain the viral nucleocapsids in the nuclei probably due to the abolishment of BFLF2 expression (Gershburg et al., 2007; Lee et al., 2008; Murata et al., 2009). Furthermore, BGLF4 interacts with

Xeroderma Pigmentosum C (XPC) to enhance the viral DNA replication and repair. Previous studies have shown that γ-herpesviruses, notably BGLF4, induce a DNA damage response in replicating cells through the phosphorylation of H2AX and the activation of the histone acetyl-transferase Tip60 (Li et al., 2011; Lu et al., 2007; Tarakanova et al., 2007). Not only does BGLF4 regulate EBV viral replication by phosphorylating BZLF1 at serine 209 (Asai et al., 2009), it does also interact with the small ubiquitin-like modifier SUMO2. This binding between SUMO2 and BGLF4 confers it the ability to suppress BZLF1 sumoylation and induces a DNA damage response (Li et al., 2012). In addition to these roles, BGLF4 has many viral targets including including the lytic DNA polymerase processivity factor (BMRF1)(Chen et al., 2000). BMRF1, which encodes the EA-D product, can be found in a phosphorylated or a hyper-phosphorylated form (Chen et al., 2000). EBV latent proteins are also targeted by BGLF4. EBNA2 is phosphorylated at the site serine 243 by BGLF4 and this blocks its ability to transactivate the LMP1 promoter (Asai et al., 2009). Owing to the down-regulation of some latent genes, it contributes to an effective lytic cycle and reduces the amount of EBV episomal genomes within the nucleus (Zhu et al., 2009). The heat shock protein Hsp90, wich is present in the tegument of EBV virions, stabilizes BGLF4 (Wang et al., 2016). Finally, BGLF4 is the only gene necessary for the expression of all late genes (McKenzie et al., 2016) (Figure 5).

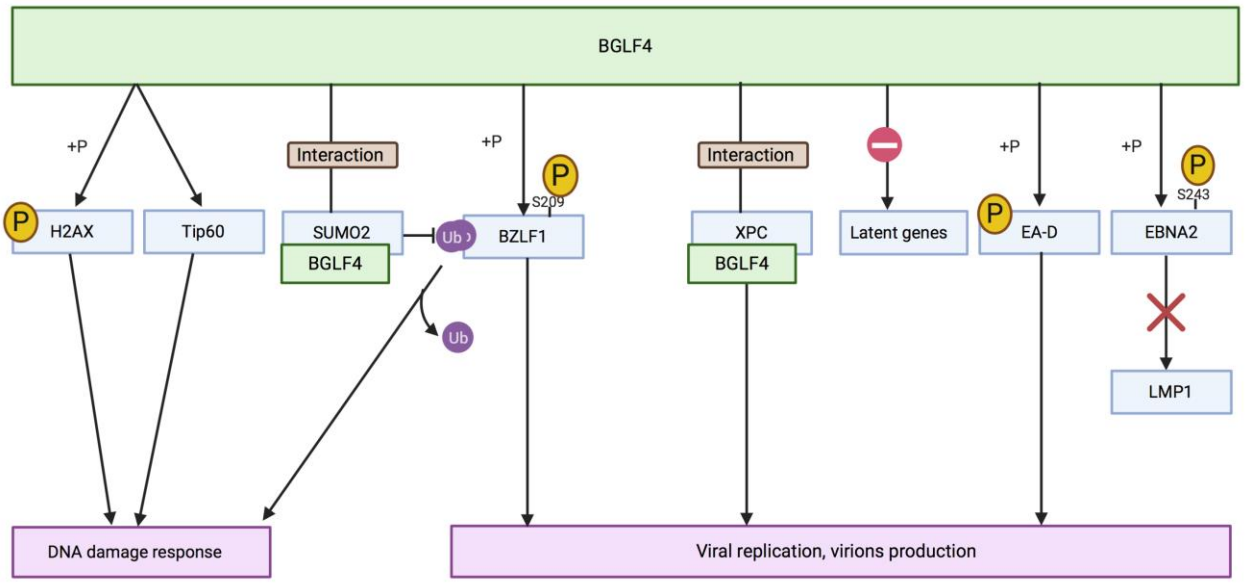


Figure 5: The specific functions of BGLF4 in EBV replication. The figure depicts the specific functions of BGLF4 during the viral replication. The process of phosphorylation is shown in the yellow circle. The red minus indicated a down-regulation step although the Red Cross means that the transactivation of the gene is not possible. Interaction between a specific protein and BGLF4 are visible

with the brown rectangles. The arrows mean an activation process or an inhibition process. Figure created on BioRender.

4.5.2.2.2. BGLF4 and its role in cellular mechanisms

In addition to its role in viral processes, BGLF4 plays a crucial role in cellular processes. BGLF4 phosphorylates and down-regulates the microtubule destabilizing protein stathmin. This kinase alters the cell microtubule dynamics (Chen et al., 2010). BGLF4 was shown to interact and suppress IRF3 transactivation activities, which lead to inhibit apoptosis and consequently an enhancement of viral replication (Wang et al., 2009).

To promote the lytic cycle, BGLF4 phosphorylates the UXT coactivator of NFκB. BGLF4 down-regulates NFκB transactivation in response to tumor necrosis factor alpha (TNF-α) and poly(I·C) stimulation. It also down-regulates NFκB-regulated cellular genes expression. Furthermore, BGLF4 attenuates the NF-κB-mediated repression of the EBV lytic transactivators, Zta and Rta (Chang et al., 2012b). To conclude, the knockdown of UXT enhances the lytic cycle, suggesting a role for UXT in maintaining the EBV latency (Figure 6).

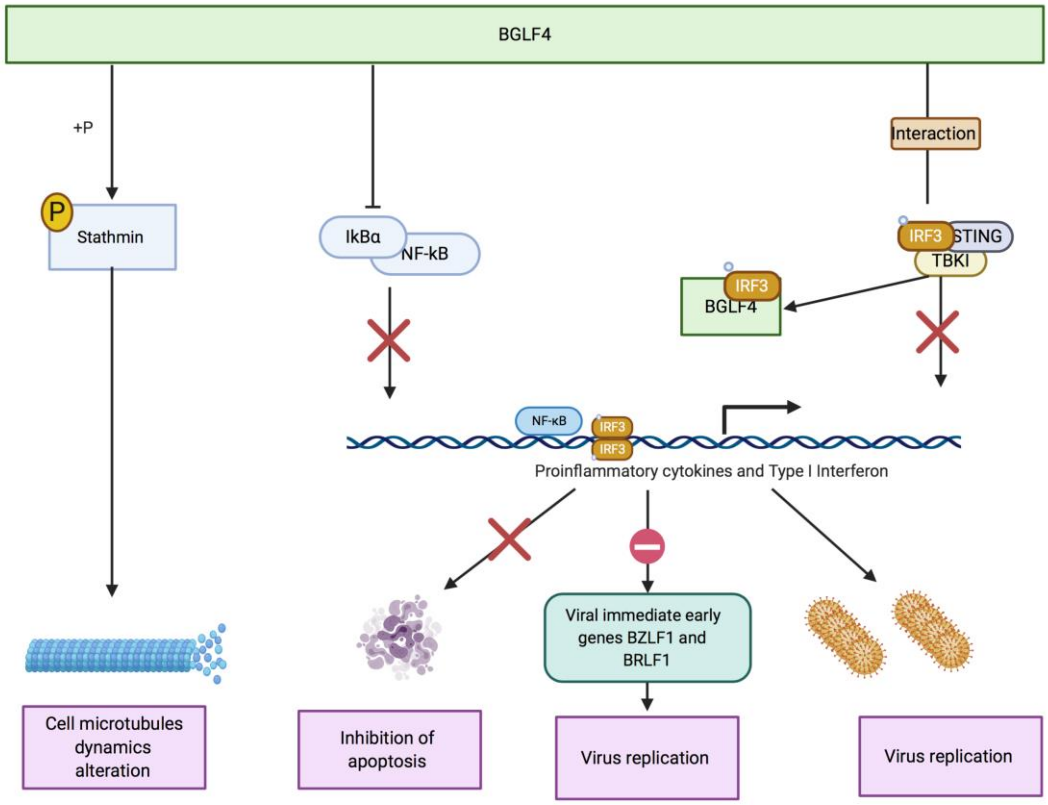


Figure 6: The specific functions of BGLF4 in cellular mechanisms. The figure depicts some specific functions of BGLF4 within the cells. The process of phosphorylation is shown in the yellow circle. The red minus indicated a downregulation step although the Red Cross means that the transactivation of the gene is not possible. Interaction between a specific protein and BGLF4 are visible with the brown rectangles. The arrows mean an activation process or an inhibition process. Figure created on BioRender.

4.6. Aims of the Thesis

The tegument proteins functions were postulated from studies of HSV-1 and HCMV tegument proteins. Recently, data on EBV tegument proteins were collected from studies in EBV B95.8 strain or Akata cells. These models need to be induced to express lytic replication and many questions remain regarding the functions of these tegument proteins in spontaneous lytic replication as well as in processes such as virions production or infectivity.

We constructed a library of recombinant viruses to study the importance of the tegument proteins during virions production, infectivity and lytic replication in LCL established from primary B cells infected with viruses.

Later, we studied BGLF4 serine/threonine protein kinase. BGLF4 from EBV Akata or B95.8 strains have been described to play role in induced replication, to affect nuclear egress, and to contribute in some extent to the cell cycle arrest. Besides, it has been demonstrated to activate some anti-viral replication drugs in vitro such as acyclovir and ganciclovir. However, the efficiency of the drugs has not been observed in clinic. Therefore, it is interesting to investigate the M81 BGLF4 function. Thus, nothing has been reported in its functions during the switch between lytic replication and latent infection.

1- What is the role of M81 BGLF4 during early post-infectious events?
2- Is BGLF4 required for the EBV M81 lytic cycle and what is its role during the switch between lytic replication and latent infection?

The aim of my thesis was to study the phenotype of BGLF4 in LCL established from infection of primary B cells with virus that carry a viral mutant genome for the expression of M81 BGLF4. Notably, we studied EBV infectivity in the absence of BGLF4 expression. The role of BGLF4 to support spontaneous lytic replication and its role during the switch between lytic replication and latent infection was also a major point of the study.

5. Material and Methods

5.1. Material

5.1.1. Bacteria

E.coli DH5α bacteria carry the following genotype: *fhuA2 lac (del) U169 phoA glnV44 Φ80' lacZ (del) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR1.*

E.coli DH10β bacteria carry the following genotype: *F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 galU galK nupG rpsL λ⁻.*

GS1783 bacteria carry the following genotype: *DH10B l cI857 Δ (cro-bioA)<>araC-PBADI-sceI.*

5.1.2. Oligonucleotides

Eurofins MWG synthesized the oligonucleotides.

Name of the primer	Goal	Sequence
BGLF4 KO fwd	M81 ΔBGLF4	5'- CTTGAGGCCAGAGACGAGCTGCGACAGGCCCTCG AGCCATT TGAGGAACATGGATGTGAATATGGCTGC GGAGTTGTAGGGATA ACAGGGTAATCGATTTATTCAACAAAG-3'
BGLF4 KO rev	M81 ΔBGLF4	5'- GTTCTGGGGAGACACTCAACTCGCCACTGCTGGA GGAGTTC GTCGGGCTCTACTCCGCAGCCGTATTCAC ATCCGTGTTCCTCAAATGCCAGTGTTACAACCAATT AACC-3'
BGLF4 rever-tant fwd	M81 ΔBGLF4 revertant	5'-CTTGAGGCCAGAGACGAGCTGCGACAGGCCCTCG AGCCATTTGAGGAACATGGATGTGAATATGGCTGCGG AGTTGTAGGGATAACAGGGTAATCGATTTATTCAA CAAAG-3'
BGLF4 rever-tant rev	M81 ΔBGLF4 revertant	5'-GTTCTGGGGAGACACTCAACTCGCCACTGCTGGA GGAGTTC GTCGGGCTCAACTCCGCAGCCATATTCA CATCCATGTTCCTCAAATGCCAGTGTTACAACAATTAACC-3'

5.1.3. Plasmids

5.1.3.1. Vector plasmid

pRK5 was used for constructing the expression plasmids that were required in this study. It places the insert behind an early gene promoter from Cytomegalovirus (CMV).

5.1.3.2. Expression plasmids

The expression plasmids were used for transient expression into the producer cell lines:

p509 contains the BZLF1 gene derived from EBV B95.8 strain and controlled by a CMV promoter.

p2080 contains the BLRF1 gene and controlled by a CMV promoter. This plasmid must be co-transfected with p509.

pRA contains the BALF4 gene (= gp110) derived from EBV B95.8 strain and controlled by a CMV promoter. This plasmid must be co-transfected with p509.

B300 contains the BGLF4 gene derived from EBV B95.8 strain and controlled by a CMV promoter.

5.1.3.3. Plasmid used for making the recombinant EBV

Pcp15 is the template of the kanamycin resistance cassette. This vector carries a region of homology with the EBV genome, which is necessary for the construction of a new recombinant EBV.

5.1.4. Recombinant EBVs (rEBV ; EBV-BACs)

B110 is the rEBV and carries the rEBV M81 wildtype.

B1671 is the rEBV and carries the rEBV M81 defective for the expression of BGLF4.

B1804 is the revertant of B1671. It carries the revertant of the rEBV M81 mutant defective for the expression of BGLF4.

B1039 is the rEBV and carries the rEBV M81 mutant defective for the expression of the Thymidine Kinase.

5.1.5. Eukaryotic cells

The **HEK 293 cell line** is a specific cell line originally generated by the transformation of embryonic epithelial kidney cells with adenovirus (ATCC CRL-1573). https://www.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-36-1-59#tab2

B-lymphocytes are primary B cells isolated from human blood samples.

Elijah negative cell line is an EBV-negative subclone of EBV-positive Elijah Burkitt's lymphoma cell line that grows in suspension.

The **EBV producer cell lines** used in this study (**rM81; ΔTK; ΔBGLF4; ΔBGLF4 rev**) were established by stable transfection of EBV-BACs into HEK293 cells supplemented with

100µg/mL hygromycin. The ΔTK, ΔBGLF4 and ΔBGLF4 rev are available on the basis of the EBV M81 strain. ΔBGLF4 and ΔBGLF4 rev were constructed with the "en passant mutagenesis" method. M81ΔBGLF4 lacks the BGLF4 tegument protein and M81ΔTK lacks the thymidine kinase. The M81 ΔBGLF4 rev expresses the BGLF4 tegument protein.

WI 38 feeder cells are primary human lung embryonic fibroblasts.

5.1.5.1. Primary cells

Peripheral blood mononuclear D19$^+$ B cells are isolated from fresh blood buffy coats by Ficoll density gradient. CD19$^+$ B cells were selected with CD19 (PanB) magnetic beads (Invitrogen).

5.1.5.2. Cell culture media

All cell lines used in the experiments were routinely grown in RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biochrom AG).

5.1.6. Antibodies

Name	Clone number	Usage for	Origin	Company
Anti α-actin	Pan AB5	WB	Mouse, monoclonal	Jackson Immuno Research Laboratories
Anti γ-tubulin	T6557	WB	Mouse, monoclonal	Sigma-Aldrich
Anti-mouse coupled to Cy3	111-165-146	IF	Goat	Dianova
Anti-mouse IgG (HRP, secondary antibody)		WB	Rabbit	Promega
Anti-rabbit coupled to Alexa 488	A11029	IF	Goat	Invitrogen
Anti-rabbit IgG (H&L) (HRP, secondary antibody)		WB	Goat	Cell signaling Technology
Anti-rat IgG (HRP, secondary antibody)		WB	Goat	Jackson Immuno Research Laboratories
BZLF1	BZ.1	WB	Mouse	Hybridoma supernatant
Gp350/220	72A1	IF, WB	Mouse	Hybridoma supernatant
BGLF4		IF, WB	Rabbit	Hybridoma supernatant (Regina Federlee & Al., 2009)
EBNA2	PE2	IF	Rat	Hybridoma

				supernatant
EA-D	MAB818	WB	Mouse, monoclonal	Chemicon
LMP1	559898	WB	Mouse, monoclonal	BC Pharmingen
Phosphor-Histone3	9716	IHC	Mouse, monoclonal	Cell Signalling Technology

5.1.7. Buffers and solutions

Buffer	Composition
Blotting Buffer	25 mM Tris, 150 mM Glycine, 20% Methanol
6X DNA loading buffer	0.25% Bromophenolblue, 40% (w/v) Sucrose, dissolved in H2O
Luria-Broth agar medium	12.8g Bacto-Agar, 2g NaCl in 400mL H_2O
Luria-Bertani liquid medium	10g Tryptone, 5g Yeast extract, 10g NaCl in H2O; pH7.0
Lysis buffer (for circle preparation)	1% SDS, 2mM EDTA, 50mM NaCl, 40mM NaOH
Low fat milk 5%	5% low fat milk in 1X PBST
Mounting buffer (IF)	90% Glycerol in PBS
PFA 4%	4% paraformaldehyde in PBS; pH 7.4
10X Phosphate Buffered Saline (PBS)	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; pH=7.4
10X Phosphate Buffered Saline Tween 20 (PBST)	1.37M Nacl, 27mM KCl, 100mM Na2HPO4, 20mM KH2PO4, 1% Tween 20
4X Protein Sample Buffer	200mM Tris-HCl pH 6.8, 8% SDS, 40% Glycine, 0.4% Bromophenolblue (with or without 4% β-Mercaptoethanol)

5X Ripa Buffer	750 mM NaCl, 2.5% NP40, 5% Sodium deoxycholate, 0.5% SDS, 25 mM EDTA, 100 mM Tris-HCl pH 7.5
10X SDS running buffer	250 mM Tris, 1.92 M Glycine, 1% SDS; pH 8.5-8.8
Seperating gel buffer	2M Tris pH 8.9
20X SSC	3M NaCl, 0.3 M Sodium Citrate; pH7.0
Stacking gel buffer	2M Tris pH 6.8
Staining buffer (IF)	10% Heat-inactivated goat serum in PBS
Staining buffer (IHC)	3% BSA in PBS
TAE	40mM Tris, 1mM EDTA, 19mM acetic acid
TBE	100mM Tris, 90mM boric acid, 1mM EDTA

5.1.8. Commercial Kits

Name	Usage for	Company
Deatachabeads	Removal of bound dynabeads	Invitrogen
Dynabeads CD19 Pan B	Isolation of human primary B-cells	Invitrogen
Nucleobond BAC100	EBV-BAC DNA preparation	Macherey-Nigel
Western Lighting Plus ECL	Western Blots	PerkinElmer

5.1.9. Chemical and reagents

Name	Usage for	Company
Acetone	IF	Sigma Aldrich

Acrylamide : 30% stock/0.8% bisacrylamide	WB	Roth
a-thioglycerol (aTG) 50µM	Cell culture	Sigma M6145
Bathocuproinedisulfonic acid (BCS) 20mM	Cell culture	Sigma B1125
B-mercaptoethanol	WB	Sigma Aldrich
BSA	WB	Sigma Aldrich
Chloramphenicol	Bacteria culture (=antibiotic)	Serva 16785
DAPI	IF, IHC	
DNA ladder	DNA gel migration	Life Technologies
dNTP mix 10mM	PCR	Invitrogen
Ethanol, molecular biology grade	Various	Sigma Aldrich
Ethidium bromide	DNA gel migration	Life Technologies
Ficoll Plus	B cells isolation	Amersham Biosciences
Heparine sodium salt	B cells isolation	Sigma Aldrich
HINGS	IF	
Hygromycin B	Cell culture (= antibiotic)	Invitrogen
Isopropanol, molecular biology grade	Various	Sigma Aldrich
Metafectene	DNA transfection in cells	Biontex Laboratories
Methanol, molecular biology grade	Various	Sigma Aldrich
Page Ruler Prestained Protein ladder	WB	Fermentas
Protease inhibitor cocktail	WB (protein samples)	Roche
RNase free water	PCR/qPCR/primers	Invitrogen
RNase inhibitor	DNA Mini-preparation/digestion	Promega

Roti-Phenol	DNA Circle preparation	Roth
Sodium Pyruvate	Stable selection of transfected cells	Thermo Scientific
Triton X-100	IF	AppliChem
Trypan Blue solution	Cell culture	Sigma Aldrich
Trypsin EDTA 0.05%	Cell culture	Invitrogen
Tween 20	WB (Wash Buffer)	GE Healthcare

5.1.10. Enzymes

Enzyme	Usage	Company
Phusio High Fidelity DNA polymerase	PCR for cloning	Thermo Scientific
Restriction Enzymes	Cloning and Checking the genomic integrity of EBV-BAC	Fermentas
Rnase A	Minipreparation of DNA	Roche
Dnase I	qPCR	Fermentas
Proteinase K	qPCR and Circle preparation of DNA	Roche
TaqMan Universal Master mixes	qPCR	Life technologies

5.1.11. Consumables, equipment and software

Name	Category	Company
0.5, 1.5 and 2ml reaction tubes	Consumables	Eppendorf
15 and 50ml Falcon tubes	Consumables	Greiner Bio-one
Amersham membrane HybondTM ECL	Consumables	GE Healthcare Life Sciences

Amersham Hyperfilm Hybond XL	Consumables	GE Healthcare Life Sciences
Bacteria plates	Consumables	Greiner Bio-one
Cell counting chamber	Consumables	Biorad
Cell culture plates and flasks	Consumables	TPP, Cellstar
Filtercards for THARMACfunnel 407	Consumables	Tharmac
Glass slides for cytospin	Consumables	Tharmac
Microscope glass slides, coverslips	Consumables	Thermo Scientific
Micro-slide 8 well plate	Consumables	Ibidi
Shandon EZ Single cytofunnel	Consumables	Thermo Scientific
Syringe-driven sterile filter unit (0.45µm)	Consumables	Millipore
Tips	Consumables	Greiner Bio-one
Applied Biosystems 7300 Real-time PCR	Equipment	UVP
Bacteria incubator	Equipment	Hereaus
CO_2 cell incubator	Equipment	Thermo Scientific
Curix60	Equipment	AGFA
Cuvettes (electroporation)	Equipment	Carl Roth
Protein quantification machine	Equipment	Tecan
Centrifuges	Equipment	Sigma, Hereaus
MineVE Blotter	Equipment	GE Healthcare Life Sciences
Light microscope DMIL	Equipment	Leica
Magnetic rack	Equipment	Applied Biosystems
Microscope Leica (motorized)	Equipment	Zeiss
Nanodrop	Equipment	GE Healthcare

		Life Sciences
Radioactivity oven	Equipment	Hybrigene Techne
Shandon Cytospin 4 centrifuge	Equipment	Thermo Scientific
T100 Thermal cycler	Equipment	Biorad
Imager 680	Equipment	Amersham
FIGI	Software	Image J

5.2. Methods

5.2.1. Bacteria culture and transformation

5.2.1.1. Bacteria culture conditions

All *E.coli* strains were cultured in LB-medium in a shaker or alternatively on LB-agar plates in an incubator in order to have individual colonies. The LB-medium or LB-agar plates were supplemented with chloramphenicol antibiotic (15µg/mL). Depending on the bacteria, the cells were cultured at 32°C or 37°C. 10% glycerol was added into the bacteria for long-term storage and the bacteria were frozen at -80°C.

5.2.1.2. Bacteria electroporation

We used electroporation to transform DNA isolated from producer cell lines into electroporation-competent DH10β bacteria. The bacteria were previously prepared by a technician and kept in 10% glycerol at -80°C. We thawed bacteria slowly on ice and 20µL of bacteria were used per electroporation. The DNA was added to the bacteria into cuvettes and the mixture was incubated on ice for 5 minutes. We performed electroporation at 1.5kV, 200Ω, 25µFd. The bacteria were immediately transferred into 2 mL of pre-warmed LB-medium and cultured for 1 hour at 37°C for recovery. Then, the bacteria were spun down by centrifugation at 3000 rpm for 10 minutes, and pelleted bacteria were-suspended and cultured on LB-agar plate supplemented with chloramphenicol antibiotic overnight to get single colony.

5.2.1.3. Construction of recombinant EBVs and related techniques

The wild type EBV strain M81 is available as a recombinant BACmid: rM81 EBV. The M81 EBV genome was cloned onto a prokaryotic F-plasmid that carries the chloramphenicol (Cam) resistance gene, the gene for green fluorescent protein (GFP) and the hygromycin resistance gene (B240). All PCR primers used for the construction of the PCR cloning of M81 ΔBGLF4 and M81 ΔBGLF4 revertant are listed in the Table 1.2. and are based on the rM81 EBV sequence (GenBank accession number KF373730.1). "En passant mutagenesis" was applied to construct M81 ΔBGLF4 and M81 ΔBGLF4 revertant respectively from rM81 EBV and M81 ΔBGLF4. Mutations were inserted in the BGLF4 gene sequence by homologous recombination of the rM81 EBV with a linear DNA fragment that encodes the kanamycin resistance gene, flanked by Flp recombination sites, and short DNA regions homologous to the regions immediately outside of the deletion to be obtained. We also applied the same method in order to construct the M81 ΔBGLF4 revertant. The sequence carrying the BGLF4 sequence was reintroduced into the M81 ΔBGLF4. The integrity of the BGLF4 sequence was confirmed by sequencing and glycerol stocks of the bacteria containing the rEBV BACs were stored at -80°C. DNA BAC-preps were performed on liquid culture following the instructions of a manufacturer's kit to get the BAC DNA necessary for the transfection of HEK 293 cells.

5.2.2. Eukaryotic cell culture and HEK 293 cell transfection

5.2.2.1. Cell culture conditions

All cells were routinely cultured at 37°C in incubators with an atmosphere of 100% humidity and 5% CO_2. Adherent cells (HEK 293 cells, producer cell lines and WI 38 cells) and LCLs were maintained in RPMI-10% FCS. HEK 293 cells were stably transfected with rEBV supplemented with hygromycin (100µg/mL). LCLs and Elijah negative cells were regularly split 1 to 5 whereas adherent cells were weekly split 1 to 10. Pre-warmed 0.05% trypsin-EDTA was used for 1 minute at 37°C for the split of producer cell lines. WI 38 feeder cells were also cultured in RPMI-10% FCS but split by using 0.25% trypsin-EDTA.

5.2.2.2. HEK 293 cells transfection

5.2.2.2.1. Plasmid transfection

3 x 10^5 HEK 293 cells per well in 6-well-plates were seeded with a final volume of 2 mL. After 24 hours of incubation, cells of one well were transfected with the following procedure: 1 µg of plasmid was resuspended in 100 µL RPMI; at the same time, 3 µL of metafectene, which is a liposomal-based transfection reagent containing cationic lipids, were resuspended in 100 µL of RPMI. After 15 minutes of incubation at room temperature, the two mixtures were mixed slowly and then left for 20 minutes at room temperature. During incubation, 1.2 mL of medium was removed from the well. The final mixture is gently added dropwise to the cells and the cells are place overnight at 37°C. One day post-transfection, the medium of the transfected cells was removed and replaced with fresh medium (RPMI-10% FBS). Analysis of the transfected cells can be performed 4 days post-transfection.

5.2.2.2.2. Stably transfected HEK293 producer cells

HEK 293 cells were seeded at a concentration of 3 x 10^5 cells per well in 6-well-plate in a final volume of 2 mL. One day after, cells of one well were transfected with the following procedure: 7 µL of BAC-DNA were resuspended in 100 µL RPMI with a cut tip; at the same time, 5 µL of metafectene, were resuspended in 100 µL of RPMI. After 15 minutes of incubation at room temperature, the two mixtures were mixed slowly and then left for 20 minutes at room temperature. In parallel, 1.2 mL of medium was removed from the well. The final mixture was gently added dropwise to the cells and the cells were incubated overnight at 37°C. One day post-transfection, efficiency of the transfection is checked under fluorescence microscope and the transfected cells were transferred in a cell culture dish (15 cm diameter) and selected by adding hygromycin antibiotic.

5.2.2.2.3. Generation of rEBV/293 producer cell lines

The transfection procedure of HEK 293 cells is described in 2.3.2.2. 3 weeks after transfection, cell clones that express the highest GFP expression were picked up and expanded in 6-well-plates in RPMI-10% FCS supplemented of hygromycin in order to keep the EBV BAC inside the cells

in the long term. The cells were tested for virus production. A qPCR and a gp350-staining were respectively performed on the supernatants and the transfected cells. The cell clones, which display on the highest viral titer and gp350 expression, were then analyzed for the rEBV genome integrity by circle preparation. The final rEBV/293 producer cell clone is selected out for the study.

5.2.3. Viruses production

The rEBV/293 producer cells were lytically induced by transfection of a plasmid containing the BZLF1 gene (p509) and a plasmid containing the BLRF1 gene (p2130). One day post-transfection, medium was exchanged and replaced by fresh RPMI-10% FCS. Four days post-transfection, the virus supernatant was harvested and filtered with a 0.45 µm cellulose filter. The virus was stored at 4°C.

5.2.4. Viral titers quantification by qPCR

Total copy number of viral genomes was determined by a qPCR measurement on the viral supernatant. In a first time, 4 µL of DNAse I were added to 44 µL of supernatant and incubated at 37 °C for 1 hour to fully remove free viral DNA in supernatant that contains the viral particles. Then, DNAse I was inactivated at 70 °C for 10 minutes. The viral envelope protected the viral DNA and DNAse I could not digest it. The viral envelope was then digested by proteinase K for the release of viral genome for qPCR analysis. 5 µL of DNAse I treated supernatant were incubated with 5 µL of proteinase K (100 µg/mL) for 1 hour at 50 °C. Then, proteinase K was denatured at 75 °C for 20 minutes. The qPCR master mix was prepared with primers and probe that encode the viral DNA polymerase BALF5 gene, mixed with the supernatant treated with proteinase K and amplified by real time PCR using a StepOnePlus machine. The sequences of the primers and the probe, the master mix components and the qPCR cycling conditions are listed in the table below.

Name	**Sequence**
EBV pol probe	5'- FAM - CATCAAGAAGCTGCTGGCGGCC – TAMRA – 3'
EBV pol forward primer	5'- AGTCCTTCTTGGCTAGTCTGTTGAC – 3'
EBV pol reverse primer	5'- CTTTGGCGCGGATCCTC – 3'

For 1 reaction

Master mix component	**Volume in µL (Final volume =25µL)**
Taqman universal master mix 2X	12.5
EBV pol forward primer 10µM	2.5
EBV pol reverse primer 10µM	2.5
EBV pol probe 20µM	1.0
H_2O	1.5
DNAse I/ proteinase K treated viral supernatant	5.0

qPCR cycling conditions

Temperature	**Time**	**Number of cycles**
50°C (initial denaturation)	2 minutes	*1
95°C (denaturation)	10 minutes	*40
95°C	15 seconds	
60°C	1 minute	

Internal controls with known copies number were included for comparison. Finally, the qPCR results were analyzed in order to give the absolute copy number of EBV genomes per mL of viral supernatant.

5.2.5. Confirmation of the genome integrity of the recombinant EBV in the stably transfected HEK293 producer cells by analysis of the rescued circular BACmid: Circle preparation method

10^7 producer cells were prepared for confirming the genome integrity of the rEBV in producer cells. Cells were washed in sterile cold PBS 1X two times prior to lysis with circle preparation

lysis buffer at room temperature for exactly 5 minutes. To neutralize the pH of the lysate, 500 µL of 1 M Tris-HCl, pH 7.1 was added drop wise into the solution, followed by 2 mL of 3M NaCl. Proteins in the lysate were incubated with proteinase K (10mg/ml) at 37°C overnight. Phenol/butanol extraction method was performed, and the viral DNA was precipitated with 2.5 volumes of absolute ethanol at -20 °C overnight. DNA was pelleted down at 4800 rpm for 30 min at room temperature, washed with ethanol 70% and dissolved in 50 µL of TE buffer. *E.coli* DH10β bacteria were electroporated with BAC DNA and the BACmid of five single colonies were analyzed by digestion with BamHI restriction enzyme. The genome integrity of these rEBV/ HEK 293 cells was compared to a control, which is the parental rM81 EBV.

5.2.6. B cells infections, transformation and binding assay

5.2.6.1. B cells infection assay

10^5 B cells were purified from buffy coats of different healthy donors and were exposed to viruses for two hours at room temperature at a multiplicity of infection (MOI) of 30 virus genomes per cell, as quantified by qPCR. The infected cells were spun down and plated in 96-well plates in RPMI-10% FCS. 3 days post-infection, cells were stained using anti-EBNA2 antibody and the percentage of EBNA-2 positive cells was counted. This measurement provides the number of infectious units per volume of applied viral supernatant.

5.2.6.2. B cells transformation assay

For transformation assays, the percentage of EBNA2 positive cells was determined within the infected cells from infection assay using immunostaining 3 days post-infection. 3 or 30 EBNA2-positive cells per well were seeded in U-bottom 96-well plates already coated with 10^3 gamma-irradiated WI38 feeder cells. The outgrowth of lymphoblastoid cell lines (LCLs) was monitored at 2 and 4 weeks post-infection.

5.2.6.3. Binding assay

The capacity for the binding of viruses-containing supernatants to target cells was analyzed by incubating Elijah cells at a MOI of 10 genome equivalents per cell with FACS and

immunostaining. Immunostaining was performed following this protocol: 10^5 Elijah cells were exposed to viral supernatants for 4 hours at 4°C on the rotator. Cells were washed 3 times with ice cold PBS and cells were dropped and stained for gp350 expression. The negative control was cells exposed to RPMI-10% only. The amount of cell-bound viral particles was then visualized under immunofluorescence microscope.

FACS analysis was performed following this protocol: cells were rolled at 4°C for 4 hours, washed 2 times with ice-cold PBS-1X and then resuspended in 100µL of cold PBS-1X. The cells were then fixed for 15 minutes with 100µL of 4% formaldehyde. Cells were washed 2 times, incubated with gp350 antibody on ice for 30 minutes, washed one time with cold PBS-1X and incubated with a mouse PE antibody for 30 minutes on ice. Cells were washed prior FACS acquisition.

5.2.7. Immunofluorescence staining

5.2.7.1. EBNA2 staining

Cells were washed 3 times and re-suspended in PBS 1X. Cells were dropped and air-dried on a glass slide. Cells were fixed in a solution composed of 50% methanol/50% acetone for 20 min at room temperature. Cells were incubated with an EBNA2 antibody for 30 min at 37°C, washed in PBS 1X three times for 5 minutes, and incubated with a mouse secondary antibody conjugated to Cy-3 for exactly 30 min at 37°C. Cells were washed in PBS three times for 5 minutes, embedded in 90% glycerol and observed with a fluorescence microscope (Leica). The slide was stored at 4°C.

5.2.7.2. BZLF1 and BGLF4 staining

Cells were washed 3 times and re-suspended in PBS 1X. Cells were dropped and air-dried on a glass slide. Cells were fixed in PBS- 4% paraformaldehyde for 20 min at room temperature, permeabilized in PBS 0.5% Triton X-100 for 2 minutes exactly and incubated with a BZLF1 or BGLF4 antibody for 30 min at 37°C. Cells were washed in PBS 1X three times for 5 minutes, and incubated with a mouse secondary antibody conjugated to Cy-3 (BZLF1) or rabbit secondary antibody coupled to Alexa 488 (BGLF4) for exactly 30 min at 37°C. Cells were washed in PBS

three times for 5 minutes, embedded in 90% glycerol and observed with a fluorescence microscope (Leica). The slide was stored at 4°C.

5.2.7.4. Gp350 staining

Cells were washed 3 times and re-suspended in PBS 1X. Cells were dropped and air-dried on a glass slide. Cells were fixed in acetone for 20 min at room temperature and incubated with a gp350 antibody for 30 min at 37°C. Cells were washed in PBS 1X three times for 5 minutes, and incubated with a mouse secondary antibody conjugated to Cy-3 for exactly 30 min at 37°C. Cells were washed in PBS three times for 5 minutes, embedded in 90% glycerol and observed with a fluorescence microscope (Leica). The slide was stored at 4°C.

5.2.8. Electron microscopy analysis

5.2.8.1. Cells microscopy

The rEBV/HEK 293 producer cells were lytically induced with the BZLF1 and BLRF1 expression plasmids. 4 days post-induction, the cells were resuspended gently in cold PBS 1X, centrifuged at 300 g for 5 minutes at room temperature. The pelleted cells were fixed with 2.5% glutaraldehyde by placing on ice for 30 minutes. After this incubation, the buffer was replaced with fresh 2.5% glutaraldehyde buffer and passed onto the DKFZ Core Facility for further preparations. Samples were post-fixed in 2% osmium tetroxide in cacodylate buffer for 2 h at 4°C, stained with 0.5% uranyl acetate for 16 h at 4°C, washed twice in distilled water, dehydrated in ethanol, and embedded in Epon resin. Ultrathin sections were examined by a Zeiss electron microscope. 20 random cells were studied for the analysis.

5.2.8.2. Virus microscopy

Supernatant (5 mL) produced by BZLF1/BLRF1 induced rEBV/HEK 293 producer cells were centrifuged for 2 hours at 30 000*G* at 4°C. The virus pellet was fixed with 2.5% glutaraldehyde, and the DKFZ Core Facility carried out further preparations as mentioned in 4.2.8.1. Ultrathin sections were examined by a Zeiss electron microscope.

5.2.10. Lymphoblastoid Cell Lines (LCLs)

5.2.10.1. Establishment of LCLs

B cells were purified from buffy coats of different healthy donors and were incubated with 2 mL of virus overnight at 37°C. The next day, virus was removed and replaced with RPMI-20% FCS until the LCL was established. The cells are then maintained in RPMI-10% FCS.

5.2.10.2. Cell growth

Cell growth was monitored for 3 days by counting the cell numbers. 3.10^5 cells from established LCL were seeded in 48-well-plates with a final volume of 1 mL RPMI-10% FCS. At 1, 2 and 3 days post-seeding, the cells were resuspended and 7 µL of cells were then used for cell counting.

5.2.11. Southern blot

A Gardella gel electrophoresis was followed by a Southern blotting to detect viral DNA in replicating lymphoblastoid cells. 5.10^5 cells were resuspended in buffer (PBS 1X, Ficoll 7%) and loaded in Gardella gel (Gardella & al., 1984) at 4°C. Lysis Buffer (TBE, SDS 1%, bromophenol blue 0.01%) was quickly layered over the cells. Electrophoresis was performed for 2 hours at 80V and then increased to 120V for 14 hours. Gel was stained with TBE/EtBr and visualized under UV light. The gel was then blotted onto a Hybond XL membrane and hybridized with a ^{32}P-labeled DNA fragment specific to the EBV gp350 gene. Lymphoblastoid cells carrying the EBV 2089 strain were used as a negative control.

5.2.12. Western blot

Proteins were extracted from cells using a standard RIPA buffer (150 mM NaCl, 0.5% NP-40, 1% Sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 20 mM Tris-HCl pH7.5, proteinase inhibitor cocktail (Roche)) on ice. Sonication was performed to shear the genomic DNA. All protein expressions were performed in reduced conditions using Laemmli Buffer except for gp350 expression analysis. A total of 50 µg of proteins were denatured for 10 min at 95°C and separated on SDS-polyacryl-amide gels. The transfer of proteins was performed by electroblotting onto a

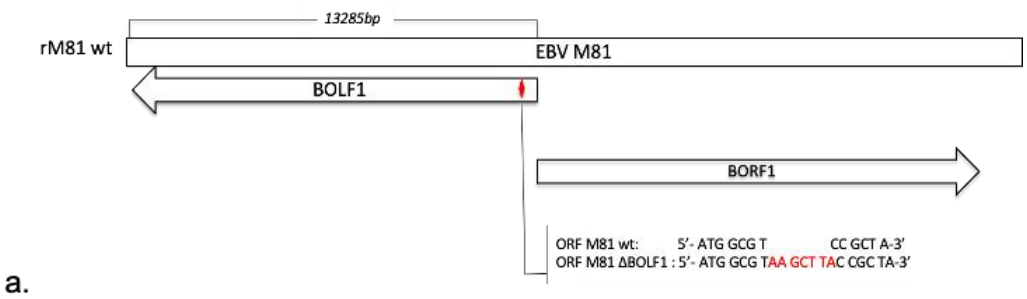
wet nitrocellulose membrane at 25V for 90 minutes. The membrane was incubated in PBST- milk 5% for 50 minutes. The primary antibody was added and the membrane was incubated at 4°C overnight. After washings in PBST, the blot was incubated for 1 hour with a suitable secondary antibody coupled to horseradish peroxidase (goat anti-mouse (Promega), goat anti-rabbit (Life technologies), or rabbit anti-goat (Santa Cruz) IgG). Bound antibodies were detected using the ECL detection reagent .

6. Results

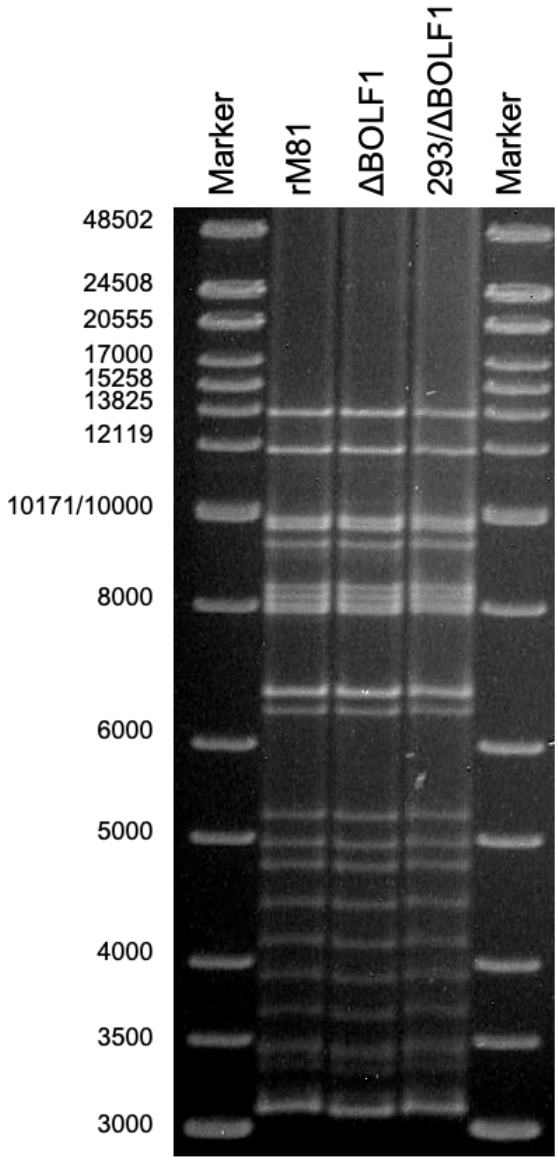
6.1. Study of EBV M81 tegument proteins functions during early lytic events

6.1.1. Construction of a library of recombinant viral genomes

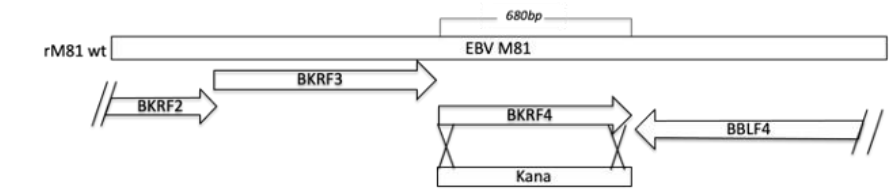
Although the EBV B95.8 tegument proteins have been extensively studied during the last few years, their role in spontaneous lytic replication is unknown (Chiu et al., 2012; Duarte et al., 2013; Gershburg et al., 2004; Masud et al., 2017, 2019). Therefore, we concentrated on the EBV M81 strain that spontaneously replicates in B cells (Tsai et al., 2013). To determine the role of the M81 tegument proteins in B cells, we infected primary B cells with M81 viruses defective for the expression of tegument proteins. These defective viruses were obtained by deletion of the BLRF2, BKRF4, BOLF1, BSRF1, BBL1 and BGLF4 genes using homologous recombination of the viral genome with selection markers. For example, we introduced a kanamycin cassette in the viral genome to delete the BLRF2 and BKRF4 genes. However, this method was not suitable for the construction of the other mutants, as a complete deletion of these genes would also delete regulatory elements that are important for the expression of neighboring genes. Indeed, the viral genes encoded by the EBV genome are frequently overlapping. Consequently, we used the “en passant mutagenesis”, a homologous recombination method, which introduces a stop codon at the beginning of the gene of interest to inhibit the protein expression (Tischer et al., 2010). This method does not influence the expression of the neighboring genes. The restriction pattern of BKRF4 mutant and BOLF1 mutant after digestion with BamHI restriction enzymes are given in Figure 7, whereas the other recombinant mutants are present in Appendix 1-3. The BGLF4 restriction pattern will be given in paragraph 5.2.1.

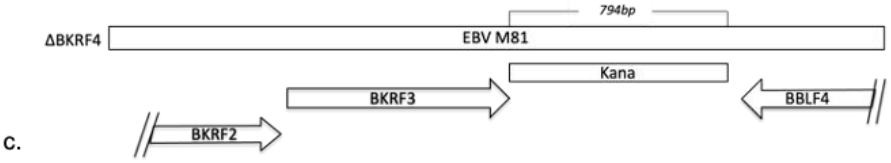

13285bp
rM81 wt
EBV M81
BOLF1
BORF1
ORF M81 wt: 5'- ATG GCG T CC GCT A-3'
ORF M81 ΔBOLF1 : 5'- ATG GCG TAA GCT TAC CGC TA-3'

a.

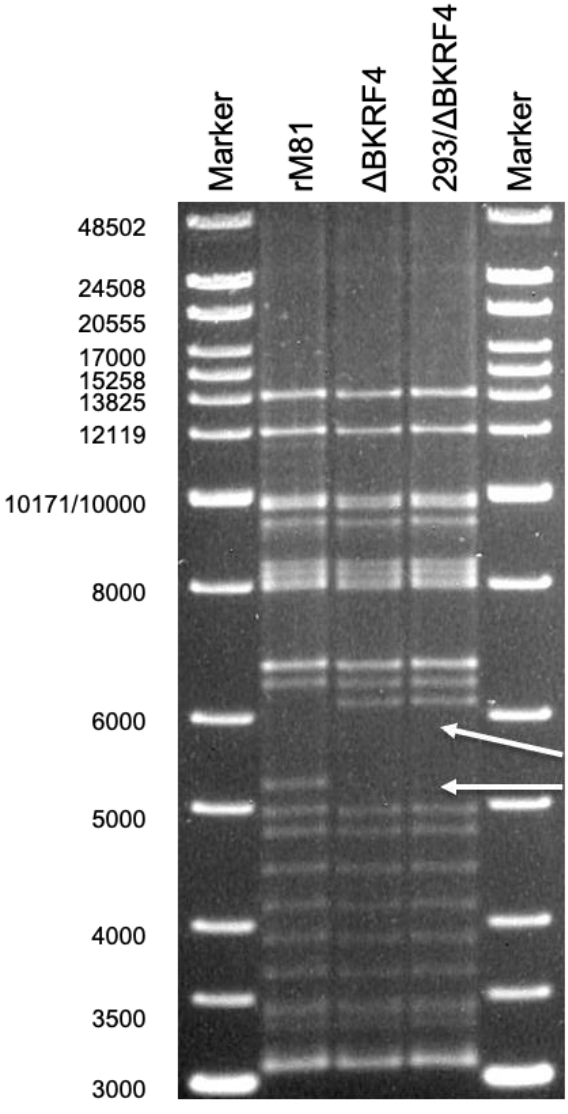

Marker
rM81
ΔBOLF1
293/ΔBOLF1
Marker
48502
24508
20555
17000
15258
13825
12119
10171/10000
8000
6000
5000
4000
3500
3000

b.





c.



d.

<u>Figure 7</u>: Generation of recombinant M81 EBV tegument protein knockouts. (a and c) Schematic representations of the genomic maps of the recombinant M81 (rM81) and tegument protein knockouts

viruses (BOLF1 KO and BKRF4 KO). The figures depict some of the viral neighboring genes of BOLF1 and BKRF4. The BOLF1 tegument protein mutant was constructed by “en passant mutagenesis” of the BAC clone M81 wild-type. The BKRF4 tegument protein mutant was constructed by homologous recombination to exchange the gene of interest against a selection marker. (b and d) Mini-preparations of DNA from the rM81 wild type genome and of tegument protein mutants derived thereof were cleaved with the restriction enzyme BamHI and separated on a 0.65% agarose gel for 27 hours at 65V. The analyses also included the BOLF1 and BKRF4 knockout genomes rescued from the 293 producer cell line in which it was stably transfected. Arrows indicate the different viral DNA fragments between the wild-type genome rM81 and the BKRF4 mutant genome (ΔBKRF4) due to the insertion of a kanamycin cassette. The numbers on the right correspond to the size of the DNA marker.

6.1.2. The tegument proteins: roles in virions production and infectivity

To be able to infect B cells and establish LCL from these cells, we began by producing viruses with the producer cell lines. The two immediate-early proteins BZLF1 and BRLF1 proteins were transiently transfected into the M81 wild-type (M81wt) and tegument protein mutant producer cells to induce the lytic cycle. Trans-complemented mutant viruses were obtained by co-transfecting plasmids encoding the different tegument proteins. Three days after transfection, supernatants were collected and a qPCR determined the number of EBV DNA copies per mL. The figure 8 shows that deletion of BLRF2, BKRF4, BBLF1, BSRF1 and BGLF4 affect viral titers. The M81 wild-type viral titer is about 7.10^7 number of EBV DNA copy per mL of supernatant. Trans-complementation of the mutants with the missing tegument protein rescued at least half of their viral titer in comparison to M81wt.

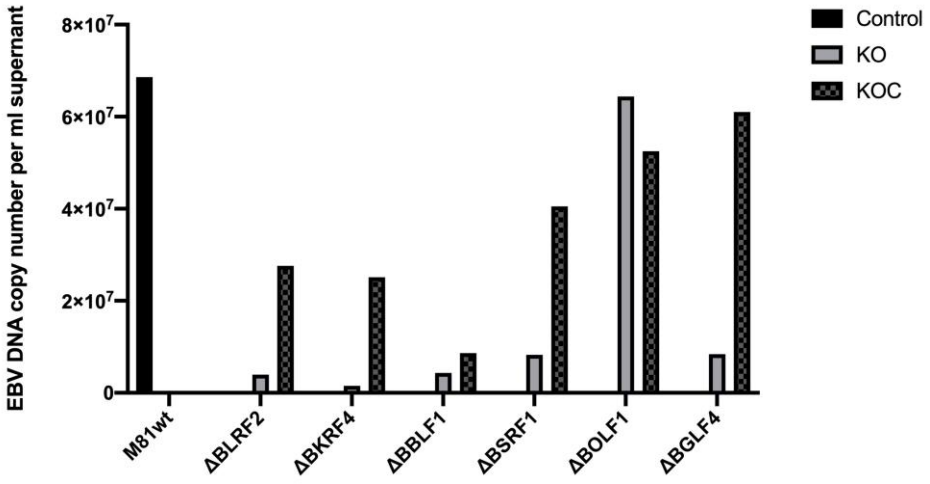


Figure 8: Role of the tegument proteins in virion production. Viral titers in various induced tegument protein mutant cell lines (ΔBLRF2, ΔBKRF4, ΔBBLF1, ΔBSRF1, ΔBOLF1, ΔBGLF4). HEK293 cells, carrying the indicated EBV genomes, were transfected with BZLF1 and BRLF1 expression vectors and harvested 3 days later. M81wt represents our control sample. KO represents the tegument protein mutant cells lines samples without trans-complementation. KOC represents the tegument protein mutant cell lines for which we trans-complemented the deleted tegument protein. Viral DNA levels per mL of supernatant were quantified using qPCR amplification of the viral BALF5 gene.

However, deletion of the BOLF1 protein did not affect virus production as 293 cells carrying the BOLF1 Knockout (ΔBOLF1) delivered similar titers in comparison to M81 wild-type. Furthermore, trans-complementation of BOLF1 mutant with BOLF1 expression plasmid did not

increase the level of virus production and even slightly decreased it. To conclude, unlike BGLF4, BLRF2, BBLF1, BSRF1 and BKRF4 tegument proteins, which are required for efficient virions production, BOLF1 does not seem to play an important role in virus progeny production.

To assess the role of the tegument proteins during EBV M81 infection, we exposed primary B cells to our virus panel. To this end, we used the same number of viral genomes to infect primary B cells. Three days post-infection, cells were collected and stained for the expression of the EBNA2 protein that is a marker of EBV latently-infected B cells. The Figure 9 shows the results obtained after infectivity assays. It revealed that while ΔBLRF2, ΔBKRF4, ΔBBLF1 viruses are nearly not infectious, the ΔBSRF1 virus showed a six-fold reduction in infection rates relative to wt. However, the BOLF1 knockout and BGLF4 knockout viruses infect B cells only two times less relative to wt controls.

Altogether, the assays indicate that the tegument proteins are necessary for an efficient EBV infectivity, but some tegument proteins are more important than others.

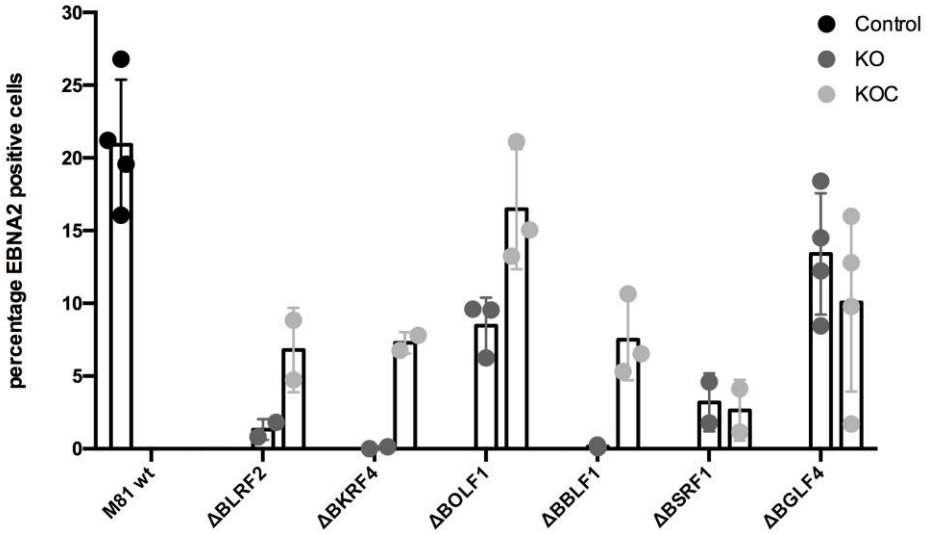
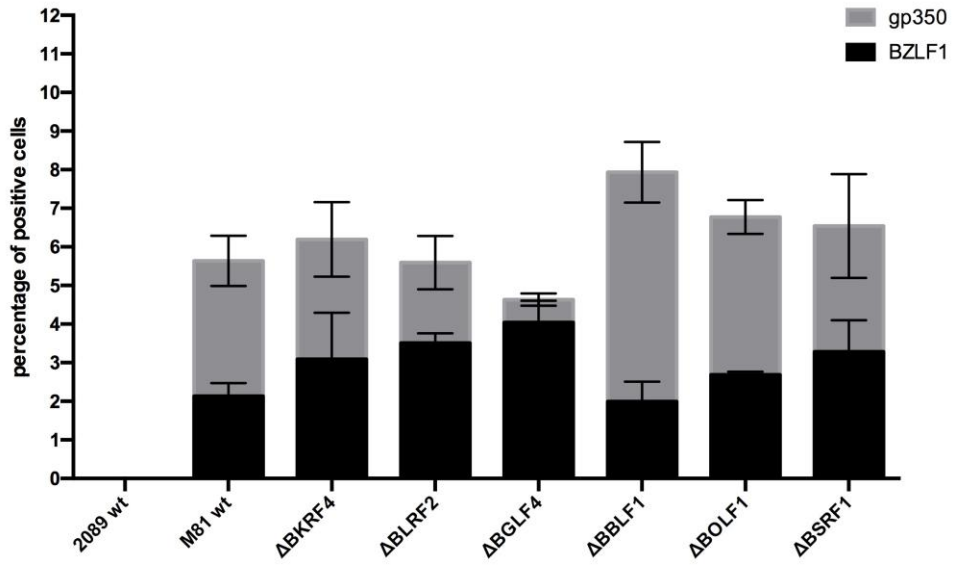


Figure 9: Role of the tegument proteins in infectivity. Primary human B cells were infected with the same number of defective viral genomes and an EBNA2 immunostaining was performed on cells 3 days post-infection to determine the number of EBNA2-positive cells. The mean and SEM values are shown. M81wt represents the positive control. KO means that cells were infected with knockout viruses and KOC means that we used trans-complemented viruses.

6.1.3. The role of the tegument protein during spontaneous lytic replication

To assess the role of tegument proteins in spontaneous lytic replication, primary B cells were infected with viruses devoid of tegument protein expression. Four weeks after infection, we examined the expression of two lytic proteins, the immediate early-lytic protein BZLF1 and the glycoprotein gp350 in primary B cells. Primary B cells were infected with 2089 viruses as negative control. EBV 2089 does not express BZLF1 or gp350 spontaneously (Tsai et al., 2013). The control, M81wt, shows that about 2% of the cells express BZLF1 and 6% express gp350. We can observe that all LCLs deleted for the expression of one tegument protein expressed BZLF1 protein in a similar level, around 2-3%. However, gp350 expression differs in LCL depending of the type of tegument protein knockout LCLs (Figure 10). Notably, ΔBGLF4 LCLs show a a decrease of gp350 expression (around 4.5 %°) in comparison of the other LCLs deleted for the expression of one tegument protein. Collectively, these data show that the tegument proteins are not involved in the initiation of spontaneous replication, but can influence its final outcome.



<u>Figure 10</u>: Expression of BZLF1 and gp350 in LCL during spontaneous lytic replication. Primary B cells were infected with viruses devoid for the expression of a tegument protein. Cells were maintained in culture for 30 days and a staining for the expression of two lytic proteins, BZLF1 and gp350 was performed at the time the cells were replicating. The mean and SEM values are shown.

6.2. The deletion of BGLF4 tegument protein affects multiple viral processes

6.2.1. Deletion of the BGLF4 gene in the EBV M81 genome by homologous recombination

We then focused our attention on the BGLF4 serine/threonine protein kinase and its involvement in infected B cells. This protein has previously been studied in detail and is known to play multiple roles in EBV lytic replication (Asai et al., 2006, 2009; Aubry et al., 2014; El-Guindy et al., 2014; Feederle et al., 2009; Gershburg et al., 2004b, 2007; Goswami et al., 2012; Li et al., 2011, 2012; Meng et al., 2010; Zhu et al., 2009). However, nothing has been reported about its role after infection with the M81 strain that undergoes spontaneous lytic replication in B cells. BGLF4 has been reported to phosphorylate some anti-viral drugs such as acyclovir and ganciclovir and render them toxic to infected cells (Meng et al., 2010). Indeed, treatment of patients with these drugs led to a reduction of EBV production in the saliva (Ljungman et al., 2007).

We inactivated the BGLF4 gene by "en passant mutagenesis" that uses homologous recombination of the viral genome to introduce stop codons in the three open reading frames at the beginning of the BGLF4 gene (M81/ΔBGLF4). This limited mutagenesis did not affect the expression of the neighboring genes BGLF5 and BGLF3.5. Furthermore, we constructed a revertant virus by reintroducing the wild-type BGLF4 gene into the M81/ΔBGLF4 genome to recover the wild-type configuration (M81/revBGLF4). All recombinants were stably introduced into HEK293 cells to generate producer cell lines, which carry intact copies of the mutant or revertant genome. These cells were respectively called 293/ΔBGLF4 and 293/Rev BGLF4 (Figure 11).

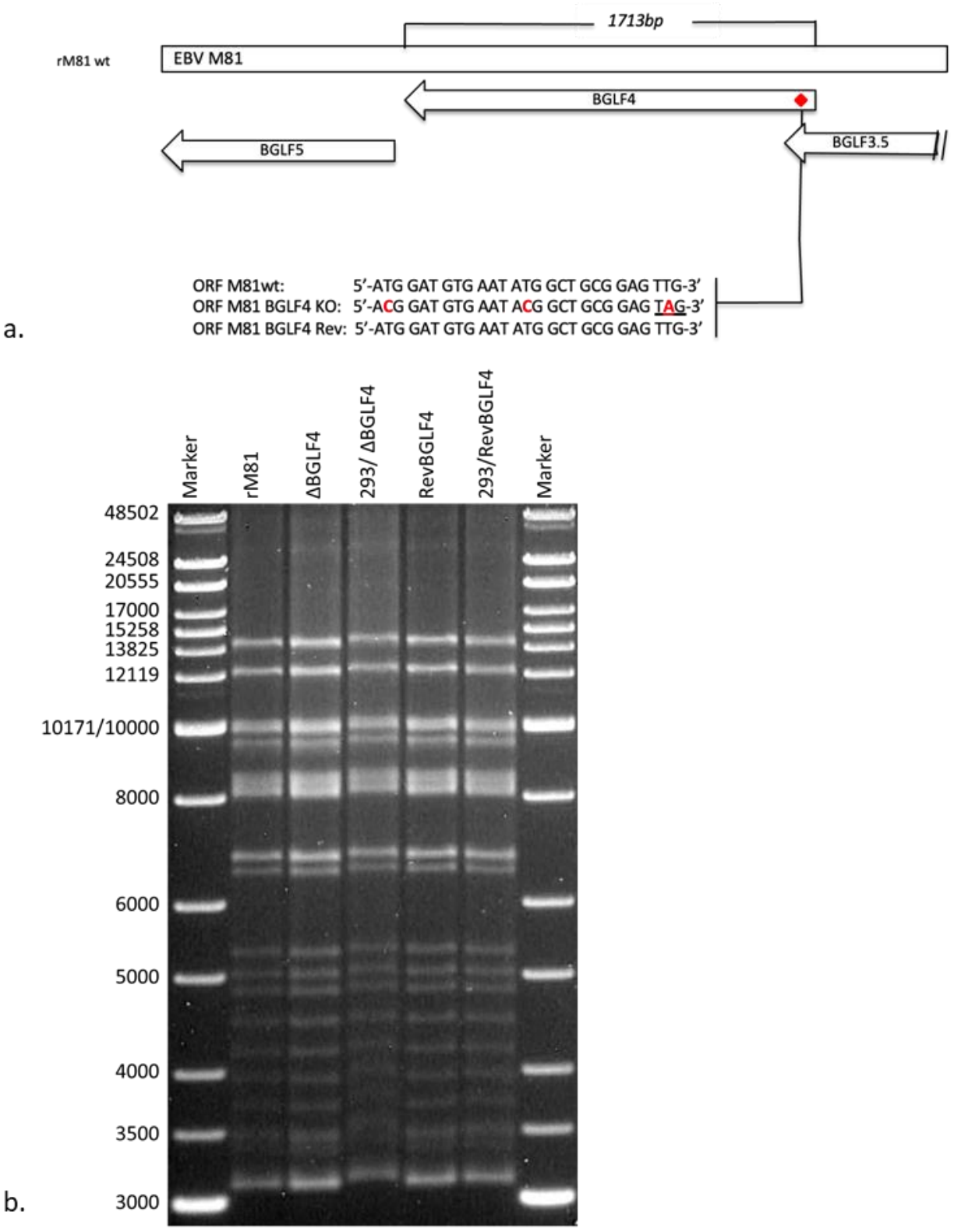
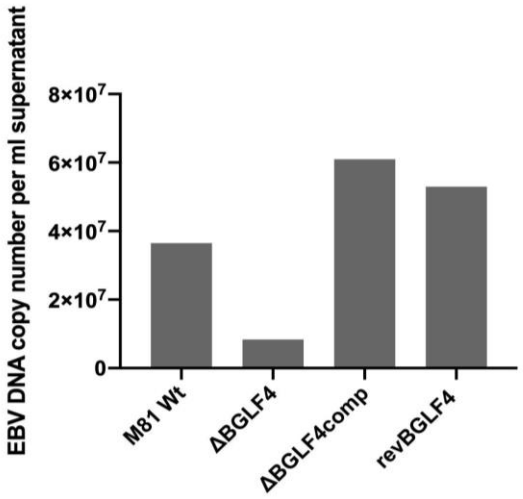


Figure 11: Generation of the recombinant M81 EBV BGLF4 Knockout and M81 EBV BGLF4 Revertant genomes. (a)Schematic representation of the genomic map of the recombinant M81 wild-type

(rM81), BGLF4 Knockout (ΔBGLF4) and BGLF4 Revertant (RevBGLF4) viruses. The figure depicts some of the viral neighboring genes of BGLF4. The BGLF4 tegument protein mutant and its Revertant were constructed by "*en passant"* homologous recombination of the BAC clone recombinant M81 wild-type. (b) Mini-preparations of DNA from the rM81, the ΔBGLF4 and the Rev BGLF4 genome were cleaved with the restriction enzyme Bam HI and separated on a 0.65% agarose gel for 27 hours at 65V. This analysis showed that the BGLF4 Knockout and the BGLF4 Revertant genome present in 293/ΔBGLF4 and 293/ Rev BGLF4 Producer cell lines have an intact structure. The numbers on the right correspond to the size of the DNA ladder.

6.2.2. BGLF4 is required for efficient virion production in epithelial cells.

We first determined the contribution of BGLF4 to EBV lytic replication in 293 cells. To this end, we transiently transfected the two immediate-early proteins BZLF1 and BLRF1 into the M81 wild-type (M81wt), BGLF4 knockout (ΔBGLF4) and BGLF4 revertant (revBGLF4) producer cells to induce the lytic cycle. Three days after transfection, supernatants were collected and a qPCR measured the number of EBV DNA copies per mL of supernatant (Figure 12).



<u>Figure 12</u>: Role of the BGLF4 tegument protein in EBV virion production. Viral titers in M81 wild-type (M81 wt), BGLF4 mutant (ΔBGLF4) and BGLF4 revertant (revBGLF4)-induced producer cell lines. HEK293 cells, carrying the indicated EBV genomes, were transfected with BZLF1 and BRLF1 expression vectors and supernatants were harvested on day 3 post-infection. Viral DNA genome copies per mL of supernatant were quantified using qPCR amplification of the viral BALF5 gene. ΔBGLF4comp indicates the producer cells that were trans-complemented for BGLF4 expression.

In the absence of the BGLF4 protein within induced cells, we noted that virus production decreased 16 times in comparison to the wild-type virus. However, complementation of the gene

in trans rescued the wild type phenotype. Similarly, 293 cells carrying the BGLF4 revertant produced virus at wild type levels. We then performed a binding assay to measure the binding ability of the viruses. Elijah EBV negative cells were used in this assay and were infected at the same multiplicity of infection as defined by qPCR (30 genomes per target cell). An immunostaining confirmed the presence of virus particles on the surface of the cells (Figure 13).

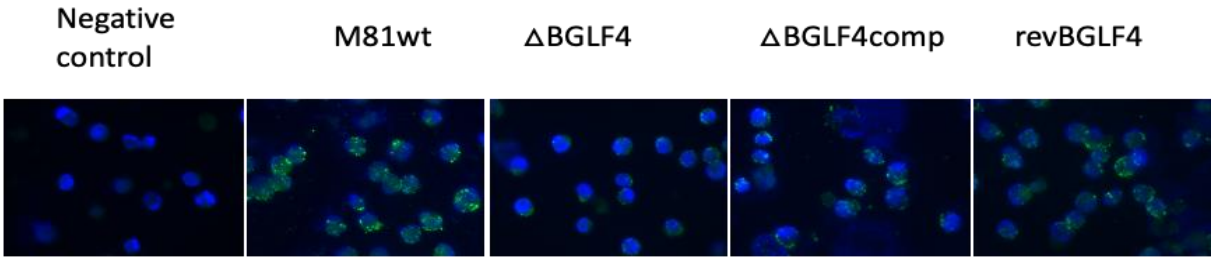


Figure 13: BGLF4 is required to bind B cells efficiency. EBV negative Elijah B cells were infected with the same number of viral genomes. The cells were exposed to culture medium only as a negative control. A gp350 immunostaining was performed to confirm the virus binding on the surface of the cells. The amount of cell-bound viral particles was visualized under immunofluorescence microscope. Cells were stained with DAPI (in blue) and gp350 expression is visible in green.M81wt is the positive control and ΔBGLF4 represents the cells infected with knockout virus. revBGLF4 represents the cells that were infected with Revertant BGLF4 virus although ΔBGLF4comp was obtained by infecting Elijah cells with trans-complemented ΔBGLF4 virus.

However, we noted that the M81/ΔBGLF4 viruses gave rise to much weaker signals than the wild type controls. The complemented viruses and the revertant showed the same level of signals, the wild type virus gave even stronger signals but in the range of variation that is observed among wild type controls. Indeed, a FACS analysis of the different viruses bound to B cells showed no difference between the wild type controls and the complemented viruses (Figure 14 a). This analysis also confirmed that the cells exposed to the BGLF4 deletion mutant displayed a much lower level of binding than the controls.

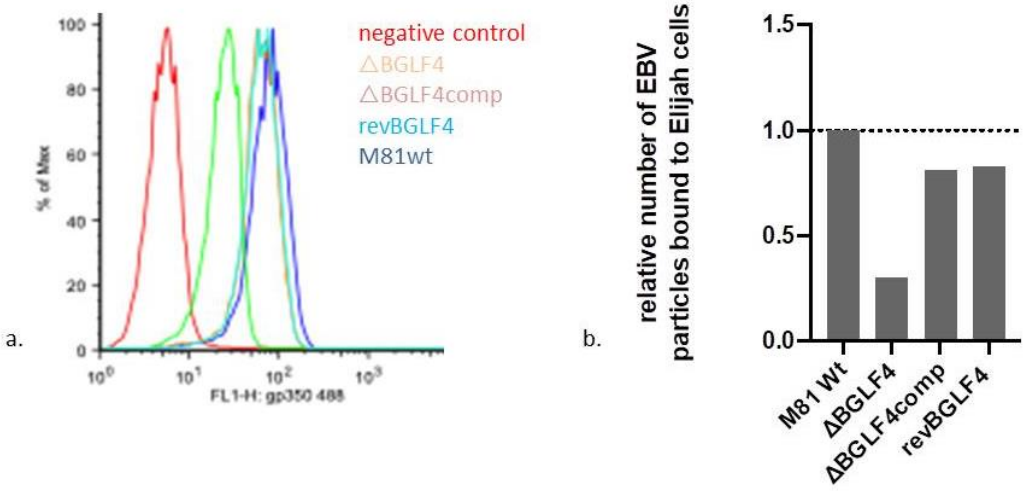


Figure 14: Number of EBV copies bound to Elijah cells. EBV negative Elijah B cells were infected with the same number of viral genomes as defined by qPCR. Elijah negative control represents the Elijah cells exposed to culture medium only. The amount of cell-bound viral particles was quantified using flow cytometry (a) and given as a relative number of viral particles bound to cell (b).

The weaker signals observed at the surface of cells exposed to M81/ΔBGLF4 viruses could either correspond to a decreased number of bound viruses and/or to a decreased gp350 expression within the virions. To distinguish between both hypotheses, we determined the number of bound viral genomes per cell (Figure 15). We then calculated the ratio between the number of viral genomes to which cells were exposed and the number of viral genomes that remained bound at the surface of cells.

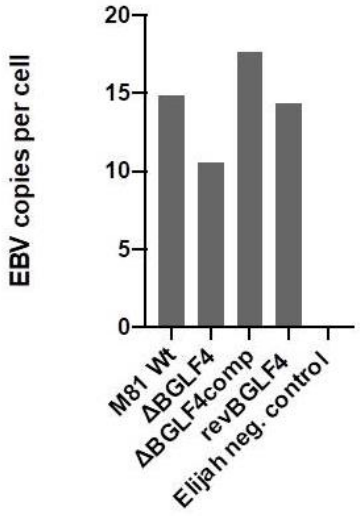
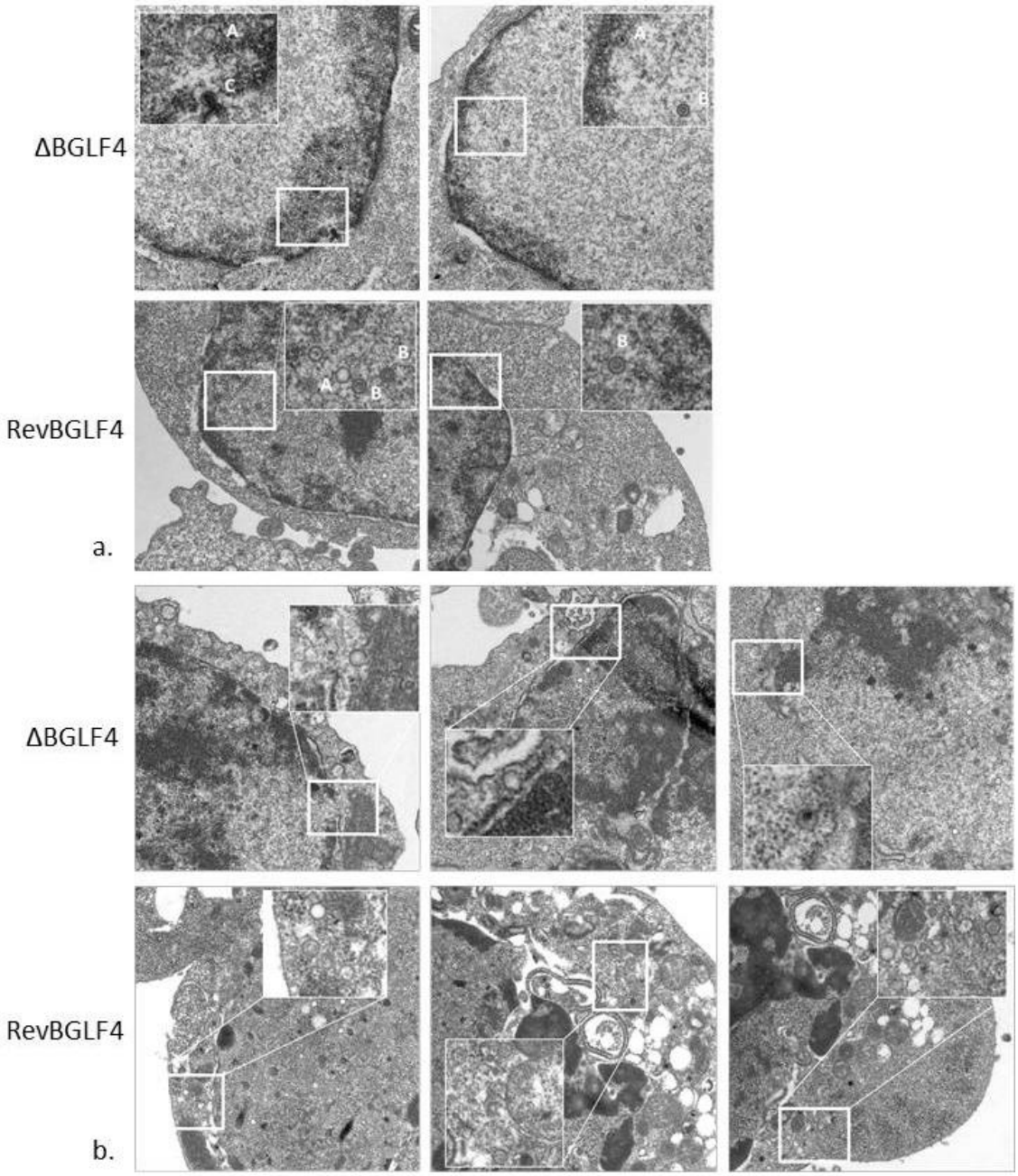


Figure 15: Number of EBV copies bound to Elijah cells. EBV negative Elijah B cells were infected with the same number of viral genomes as defined by qPCR. Elijah negative control represents the Elijah cells exposed to culture medium only. The amount of EBV copies bound to Elijah cells was quantified by flow cytometry and estimated by qPCR.

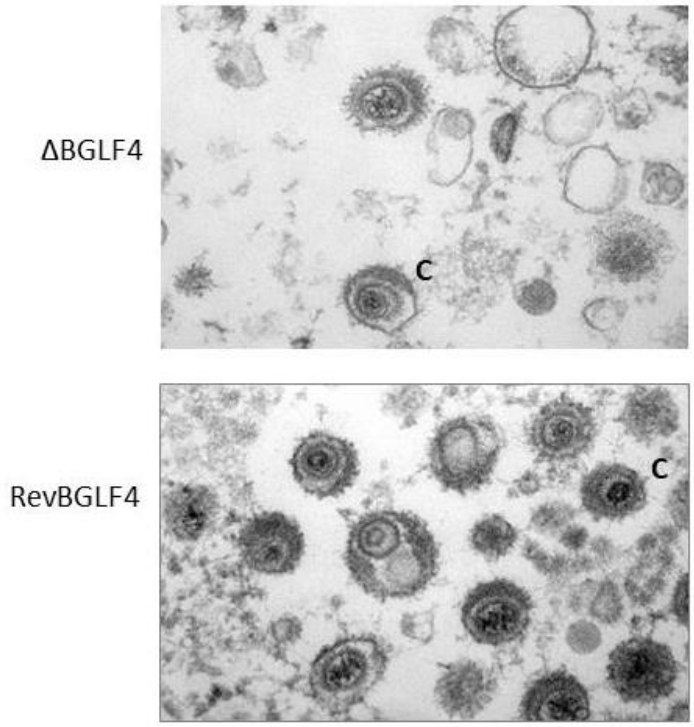
The relative number of EBV particles that bound to the surface of Elijah cells is shown in Figure 14 b. M81 wild-type, trans-complemented BGLF4 mutant and BGLF4 revertant viruses display similar binding properties. Indeed, trans-complemented BGLF4 and BGLF4 revertant viruses show a close relative number of EBV particles bound on the surface of Elijah cells. BGLF4 mutant infected Elijah cells showed a loss of approximately 1/3 of binding ability compared to the wild-type. However, this decrease in binding is less pronounced that suggested by the FACS analysis (Figure 14 a). Thus, the absence of BGLF4 leads both to a decrease in gp350 expression and to a reduced binding. Both events are related as gp350 is the glycoprotein that allows virus binding to B cells (Birkenbach et al., 1992). Thus, BGLF4 is required both for efficient virion production and efficient binding.

6.2.3. The role of BGLF4 in virus particles production in epithelial cells

We determined the role of BGLF4 in virion maturation. To this end, we performed an electron microscopy analysis on HEK293/rEBV knockout producer cells that were previously induced with the immediate early genes BRLF1 and BZLF1, together with its revertant. The cells were fixed and visualized using electron microscopy (Figure 16 a and b). In parallel, cell supernatants were centrifugated for 2 hours at 4°C and the virions pellets were fixed before analysis (Figure 17).



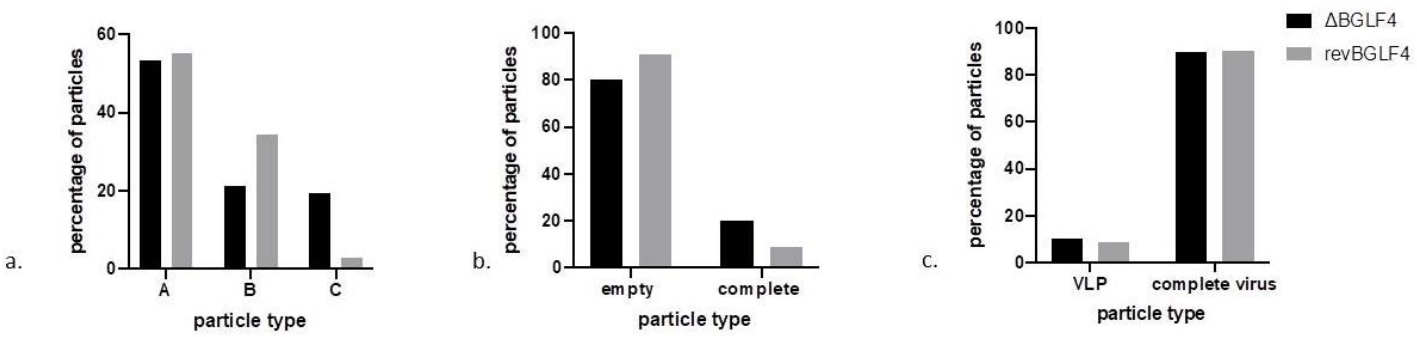
<u>Figure 16</u>: Production of infectious mature virions in BGLF4 knockout and BGLF4 revertant within the cells. HEK293 cells, carrying the indicated EBV genomes, were transfected with BZLF1 and BRLF1 expression vectors and harvested at day 3. Electron micrographs of (a-b) induced cells and (c) viral particles in pelleted supernatant are shown.



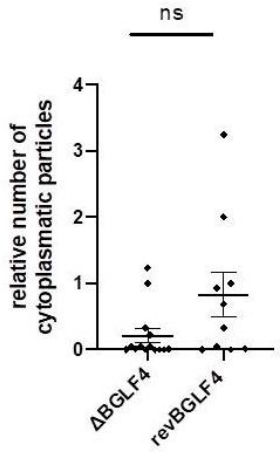
<u>Figure 17</u>: Production of infectious mature virions in BGLF4 knockout and BGLF4 revertant in the supernatant. HEK293 cells, carrying the indicated EBV genomes, were transfected with BZLF1 and BRLF1 expression vectors and harvested at day 3. Electron micrographs of viral particles in pelleted supernatant are shown.

These assays allowed the investigation of every virus maturation step from capsid assembly in the nucleus to release of the mature virion in the supernatant. Ten cells undergoing virus production were analyzed for the presence of virions within the nucleus (Figure 16 a) or in the cytoplasm (Figure 16 b). These virus particles are present in various forms within the cells. In the nucleus, the forms A and B represent immature capsids devoid of viral DNA and the form C corresponds to a mature capsid that has incorporated the viral genome. In the cytoplasm and in the supernatant (Figure 16 b and 17), defective forms that lack DNA, also known as defective particles or virus-like particles (VLPs), can be identified next to wild type particles that contains packaged viral DNA. Comparison between producer cells infected by the BGLF4 deletion mutant and its revertant showed that the revertant (Figure 18 a) had on average less form C capsids in the nucleus. Similarly, virions in the cytoplasm were more frequently defective in the producer cells infected with the revertant (Figure 18 b). The free mature virions in the supernatant were similar in both

group of cell lines (Figure 18 c), although we noted that the number of viral particles per EM grid was much higher in the wild type control. We then determined the ratio between the number of particles present in the cytoplasm and those present in the nucleus of replicating cells and found that this ratio was much higher in cells infected by the revertant (Figure 19). Altogether, this suggests that BGLF4 facilitates primary egress from the nucleus to the cytoplasm and that mature forms of capsids or of completed virions are less visible in the control infected cells because the mature virions can successfully egress.



<u>Figure 18</u>: Comparison of the release of infectious mature virions in BGLF4 knockout and BGLF4 revertant. HEK293 cells, carrying the indicated EBV genomes, were transfected with BZLF1 and BRLF1 expression vectors and harvested at day 3. Quantitative analyses of viral structures in (a) the nucleus, (b) the cytoplasm of the induced cells and in (c) the pelleted supernatant were performed on 10 cells.



<u>Figure 19</u>: Release of infectious mature virions between the cytoplasm and the nucleus of BGLF4 knockout and BGLF4 revertant. HEK293 cells, carrying the indicated EBV genomes, were transfected

with BZLF1 and BRLF1 expression vectors and harvested at day 3. The relative number of cytoplasmatic infectious particles is shown on analyses performed on 10 epithelial cells.

6.3. The role of BGLF4 in infection of primary B cells.

6.3.1. LCLs infected with a virus that lacks BGLF4 show a decrease in gp350 expression.

To determine the global role of BGLF4 in B cell spontaneous lytic replication, we investigated virions production from infected B cells and the ability of these viruses to infect resting primary B cells. For this purpose, we collected supernatants of LCLs transformed by M81. We then infected primary B cells with the same volume of supernatant and 3 days later, we counted the number of latently infected B cells, as determined by EBNA2 expression (Figure 20). We found that 1.4% of the M81 wild-type infected cells expressed EBNA2, whilst 0.3% of the BGLF4 mutant infected cells were latently infected. Therefore, the absence of BGLF4 reduced, but not completely suppressed the ability of the virus to be propagated from an infected cell to a non-infected B cell (p-value 0.002).

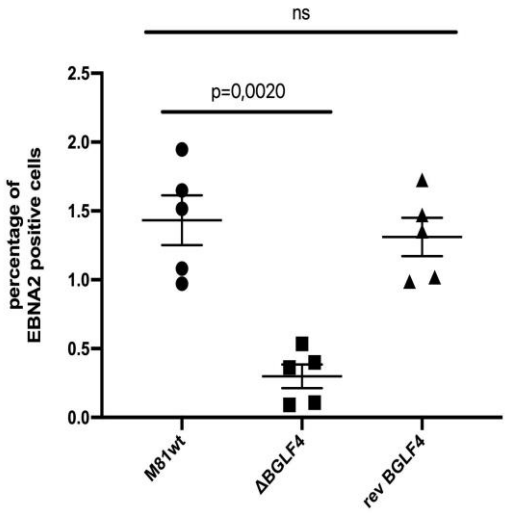


Figure 20: EBV infectivity assay. This graph depicts the result of infectivity assays performed by exposure of primary B cells to supernatants from wild-type, BGLF4 mutant and BGLF4 revertant LCLs. Means and SEM values are indicated.

We then attempted to explain the decreased ability of the BGLF4 deletion mutant to be propagated to EBV-negative B cells at the molecular level. We focused our attention on the lytic protein production and on the viral DNA replication.

We first studied the expression of various lytic proteins during viral replication, which usually begins between day 21 and 35 post-infection in 5 LCLs established from random donors. We established LCLs that carried the genome of BGLF4 mutant or revertant BGLF4 mutant genome, as well as wild type controls. After 4 weeks, cells were stained (Figure 21) and we determined the percentage of cells that express BZLF1 or gp350 (Figure 22 and 24). We also stained infected cells with an antibody specific for BGLF4 (Figure 21) or BGLF5.

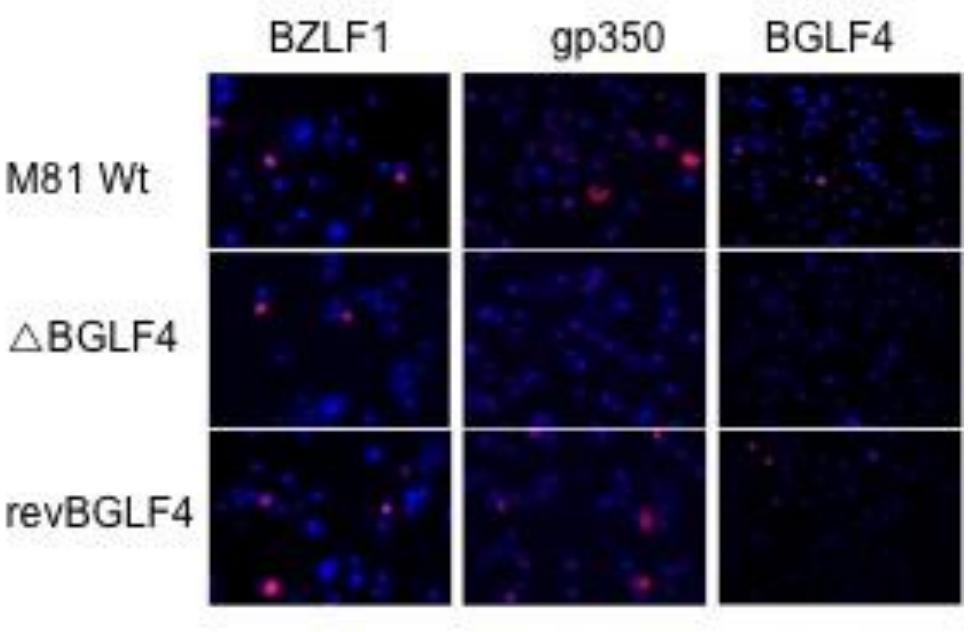


Figure 21: Expression of lytic proteins at four weeks post-infection in immunostaining. Infected B cells were cultured *in vitro* for four weeks and stained for the expression of BZLF1, gp350, BGLF4 and BGLF5 lytic proteins.

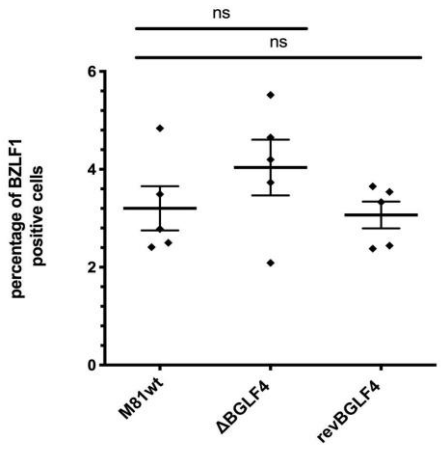


Figure 22: Expression of the BZLF1 lytic protein at four weeks post-infection in immunostaining. Infected B cells were cultured *in vitro* for four weeks and stained for BZLF1 protein expression. The number of positive cells was counted and analyses were performed for the expression of BZLF1 protein. The mean and SEM values are shown.

Approximately 3% (on average) of the B cells infected with wild-type virus or the BGLF4 revertant, and 4% of the BGLF4 mutant cells expressed BZLF1 protein, but this difference was not significant (Figure 22).

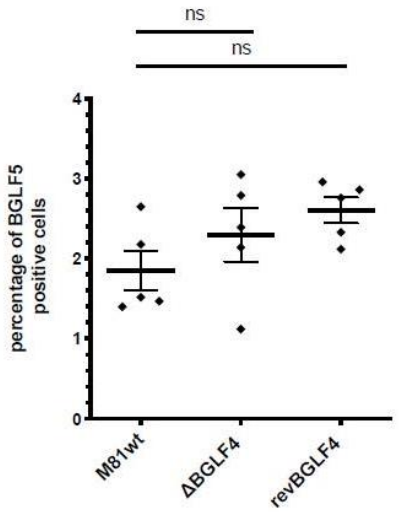


Figure 23: Expression of the BGLF5 lytic exonuclease at four weeks post-infection in immunostaining. Infected B cells were cultured *in vitro* for four weeks and stained for BGLF5 protein expression. The number of positive cells was counted and analyses were performed for the expression of BGLF5 protein. The mean and SEM values are shown.

The BGLF5 protein was expressed in 1.8% of the cells infected with wild-type virus and in 2.6% after infection with the BGLF4 revertant virus cells displayed BGLF5 expression (Figure 23). Our results showed that 2.3% of the cells infected with the BGLF4 deletion mutant were positive for BGLF5 expression. The BZLF1 and BGLF5 protein expression were not significantly different in wild-type, BGLF4 mutant and BGLF4 revertant cells (according the t-test).

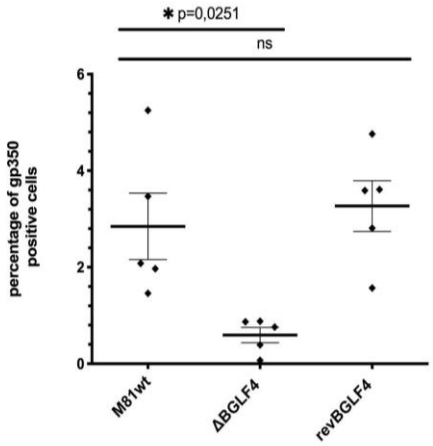


Figure 24: Expression of the gp350 lytic glycoprotein at four weeks post-infection in immunostaining. Infected B cells were cultured *in vitro* for four weeks and stained for gp350 protein expression. The number of positive cells was counted and analyses were performed for the expression of gp350 protein. The mean and SEM values are shown.

Conversely, a t-test statistical test pointed to a significant difference in gp350 expression between the wild-type and the BGLF4 knockout: 2.8 % of the wild-type and 3.2% of the BGLF4 revertant cells expressed gp350 protein, whereas 0.6% of the BGLF4 knockout cells were positive for gp350 (Figure 24).

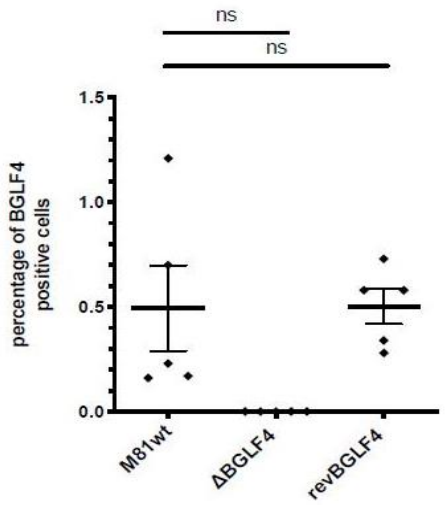


Figure 25: Expression of the BGLF4 lytic protein kinase at four weeks post-infection in immunostaining. Infected B cells were cultured *in vitro* for four weeks and stained for BGLF4 protein expression. The number of positive cells was counted and analyses were performed for the expression of BGLF4 protein. The mean and SEM values are shown.

Staining with antibodies against BGLF4 showed that 0.5% of the B cells infected with wild-type M81 and with the BGLF4 revertant virus expressed the BGLF4 protein. As expected, B cells infected with the BGLF4 null mutant did not express BGLF4 (Figure 25).

We extended these observations with a western blot analysis performed with protein extracts from 3 out of 5 LCL samples and stained with antibodies specific for BZLF1, BRLF1, BGLF4, BGLF5, BMRF1, BFLF1, BFRF2 and gp350 (Figure 26). The first two proteins are immediate early proteins, the following five proteins are early proteins, the last being a late lytic protein.

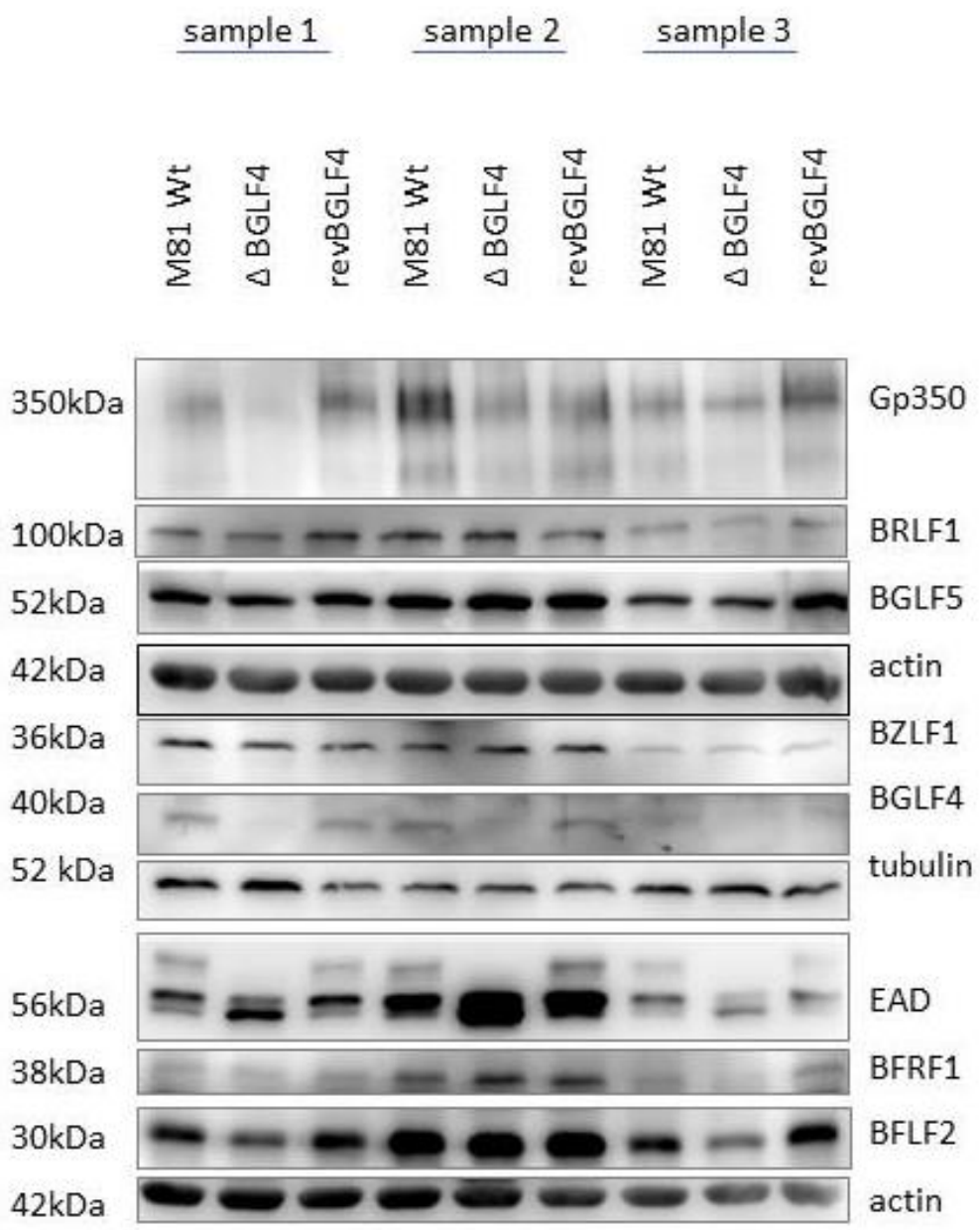


Figure 26: Immunoblot on three sets of LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Four weeks after infection of B cells, cells from three donors were analyzed for the expression of lytic proteins with specific antibodies. Antibodies against tubulin and actin were used are protein loading controls.

As expected, BGLF4 is deleted in the BGLF4 mutant cell lines (Figure 26 and 27).

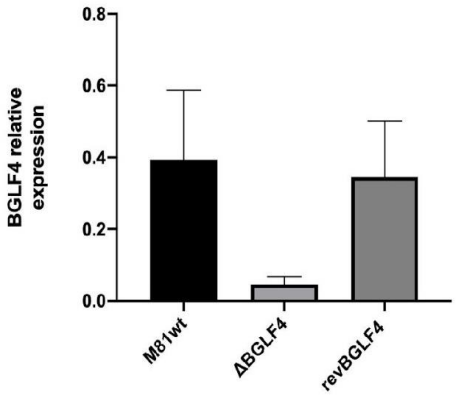


Figure 27: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Expression level of BGLF4 serine/threonine protein kinase in lymphoblastoid cell lines determined by the western blots shown in Figure 24 was quantified after scanning of the signals and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

The expression of the BMRF1-encoded gene product, EA-D, was also analyzed. This protein exists in various phosphorylated forms that give rise to signals of increasing size (Feederle et al., 2009). This pattern was visible on our blots (Figure 26). Deletion of BGLF4 led to a disappearance of the hyperphosphorylated EA-D, confirming that EA-D is a target of BGLF4. However, the global expression of EA-D is similar after infection with wild-type M81, the BGLF4 deletion mutant or the BGLF4 revertant (Figure 28).

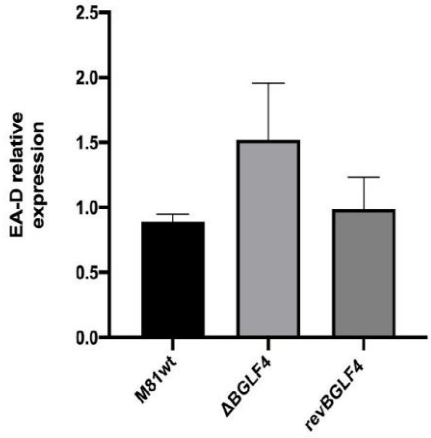


Figure 28: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Expression level of EA-D protein in lymphoblastoid cell lines

determined by the western blots shown in Figure 24 was quantified after scanning of the signals corresponding to the non-phosphorylated protein forms and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

We did not observe any statistically significant differences in BZLF1 expression between wild-type, mutant and revertant BGLF4 LCLs (Figure 29).

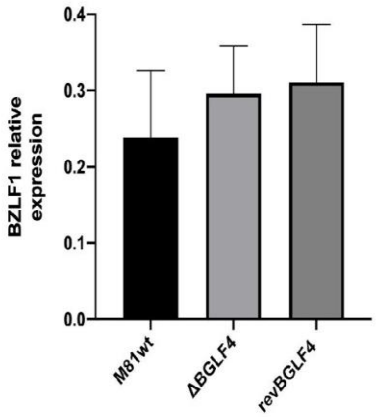


Figure 29: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Expression level of BZLF1early lytic protein in lymphoblastoid cell lines determined by the western blots shown in Figure 24 was quantified after scanning of the signals and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

As it was previously found that BGLF4 deletion affects the nuclear egress, we studied BFRF1 and BFLF2 protein expression during viral replication in LCL, the two proteins that govern this process (Figure 26) (Gonnella et al., 2005; Granato et al., 2008). We indeed noticed a decreased BFLF2 expression (Figure 30) after infection with the BGLF4 deletion mutant relative to wild-type controls. However, the expression of BFRF1 did not vary significantly between the BGLF4 mutant and BGLF4 revertant lymphoblastoid cells during spontaneous replication and there was even a trend of higher expression after infection with the deletion mutant (Figure 31).

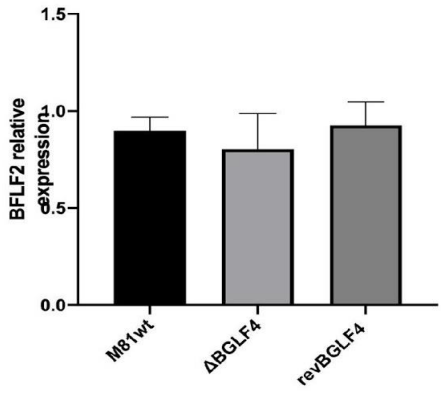


Figure 30: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Expression level of BFLF2 protein in lymphoblastoid cell lines determined by the western blots shown in Figure 24 was quantified after scanning of the signals and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

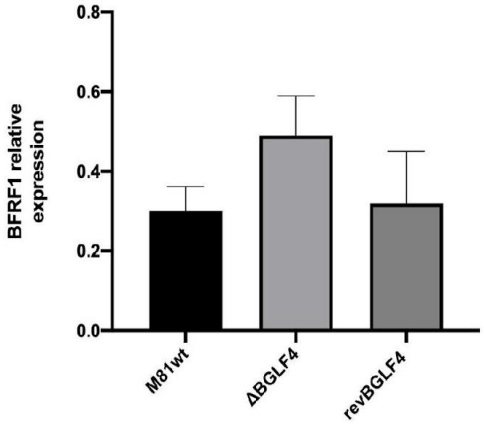


Figure 31: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Expression level of BFRF1 protein in lymphoblastoid cell lines determined by the western blots shown in Figure 24 was quantified after scanning of the signals and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

The main feature of our analysis was the decreased gp350 expression in B cells infected with the M81 BGLF4 deletion mutant, that was halved relative to wild type controls (M81 wild-type and BGLF4 revertant LCL) (Figure 26 and 32).

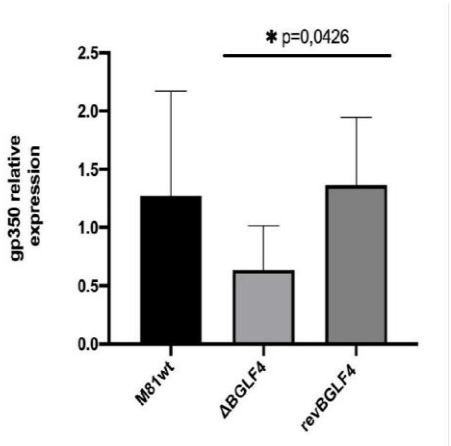


Figure 32: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. The expression level of the gp350 lytic glycoprotein in lymphoblastoid cell lines determined by the western blots shown in Figure 24 was quantified after scanning of the signals and densitometry with ImageJ. The bar plots show the average expression levels of these proteins, together with the standard deviation.

6.3.2. The role of BGLF4 in viral DNA lytic replication

We then investigated the role of M81 BGLF4 protein in viral replication. A gardella gel coupled to Southern Blot was performed to quantify the amount of linear EBV DNA by using a probe specific to the BLLF1 gene (Figure 33). This type of electrophoresis allows separation of circular and linear genomes and thus to quantify lytic DNA production.

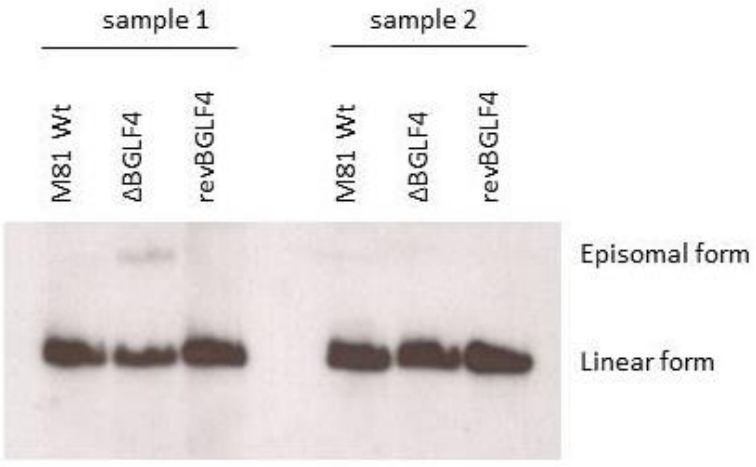


Figure 33: Role of BGLF4 in viral replication. A gardella gel electrophoresis was coupled to Southern blot analysis using a gp350 specific probe. We distinguish the episomal form (upper band) from linear form (lower band), which represents the viral replicating DNA.

The deletion of BGLF4 only marginally influenced the production of linear genomes relative to the wild-type controls and revertants during spontaneous lytic replication, as shown by signal quantification (Figure 34).

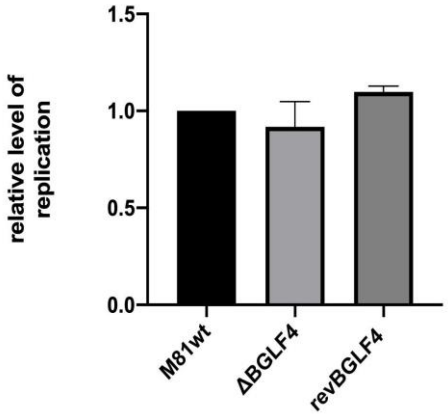
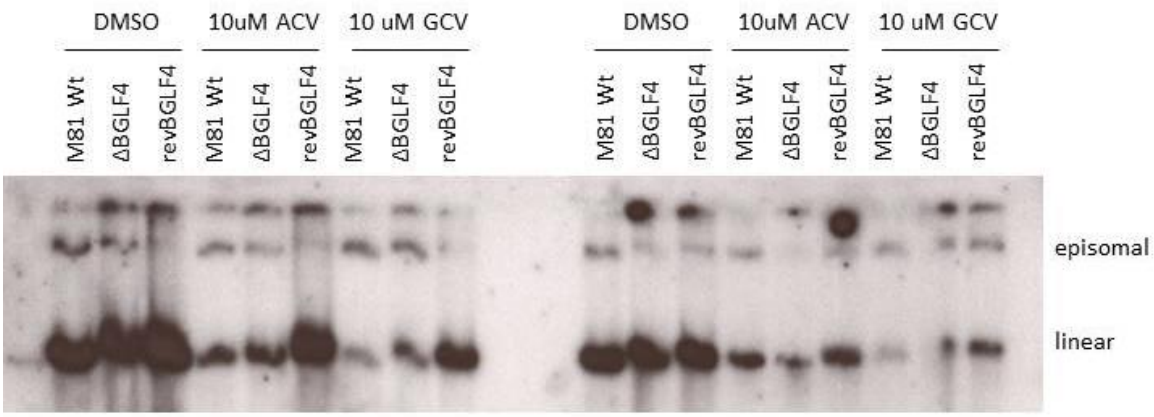


Figure 34: Role of BGLF4 in viral replication. Relative replication expression in comparison of the wild-type replication level. The mean and SEM values are indicated.

6.3.3. BGLF4 does not influence the sensitivity of infected B cells to Acyclovir or Gancyclovir.

BGLF4 has been previously reported to phosphorylate and as a consequence activate the antiviral drugs acyclovir and ganciclovir (Meng et al., 2010). Other cellular kinases add additional phosphate residues to the drug that becomes highly toxic to the cell, thereby eliminating the infected cell. This ability to activate anti-viral drugs is shared by the HCMV and HSV homologs of BGLF4 protein (Marschall et al., 2002; Shiraki, 2018; Talarico et al., 1999). We wished to evaluate the role of BGLF4 in the sensitivity of acyclovir and ganciclovir in infected B cells. Therefore, we treated lymphoblastoid cells that were produced at high levels with 10µM acyclovir or ganciclovir for 7 days. DMSO-treated cells were used as negative control. We evaluated replication using a Gardella gel analysis with a gp350 specific probe (Figure 35).



<u>Figure 35</u>: Effect of BGLF4 in activation of antiviral drugs. A gardella gel electrophoresis was coupled to Southern blot analysis using a gp350 specific probe. We distinguish the episomal form (upper band) from linear form (lower band), which represents the viral replicating DNA. Acyclovir is indicated with the ACV abbreviation although Gancyclovir is written GCV.

The treatment of replicating cells with acyclovir (ACV) or ganciclovir (GCV) decreased the viral replication. However, the deletion of BGLF4 did not show any differences in the inhibition of viral replication in cells treated with acyclovir in comparison to wild type controls. Collectively, this result suggests that BGLF4 plays a minor role in the activation of acyclovir or ganciclovir.

6.4. BGLF4 deletion plays a minor role in latently infected cells.

6.4.1. The deletion of BGLF4 expression does not affect cell proliferation.

Paladino and colleagues reported that BGLF4 is one of the proteins which contribute to the cell cycle arrest in G1/S phase in replicating cells (Paladino et al., 2014). Because BGLF4 is present in virions and B cells are covered with these virions after infection with M81, it is theoretically possible that BGLF4 interferes with latently-infected B cells. To verify this hypothesis, we seeded 3.10^5 cells in well-plates and measured the growth rate for 3 days (Figure 36).

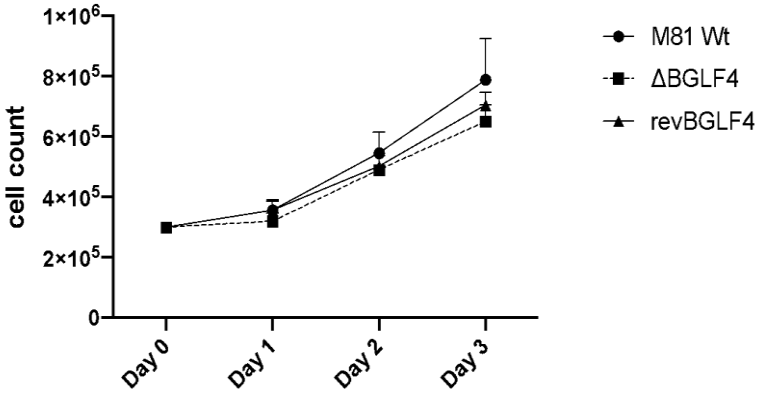


Figure 36: BGLF4 and its role in cell growth. 3.10^5 lymphoblastoid cells per well were seeded in 24-well plate. Cell growth was established by counting the cells for 3 days. Five LCLs were used in this study.

Our study did not show a statistically significant difference in cell growth between wild-type, BGLF4 mutant and BGLF4 revertant LCLs We conclude that M81 BGLF4 is not involved in proliferation of established cell lines.

6.4.2. The deletion of BGLF4 expression decreases LMP1 and EBNA2 protein expression.

In parallel, we monitored latent protein expression in B cells transformed by the BGLF4-null mutant. We first assessed LMP1 expression during lytic replication. This protein plays an essential role in B cell transformation, cell signaling regulation and apoptosis (El-Sharkawy et al., 2018; Ramakrishnan et al., 2011). We also studied the expression of other latent proteins such as EBNA2 and EBNA1 (Kempkes et al., 1995; Rawlins et al., 1985). Interestingly, BGLF4 has been reported to phosphorylate EBNA2 on serine 243 and that this modification blocks the ability of this protein to transactivate the LMP1 promoter in HeLa cells (Yue et al., 2005).

We performed a western blot with antibodies specific for LMP1 and EBNA2 and found a decrease in expression of latent proteins in B cells infected with the BGLF4 deletion mutant (Figure 37).

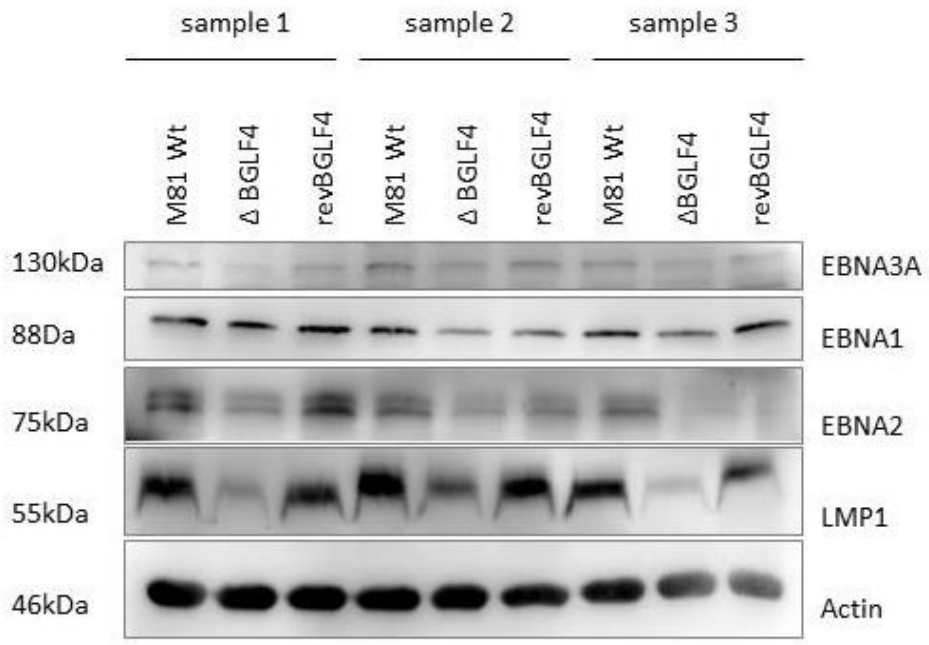
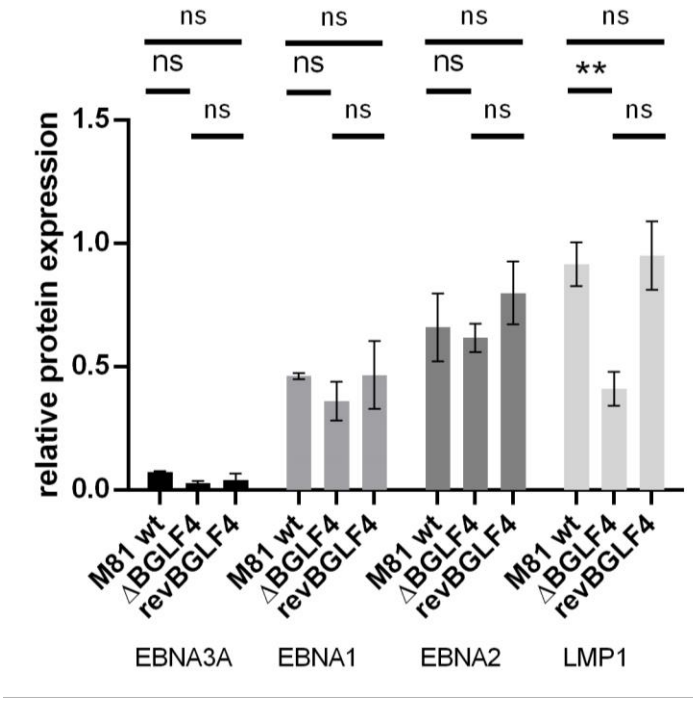


Figure 37: Immunoblot on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. Four weeks after infection of B cells, cells from three donors were analyzed for the expression of latent proteins with specific antibodies.

However, with only 3 samples, we were not able to get a statistically significant difference of their expression between the wild-type, the BGLF4 null mutant and the BGLF4 revertant cells, except for LMP1 expression between the wild type and the BGLF4 deletion mutant (Figure 38). The standard variation between the results obtained with different primary samples is not surprising and explains the absence of statistical significance. It will be necessary to test more samples to be able to conclude whether or not BGLF4 influences the expression of latent proteins.

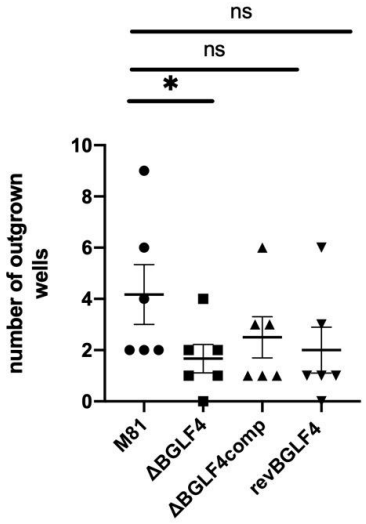


<u>Figure 38</u>: Immunoblot analyses on LCLs established from B cells infected with wild-type, BGLF4 knockout and BGLF4 revertant viruses. A relative quantification was performed on the immunoblots to measure the protein level. Mean and SEM values are indicated.

6.4.3. BGLF4 may have a limited influence on B cell transformation

The observation of a decreased LMP1 expression in cells infected by a virus that lack the BGLF4 protein suggests that BGLF4 might have an indirect effect on B cell transformation. To test this hypothesis, primary B cells were infected with viruses and 3 days post-infection, six EBNA2 positive cells were seeded per well. The number of outgrown wells was counted 2- and 4-weeks post-seeding (Figure 39).

a.



b.

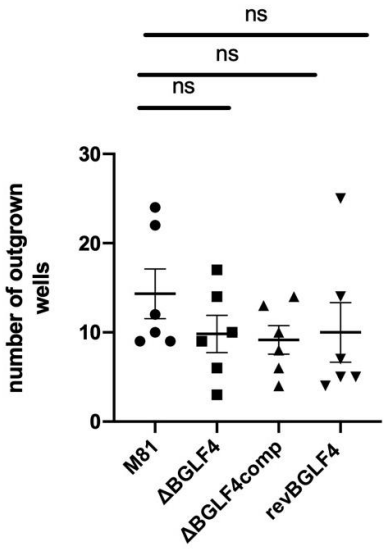


Figure 39: BGLF4 and its role in B cells transformation. Primary human B cells were infected with same number of viral genomes and stained for EBNA2 expression 3 days post-infection. 6 EBNA2-positive cells per well were seeded in 48 wells from a 96-U-well plate coated with 10^3 gamma-irradiated WI38 feeder cells. The number of outgrown wells containing lymphoblastoid cell clones (LCLs) was monitored at two- (a) and four- weeks (b) post-infection and is given as a dot-plot. The mean and SEM values are shown on the graphs.

After two weeks, the number of outgrown wells differs significantly between the wild-type and BGLF4 mutant (Figure 39). 1.7 wells that contained BGLF4 knockout cells were outgrowing although 4.2 wells containing wild-type cells were outgrowing at two-week post-infection. However, this observation is not anymore seen 4 weeks post-seeding. We conclude that BGLF4

might be involved in EBV-mediated transformation at an early time of infection but this effect disappears with time, possibly because BGLF4 is present in infected cells only at the beginning of the infection. However, we also observed a reduced transformation with the revertant. Altogether, there is no solid evidence that BGLF4 plays a role in EBV-mediated B cell transformation.

We conclude from this set of experiments that BGLF4 is important for optimal lytic protein expression, in particular of gp350. At the cellular level, we found that BALF4 is important both for efficient spontaneous virus production in B cells as well as efficient B cell infection. As a consequence, the absence of BGLF4 reduces the propagation of the virus to B cells and thus possibly the size of the B cells reservoir in vivo.

7. Discussion

Tegument proteins are equipped with complex enzymatic functions that were recently recognized to be crucial for viral infection, but that can also be involved in the neoplastic potential of the virus (Whitehurst et al., 2015). For example, the EBV BNRF1 tegument protein has been shown to induce genetic instability and centrosome amplification within infected cells, two risk factors for the development of tumors in humans (Shumilov et al., 2017). Furthermore, Ho and colleagues identified the EBV BKRF4 tegument protein as a DNA damage response inhibitor in EBV-positive gastric tumors (Ho et al., 2018). It suggests another potential role for the tegument proteins in gastric carcinomas development. It is possible that other EBV tegument proteins are involved in the development of cancers through yet uncharacterized mechanisms. Our project was to investigate the function of some EBV tegument proteins in a cellular system as close to human infection *in vivo* as possible.

7.1. Preliminary studies involving the functions of EBV M81 tegument proteins during early lytic events and spontaneous replication

Detailed studies of the functions served by the tegument proteins were performed in HCMV and HSV-1(Gershburg et al., 2015; Gregory et al., 2011; Metrick et al., 2020; Sathish et al., 2009; Schauflinger et al., 2011). In comparison, EBV tegument proteins are poorly studied. Moreover, some proteins such as EBV BKRF4 or BRRF2 tegument proteins are not conserved in all herpesviruses but only in gammaherpesviruses (Masud et al., 2017; Watanabe et al., 2017). The absence of these proteins in alpha- and betaherpesviruses suggests unique roles in gammaherpesviruses (He et al., 2020; Ho et al., 2018).

Another potential limitation of previous studies is that researchers studied the EBV tegument proteins in artificial models, which can introduce a bias in the results. For example, some researchers inhibited viral tegument protein with siRNAs but this inhibition was only partial (Gershburg et al., 2007). Another example is BBLF1 that was studied in an EBV-positive Burkitt's lymphoma cell line infected by the P3HR1 strain (Chiu et al., 2012). In this study, the authors inhibited BBLF1 tegument protein with siRNA and induced the lytic replication with TPA and sodium butyrate. However, they still observed expression of BBLF1 in the cells after

the induction of the lytic replication, suggesting that inhibition of tegument protein with BBLF1 targeting small interfering RNA (siRNA) is an imperfect way to study the functions of EBV tegument proteins. Finally, Gershburg et al. also used a partial knockdown of BGLF4 based on a BGLF4 siRNA (Gershburg et al., 2004a). Other scientists used cells infected by the B95.8 strain to study the function of tegument proteins (Asai et al., 2006; Masud et al., 2017; Yanagi et al., 2019). However, lytic replication in this case has to be induced by TPA or sodium butyrate. Another problem is that different EBV strains were used to study the EBV tegument proteins: Akata strain, B95.8 strain, P3HR1 strain, Raji strain (Chiu et al., 2012; Masud et al., 2019). These strains sometimes show polymorphisms in the protein or nucleic acids sequences. These differences can modulate the functions served by the different alleles. In the case of non-coding RNAs for example, it has been previously described that M81 EBER2 increases the lytic replication in infected B cells although B95.8 EBER2 lacks this function (Li et al., 2019b).

In contrast, we used the EBV M81 strain to generate all the virus mutants that allows the study of spontaneous lytic replication in infected primary B cells, a model that is as close as possible to infection in humans. The EBV M81 strain was isolated from a carcinoma that was developed in a Chinese patient. It differs from EBV B95.8 or Akata strains by its ability to spontaneously replicate within B cells *in vitro* at high level (Tsai et al., 2013). However, the structure of the mature M81 virion is similar to the structure of other EBV strains. The results obtained with M81 can thus be extended to other strains.

Our screen of EBV M81 mutants lacking tegument proteins showed that BKRF4, BLRF2, BBLF1, BGLF4, BSRF1 deletion decreased the viral titers but trans-complemention with expression plasmids encoding the missing proteins rescued the viral titers to wild-type levels. Similar results were previously published using B95.8 or Akata strains (Chiu et al., 2012; Duarte et al., 2013; Gershburg et al., 2007; Masud et al., 2017; Yanagi et al., 2019). Protein alignments were realized to compare the protein sequences of these tegument proteins in different strains (Appendix 4-9). No differences were observed in BLRF2, BGLF4 and BSRF1 proteins, suggesting that the functions of these tegument proteins are conserved between the different EBV strains (appendix 4, 6, 8). Three substitutions were observed in EBV B95.8 BKRF4 amino acid sequence and one polymorphism is noted in EBV B95.8 BBLF1 amino acid sequence in comparison with EBV M81 and Akata strains, but our results are similar with those already

published in the literature (appendix 5 and 7. The exception here is BOLF1, whose deletion and amino acid substitution, did not affect viral titers in our hands. The protein sequence of BOLF1 differs in a few amino acids between B95.8, M81 and the Akata strain (appendix 9). It has been published that B95-8 and Akata BOLF1 protein were not involved in virions production (Masud et al., 2019). We conclude that the polymorphisms of BOLF1 have no influence on virus production. However, the BSRF1 gene was previously studied in the context of the B95-8 strain (Yanagi et al., 2019). In contrast to our results, these authors found that B95.8 BSRF1 is not involved in virion progeny production. In this case, an alignment performed between B95.8 and M81 BSRF1 protein did not show difference in amino acid sequence (Appendix 8), suggesting that the discrepancy with our results cannot be explained by polymorphisms. Moreover, our results are in line with those published on the HSV-1 UL51 gene as well as on the HCMV UL71 gene showing that BSRF1 homologs genes are essential for virions production (Kato et al., 2018; Nozawa et al., 2005; Schauflinger et al., 2011).

We then assessed the infectivity for each tegument protein knockout virus. We found that BKRF4, BLRF2, BBLF1, BGLF4, BSRF1 and BOLF1 are all necessary to obtain wild type level infections. We confirmed the results of former studies with BLRF2, BKRF4, and BGLF4 knockouts (Duarte et al., 2013; Feederle et al., 2009; Masud et al., 2017), suggesting that the M81 background does not change the role of tegument proteins in infectivity. So far, we have performed the only study showing that BSRF1 is involved in EBV infectivity. However, although we could partly rescue the EBV infectivity by using trans-complemented viruses for all tegument proteins, we were unable to rescue fully the wild type phenotype in the case of BSRF1 tegument protein. More work is necessary to understand the role of this protein in infection and to exclude that the BSRF1 carries unwanted mutations.

Finally, we performed an analysis of the spontaneous lytic replication in B cells infected with the deletion mutants. To this end, we generated lymphoblastoid cell lines by infecting primary B cells with knockout viruses. After four weeks, we studied the expression of the two lytic proteins BZLF1 and gp350. The spontaneous lytic replication is well established at 28 days post-infection. We performed immunostaining on cells and quantified the BZLF1 and gp350 positive cells by counting. We could not detect major differences relative to wild type infected cells. Thus, our

analyses suggest that most of the screened tegument proteins do not play a role in spontaneous viral replication in B cells.

We then focused our attention on the EBV M81 BGLF4 tegument protein and its role in induced and spontaneous replication and virus production. The functions served by the BGLF4 tegument protein have been extensively studied since 1989 (Asai et al., 2006, 2009; Chang et al., 2012a, 2015, 2012b; Chen et al., 2000, 2010; El-Guindy et al., 2014; Feederle et al., 2009; Gershburg et al., 2004a, 2007; Iwahori et al., 2009; Kato et al., 2001, 2003; Kawaguchi et al., 2003; Kuny et al., 2010; Lee et al., 2007, 2008; Li et al., 2011, 2012; Lu et al., 2007; Meng et al., 2010; Murata et al., 2009; Sun et al., 2013; Wang et al., 2005, 2009, 2010; Yang et al., 2008; Yue et al., 2005; Zhang et al., 2019; Zhu et al., 2009) . Previous studies used artificial models such as siRNA to inhibit BGLF4 expression in forced replication of the virus contained in Akata cells or in cells infected by the EBV B95.8 strain. To understand the functions of EBV M81 BGLF4 tegument protein in spontaneous lytic replication, we constructed a recombinant virus devoid of this protein and a revertant virus thereof that rescues the BGLF4 expression. This serine/threonine protein kinase has been involved in multiple viral processes such as virions production (Feederle et al., 2009; Gershburg et al., 2007; Lee et al., 2007), viral replication (Gershburg et al., 2004; Lu et al., 2007; Yang et al., 2008), viral infectivity and nuclear egress (Gershburg et al., 2004). BGLF4 has pleiotropic effects on lytic products. It stimulates late protein production at the RNA and protein level, facilitates nuclear import of lytic proteins, but also potentiates the sustained expression of BZLF1, phosphorylates EBV lytic proteins such as BGLF4 was also found to influence cellular processes such as the DNA damage response, cell cycle regulation in particular of the mitosis (Paladino et al., 2014), nucleus disassembly though its targeting of lamins (Lee et al., 2008), and host immune response (Wang et al., 2009). Most investigations were performed in various *in vitro* models.

However, two major questions remain: what is the precise role of BGLF4 during induced and spontaneous lytic replication? And which of the numerous previously described functions of BGLF4 are mainly responsible for the mutant phenotype?

We used 293 cells as a producer cell line in order to generate large numbers of lytically induced cells and be able to investigate multiple parameters including viral DNA replication, viral gene expression and virus production. In parallel, we used primary B cells infected by EBV to study

virus propagation in a more physiological model, although it does not allow detailed molecular investigations.

Our study confirmed that M81 BGLF4 protein is involved in virion production *in vitro* both in 293 cells and in B cells but also uncovered a so far poorly investigated effect on viral infectivity.

7.2. Functions of EBV M81 BGLF4 tegument protein during viral spontaneous lytic replication *in vitro*

In all donors we analyzed, we found that the established LCLs supported the inception of spontaneous lytic replication with BZLF1 expression. It has been previously demonstrated in EBV-positive Akata cells, an EBV-infected Burkitt's lymphoma cell line, that BGLF4 forms a stable complex with BZLF1 and phosphorylates BZLF1 on serine 209 (Asai et al., 2009). This phosphorylation induces a down-regulation of BZLF1 transactivation on its own promoter thus reducing transcription and translation of the protein. Thus, deletion of BGLF4 should lead to an increase in the concentration of BZLF1 protein in cells undergoing replication (Asai et al., 2009; Gershburg et al., 2007). More recent work has suggested that BGLF4 activates ATM to induce phosphorylation of KAP1, a negative regulator of BZLF1 expression. In that model, BGLF4 deletion should decrease BZLF1 expression. Our results contradict both views as BGLF4 deletion did not affect BZLF1 protein expression at all in spontaneously replicating cells. The EA-D protein that is encoded by the BMRF1 DNA polymerase processivity factor, is known to be phosphorylated by BGLF4 (Gershburg and Pagano, 2002). Therefore, we tested EA-D protein expression in cells infected by either wild-type virus or the BGLF4 revertant. Furthermore, as we did not observe changes in BZLF1 expression, it was important to validate the identity of our mutant by assessing EA-D protein expression. Indeed, while the BMRF1 hyperphosphorylated form was not seen in B cells infected by BGLF4 knockout, we found that EA-D was clearly hyper-phosphorylated in cells infected by wild-type and BGLF4 revertant, as previously reported. Altogether, these observations suggest that a stable complex between BGLF4 and BZLF1, if it takes place, has no effect on B cells infected by the M81 strain. Whether BZLF1 is phosphorylated by another viral or cellular kinase, and whether BZLF1 protein expression is regulated by an unknown yet mechanism remains unknown. We then assessed the expression of

early lytic proteins. In particular, we wanted to determine the role of M81 BGLF4 in nuclear egress. It has been previously published that BGLF4 plays a role in this process as BGLF4 knockdown cells retain the viral nucleocapsids in the nuclei. This could be due to the abolishment of BFLF2 expression (Gershburg et al., 2007; Lee et al., 2008; Murata et al., 2009). The few number of replicating cells did not allow us to study the viral particles produced by lymphoblastoid cells. Therefore, we used 293 producer cells in which the replication was induced by transfection of BZLF1 and BRLF1. For this purpose, we analyzed ten cells per sample and counted the different forms of virions that were produced. We clearly observed immature virions, which were represented by the forms A and B and mature virions, which were represented by the form C. Our analyses confirmed that BGLF4 is required for the efficient transport of nucleocapsid to the cytoplasm. Indeed, we noticed that the form C of the virions is retained more frequently within the nucleus of the BGLF4 mutant producer cells, in comparison to its revertant. Therefore, this function seems to be conserved through the different EBV strains. Furthermore, we analyzed the protein expression of BFLF2 and BFRF1, which form the herpesviral core nuclear egress complex (Gonnella et al., 2005; Lake and Hutt-Fletcher, 2000). We noted that BFLF2 was decreased in two out of three BGLF4 Knockout LCLs whereas BFRF1 is unaffected by the deletion of BGLF4. This result indicates that BGLF4 likely up-regulates the expression of BFLF2, as was found in the context of the B95.8 strain (Feederle et al., 2009). However, it seems that BGLF4 does not modulate the expression of BFRF1 in M81 strain. This result also confirms the role of BGLF4 in the down-regulation of BFLF2 (Feederle et al., 2009) but does not show evidence for this role on the regulation of BFRF1. More LCLs need to be analyzed to confirm this result. It is possible that BFLF2 alone governs primary egress in infected B cells, but the previously reported phosphorylation of lamin C by BGLF4 might also play a role (Lee et al., 2008). Another possibility is that the absence of BGLF4 reduces the nuclear import of lytic proteins, thereby reducing the availability of virus building blocks resulting in a reduced virus production (Chang et al., 2015).

Finally, we studied the expression of the surface glycoprotein gp350. Based on studies performed on 293 producer cell lines performed in several groups, including our, that showed a reduced late protein transcription and translation in the absence of the viral kinase, we wanted to confirm that BGLF4 is required for the proper expression of gp350 protein in infected B cells (El-Guindy et al., 2014; Feederle et al., 2009; McKenzie et al., 2016). We confirmed indeed that BGLF4 up-

regulates the expression of the late lytic protein gp350, a protein that allows the virus to bind to B cells. BGLF4 activates the expression of BGLF3, a member of the prereplication complex that is necessary for late protein transcription initiation (El-Guindy et al., 2014; Li et al., 2019; McKenzie et al., 2016). It is interesting to note that gp350 has been involved in virion secondary egress, an effect that might contribute to the reduced virus production after BGLF4 deletion. Another possible mechanism leading to gp350 expression involves the EB2 protein, also called SM protein, an RNA-binding protein, which is conserved in the other human herpesviruses (Boyer et al., 2002; Gupta et al., 2000; Tunnicliffe et al., 2018; Winkler et al., 1994). This protein is an early lytic protein and is indispensable for the virus. Notably, EB2 is important for virus production (Gruffat et al., 2002). EB2 protein is also involved in multiple processes such as RNA processing, unspliced mRNA translation and is also associated to intranuclear assembly of EBV capsids (Batisse et al., 2005). Importantly, late EBV mRNAs are targets of EB2, among which we can find gp350 protein. EB2 is phosphorylated by CK2 (Medina-Palazon et al., 2007), which later allows the production of infectious particles. It might be possible that BGLF4 phosphorylates also the EB2 protein. This would explain the link between the deletion of BGLF4 and the decrease of gp350 protein and subsequently, the decrease of virus binding and viral infectivity. Han and colleagues gave another argument in favor of EB2 involvement in viral replication regulation even in absence of BGLF4 (Han et al., 2009). They published that the BGLF5 transcript is enhanced in EB2 immunoprecipitates from EB2 transfected P3HR1-ZHT cells but not in EB2 immunoprecipitates from B95.8-ZHT cells.

7.3. Functions of EBV M81 BGLF4 tegument protein during early lytic post-infectious events

We then investigated the impact of the viral kinase on B cell infection. In principle, this could be ascribed to a reduced viral binding, viral fusion, or to viral transport to the nucleus. BGLF4 is known to be incorporated into the mature infectious particle and has been shown to phosphorylate stathmin, a protein involved in microtubule dynamics that could influence transport of the capsid to the nucleus (Chen et al., 2010). Another possibility is that BGLF4 blocks the effect of SAMHD1, a protein involved in innate immunity against viruses (Zhang

2019). BGLF4 is known to inactivate SAMHD1 during lytic replication but could also act during another phase of the infection. However, multiple assays, including a binding assay and a FACS analysis, indicated that viruses generated by cells carrying the BGLF4 null mutant have both a reduced binding ability and a reduced gp350 content. Considering that gp350 is essential for binding to B cells, we conclude that this defect is at the basis of the reduced infectivity for B cells. These results are in line with a previous study that showed that BGLF4 is required for potent EBV infectivity (Feederle et al., 2009). The involvement of BGLF4 in EBV infectivity was confirmed with B cells undergoing spontaneous lytic replication, although we could only perform a global analysis of virus production and propagation in B cells. Whether the reduction in infectivity observed in the BGLF4 deletion mutant can be ascribed exclusively to the reduced late gene expression remains unclear at this stage of the work, but complementation of the BGLF4 defective virions with gp350 should be able to settle the case.

7.4. EBV M81 BGLF4 tegument protein and the activation of antiviral drugs

Similar to its herpesviruses homologs, BGLF4 has been shown to phosphorylate nucleosides analogues to inhibit the viral replication (Marschall et al., 2002; Meng et al., 2010; Talarico et al., 1999). Notably, acyclovir (ACV) and ganciclovir (GCV) need to be mono-phosphorylated by BGLF4 in a first step before being di-phosphorylated and tri-phosphorylated by cellular kinases. At this point, the drugs are active and can inhibit the viral DNA synthesis (Meng et al., 2010). These results were obtained from studies on producer cell lines carrying B95.8 viruses. The efficiency of these drugs has been confirmed in clinical studies that show a reduction in viral load in the saliva (Ljungman et al., 2007). We tested whether BGLF4 is able to phosphorylate ACV and GCV in B cells infected by M81. To this end, we established LCLs and performed a 7-day treatment time with these drugs. First, no differences were observed in the viral replication levels between our samples. This suggests that M81 BGLF4 does not play a crucial role in viral DNA replication, confirming our previous results obtained with immunostainings and inmmunoblots. Second, we did not observe differences between the BGLF4 mutants and wild-type cells that were treated either with acyclovir or ganciclovir. This suggests that BGLF4 is not the major

protein kinase involved in the mono-phosphorylation process of the antiviral drugs. It may be possible that the EBV thymidine kinase, called TK, is responsible for drug phosphorylation.

7.5. Connection between EBV M81 BGLF4 tegument protein and latent proteins

We analyzed the impact of BGLF4 on some major latent proteins such as EBNA2, LMP1, EBNA1 and EBNA3A. Previous studies showed that the third exon of LMP1, EBNA2 and the EBNA1 proteins were found to be substrates of BGLF4 (Zhu et al., 2009). In fact, LMP1 was found to be a substrate of BGLF4, although EBNA2 and EBNA1 were found to be substrates of both EBV BGLF4 and the CDK1/cyclin B (Zhu et al., 2009). Because EBNA2 is required to transactivate LMP1 expression, the impact of BGLF4 on latent protein might be both direct and indirect (Wang et al., 1990). Yue and colleagues observed that BGLF4 hyper-phosphorylates EBNA2 on its serine 243 in HeLa cells (Yue et al., 2005). This leads to the suppression of the LMP1 promoter transactivation and then to a decrease of LMP1 expression. It suggests a tight balance between the BGLF4 mediated phosphorylation of EBNA2 and LMP1 transactivation. We studied the expression of EBNA2 and found that the absence of BGLF4 tends to reduce EBNA2 expression, in particular of its higher molecular weight that might corresponds to the hyperphosphorylated form. More samples need to be investigated to see whether statistical significance can be reached. Moreover, we observed also a statistically significant decrease in LMP1 expression that would fit with the proposed model. LMP1 is an oncogene, which is especially important for the transformation of cells by EBV. It also down-regulates anti-apoptotic proteins (Kawanishi, 1997). Finally, the protein EBNA3A and EBNA1 were not affected in mutant cells, suggesting that BGLF4 does not modulate their expression or another signaling pathway compensates their loss of expression due to BGLF4 deletion.

We attempted to study the potential consequences of the decrease in latent protein expression in terms of cell growth. We performed transformation assay that detected mildly reduced transformation efficiency in the absence of BGLF4 expression, but the effect was weak. We also performed a growth curve of cells from established LCLs and counted the number of cells for three days. We did not observe significant difference in the growth rate of the cells in M81 wild-type, BGLF4 mutant and BGLF4 revertant cells. We conclude that the decrease in latent protein

expression is not pronounced enough to affect cell growth. It remains possible that cells infected by the BGLF4 deletion mutant is more sensitive to stress, for example induced by pro-apoptotic agents.

The observation that growing LCLs has altogether a reduced LMP1 expression is remarkable as only a small subset of cells is undergoing lytic replication during which BGLF4 is expressed. It remains possible that even latently infected B cells express low levels of BGLF4. However, BGLF4 is also present in virions. Therefore, very cells will have an initial contact with the viral kinase. Although unlikely, it remains theoretically possible that the effect of BGLF4 on the latent proteins is transmitted to daughter cells after cell division.

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9. Appendix

Appendix 1: BBLF1 recombinant genome

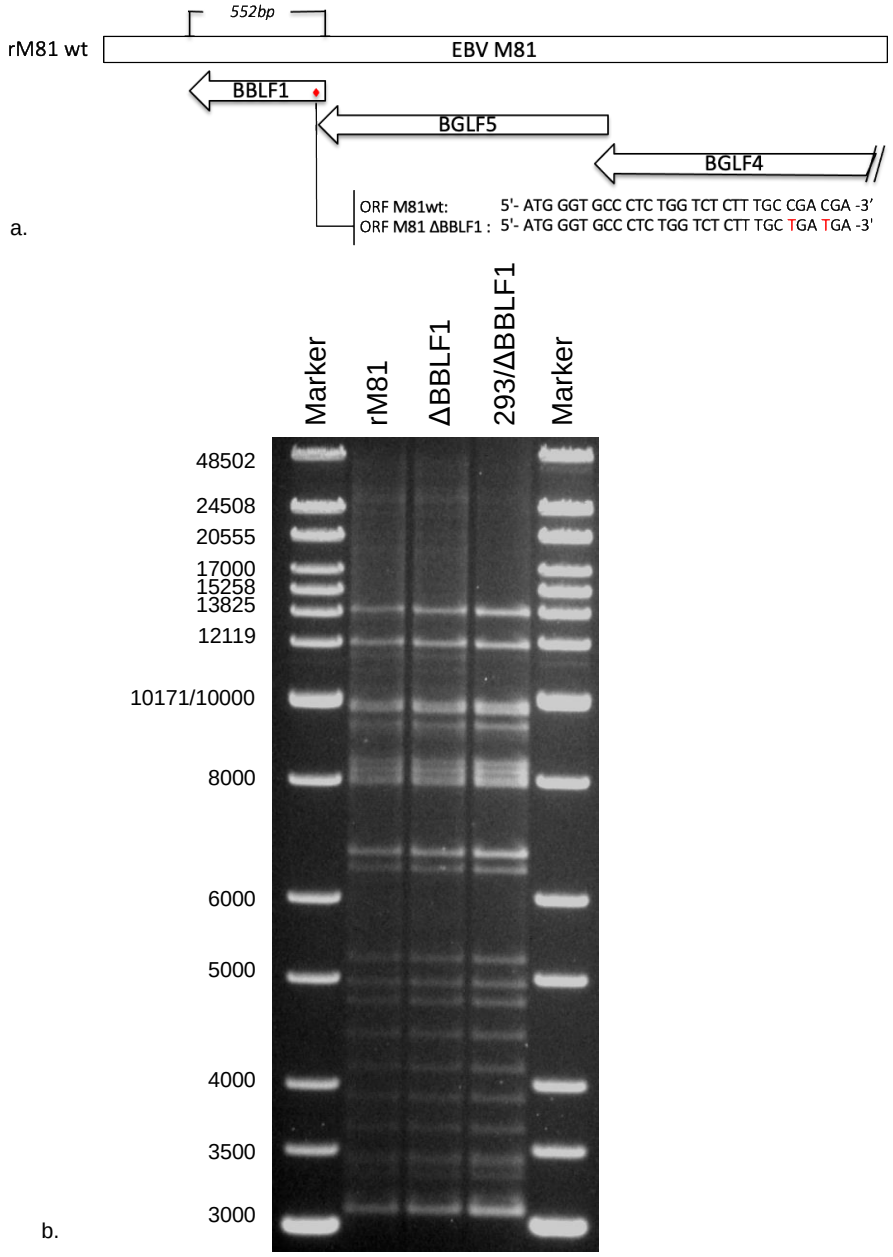


Figure 1: Generation of the recombinant M81 EBV BBLF1 Knockout. (a) Schematic representation of the genomic map of the recombinant M81 (rM81) and BBLF1 Knockout (ΔBBLF1) viruses. The figure depicts some of the viral neighboring genes of BBLF1. The BBLF1 tegument protein mutant was constructed by "*en passant*" homologous recombination of the BAC clone M81 wild-type. (b) Mini-preparations of DNA from the rM81 and ΔBBLF1 were cleaved with the BamHI restriction enzyme.

Appendix 2: BSRF1 recombinant genome

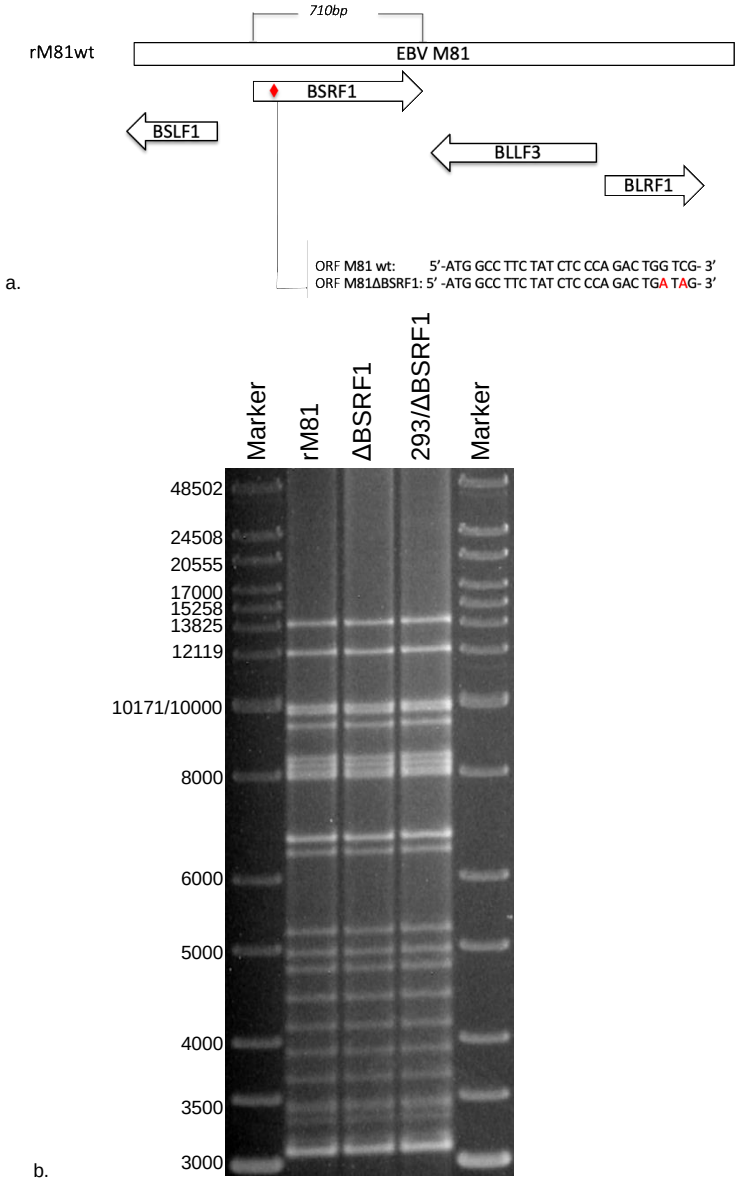


Figure 1: Generation of the recombinant M81 EBV BSRF1 Knockout. (a) Schematic representation of the genomic map of the recombinant M81 (rM81) and BSRF1 Knockout (ΔBSRF1) viruses. The figure depicts some of the viral neighboring genes of BSRF1. The BSRF1 tegument protein mutant was constructed by "*en passant*" homologous recombination of the BAC clone M81 wild-type. (b) Mini-preparations of DNA from the rM81 and ΔBSRF1 were cleaved with the BamHI restriction enzyme.

Appendix 3: BLRF2 recombinant genome

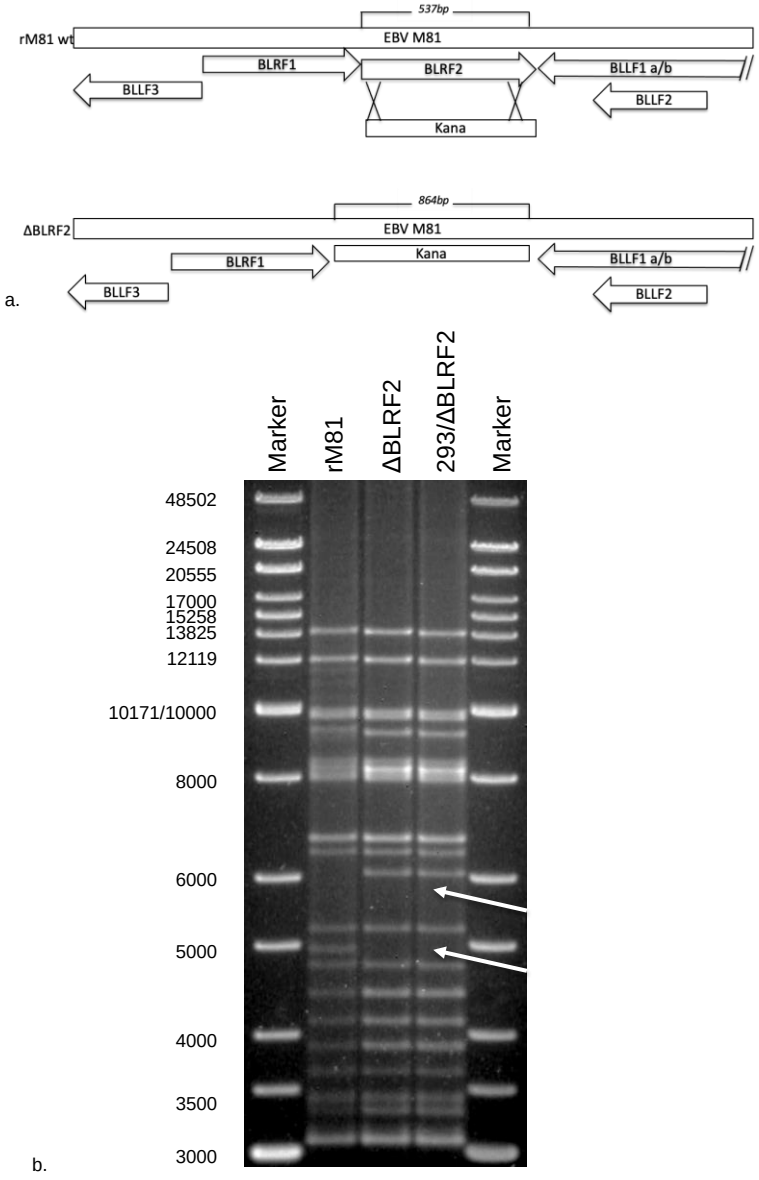


Figure 1: Generation of the recombinant M81 EBV BLRF2 Knockout. (a) Schematic representation of the genomic map of the recombinant M81 (rM81) and BLRF2 Knockout (ΔBLRF2) viruses. The figure depicts some of the viral neighboring genes of BLRF2. The BLRF2 tegument protein mutant was constructed by insertion of a kanamycine cassette via homologous recombination of the BAC clone M81 wild-type. (b) Mini-preparations of DNA from the rM81 and ΔBLRF2 were cleaved with the BamHI restriction enzyme.

Appendix 4: EBV BGLG4 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BGLF4

MDVNMAAELSPTNSSSSGELSVSPEPPRETQAFLGKVTVIDYFTFQHKHLKVTNIDDMTETLYV
KLPENMTRCDHLPITCEYLLGRGSYGAVYAHADNATVKLYDSVTELYHELMVCDMIQIGKATA
EDGQDKALVDYLSACTSCHALFMPQFRCSLQDYGHWHDGSIEPLVRGFQGLKDAVYFLNRHC
GLFHSDISPSNILVDFTDTMWGMGRLVLTDYGTASLHDRNKMLDVRLKSSKGRQLYRLYCQRE
PFSIAKDTYKPLCLLSKCYILRGAGHIPDPSACGPVGAQTALRLDLQSLGYSLLYGIMHLADSTH
KIPYPNPDMGFDRSDPLYFLQFAAPKVVLLEVLSQMWNLNLDMGLTSCGESPCVDVTAEHMSQ
FLQWCRSLKKRFKESYFFNCRPRFEHPHLPGLVAELLADDFFGPDGRRG*

EBV Akata BGLF4

MDVNMAAELSPTNSSSSGELSVSPEPPRETQAFLGKVTVIDYFTFQHKHLKVTNIDDMTETLY
VKLPENMTRCDHLPITCEYLLGRGSYGAVYAHADNATVKLYDSVTELYHELMVCDMIQIGKA
TAEDGQDKALVDYLSACTSCHALFMPQFRCSLQDYGHWHDGSIEPLVRGFQGLKDAVYFLNR
HCGLFHSDISPSNILVDFTDTMWGMGRLVLTDYGTASLHDRNKMLDVRLKSSKGRQLYRLYC
QREPFSIAKDTYKPLCLLSKCYILRGAGHIPDPSACGPVGAQTALRLDLQSLGYSLLYGIMHLA
DSTHKIPYPNPDMGFDRSDPLYFLQFAAPKVVLLEVLSQMWNLNLDMGLTSCGESPCVDVTA
EHMSQFLQWCRSLKKRFKESYFFNCRPRFEHPHLPGLVAELLADDFFGPDGRRG*

EBV M81 BGLF4

MDVNMAAELSPTNSSSSGELSVSPEPPRETQAFLGKVTVIDYFTFQHKHLKVTNIDDMTETLY
VKLPENMTRCDHLPITCEYLLGRGSYGAVYAHADNATVKLYDSVTELYHELMVCDMIQIGKA
TAEDGQDKALVDYLSACTSCHALFMPQFRCSLQDYGHWHDGSIEPLVRGFQGLKDAVYFLNR
HCGLFHSDISPSNILVDFTDTMWGMGRLVLTDYGTASLHDRNKMLDVRLKSSKGRQLYRLYC
QREPFSIAKDTYKPLCLLSKCYILRGAGHIPDPSACGPVGAQTALRLDLQSLGYSLLYGIMHLA
DSTHKIPYPNPDMGFDRSDPLYFLQFAAPKVVLLEVLSQMWNLNLDMGLTSCGESPCVDVTA
EHMSQFLQWCRSLKKRFKESYFFNCRPRFEHPHLPGLVAELLADDFFGPDGRRG*

EBV BGLF4 amino acid sequences alignment

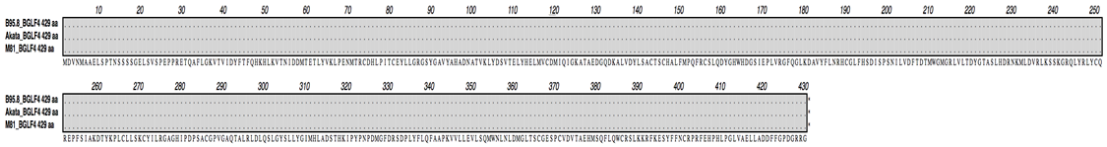


Figure 1: BGLF4 amino acid sequences and BGLF4 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BGLF4 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows no polymorphism between the B95.8, Akata and M81 strains in EBV.

Appendix 5: EBV BKRF4 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BKRF4

MAMFLKSRGVRSCRDRRLLSDEEEETSQSSSYTLGSQASQSIQEEDVSDTDESDYSDEDEEIDLE
EEYPSDEDPSEGSDSDPSWHPSDSDESDYSESDEDEATPGSQASRSSRVSPSTQQSSGLTPTPSFSR
PRTRAPPRPPAPAPVRGRASAPPRPPAPVQQSTKDKGPHRPTRPVLRGPAPRRPPPPSSPNTYNKH
MMETTPPIKGNNNYNWPWL*

EBV Akata BKRF4

MAMFLKSRGVRSCRDRRLLSDEEEETSQSSSYTLGSQASQSIQEEDVSDTDESDYSDEDEEIDLE
EEYPSDEDPSEGSDSDPSWHPSDSDESDYSESDEDEATPGSQASRSSRVSPSTQQSSGLTPTPSFSR
PRTRAPPRPPAPAPVRGRASAPPRPPAPVPQSTKDKVPNRPTRPVLRGPAPRRPPPPSSPNTYNKH
MMETTPPIKGNNNYNWPWL*

EBV M81 BKRF4

MAMFLKSRGVRSCRDRRLLSDEEEETSQSSSYTLGSQASQSIQEEDVSDTDESDYSDEDEEIDLEEE
YPSDEDPSEGSDSDPSWHPSDSDESDYSESDEDEATPGSQASRSSRVSPSTQQSSGLTPTPSFSRPRT
RAPPRPPAPAPVRGRASAPPRPPAPVPQSTKDKVPNRPTRPVLRGPAPRRPPPPSSPNTYNKHMMET
TPPIKGNNNYNWPWL*

EBV BKRF4 amino acid sequences alignment

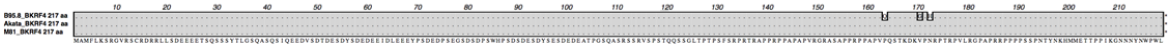


Figure 1: BKRF4 amino acid sequences and BKRF4 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BKRF4 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows three polymorphisms between the EBV B95.8 strain and the EBV Akata and M81 strains.

Appendix 6: EBV BLRF2 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BLRF2

MSAPRKVRLPSVKAVDMSMEDMAARLARLESENKALKQQVLRGGACASSTSVPSAPVPPPEPLTA
RQREVMITQATGRLASQAMKKIEDKVRKSVDGVTTRNEMENILQNLTLRIQVSMLGAKGQPSPGE
GTRPRESNDPNATRRARSRSRGREAKKVQISD*

EBV Akata BLRF2

MSAPRKVRLPSVKAVDMSMEDMAARLARLESENKALKQQVLRGGACASSTSVPSAPVPPPEPLTA
RQREVMITQATGRLASQAMKKIEDKVRKSVDGVTTRNEMENILQNLTLRIQVSMLGAKGQPSPGE
GTRPRESNDPNATRRARSRSRGREAKKVQISD*

EBV M81 BLRF2

MSAPRKVRLPSVKAVDMSMEDMAARLARLESENKALKQQVLRGGACASSTSVPSAPVPPPEPLTA
RQREVMITQATGRLASQAMKKIEDKVRKSVDGVTTRNEMENILQNLTLRIQVSMLGAKGQPSPGE
GTRPRESNDPNATRRARSRSRGREAKKVQISD*

EBV BLRF2 amino acid sequences alignment

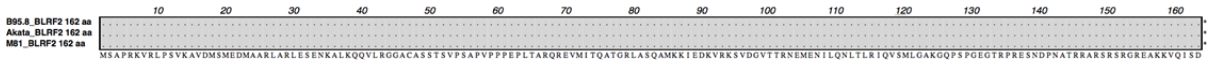


Figure 1: BLRF2 amino acid sequences and BLRF2 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BLRF2 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows no polymorphism between the B95.8, Akata and M81 strains in EBV.

Appendix 7: EBV BBLF1 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BBLF1

MGALWSLCRRRVNSIGDVDGGIINLYNDYEEFNLETTKLIAAEEGRACGETNEGLEYDEDSENDE
LLFLPNKKPN*

EBV Akata BBLF1

MGALWSLCRRRVNSIGDVDGGIINLYNDYEEFNLETTKLIAVEEGRACGETNEGLEYDEDSENDE
LLFLPNKKPN*

EBV M81 BBLF1

MGALWSLCRRRVNSIGDVDGGIINLYNDYEEFNLETTKLIAVEEGRACGETNEGLEYDEDSENDE
LLFLPNKKPN*

EBV BBLF1 amino acid sequences alignment

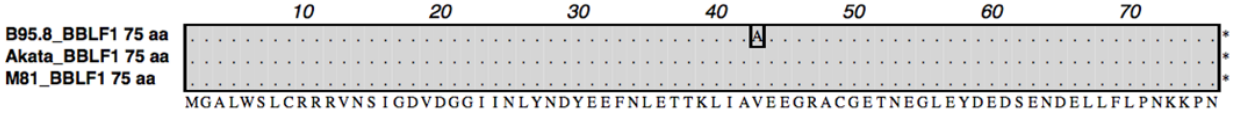


Figure 1: BBLF1 amino acid sequences and BBLF1 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BBLF1 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows a unique polymorphism between the EBV B95.8 strain and the EBV M81 and Akata strains.

<u>Appendix 8</u>: EBV BSRF1 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BSRF1

MAFYLPDWSCCGLWLFGRPRNRYSQLPEEPETFECPDRWRAEIDLGLPPGVQVGDLLRNEQTM
GSLRQVYLLAVQANSITDHLKRFDAVRVPESCRGVVEAQVAKLEAVRSVIWNTMISLAVSGIEM
DENGLKALLDKQAGDSLALMEMEKVATALKMDETGAWAQEISAVVSSVTAPSASAPFINSAFEP
EVPTPVLAPPPVVRQPEHSGPTELALT*

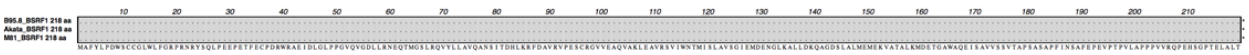
EBV Akata BSRF1

MAFYLPDWSCCGLWLFGRPRNRYSQLPEEPETFECPDRWRAEIDLGLPPGVQVGDLLRNEQTM
GSLRQVYLLAVQANSITDHLKRFDAVRVPESCRGVVEAQVAKLEAVRSVIWNTMISLAVSGIEM
DENGLKALLDKQAGDSLALMEMEKVATALKMDETGAWAQEISAVVSSVTAPSASAPFINSAFEP
EVPTPVLAPPPVVRQPEHSGPTELALT*

EBV M81 BSRF1

MAFYLPDWSCCGLWLFGRPRNRYSQLPEEPETFECPDRWRAEIDLGLPPGVQVGDLLRNEQTM
GSLRQVYLLAVQANSITDHLKRFDAVRVPESCRGVVEAQVAKLEAVRSVIWNTMISLAVSGIEM
DENGLKALLDKQAGDSLALMEMEKVATALKMDETGAWAQEISAVVSSVTAPSASAPFINSAFEP
EVPTPVLAPPPVVRQPEHSGPTELALT*

EBV BSRF1 amino acid sequences alignment



<u>Figure 1</u>: BSRF1 amino acid sequences and BSRF1 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BSRF1 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows no polymorphism between the EBV B95.8 strain and the EBV M81 and Akata strains.

Appendix 9: EBV BOLF1 amino acid sequences alignement in B95.8, Akata and M81 strains.

EBV B95.8 BOLF1

MASAMESDSSGGSGGADAQPPLAEVDGGLARVTRQLLLSGDDPAARLRALMPLELGIFGLGDLAQP
VLVRDFLNTLTLMSGHAYPAAVLRHHAYYLLRAASFSRRSFGLGHLEAALDVLASSLPPTTASPATDD
PLDGSRLIAETRALAAAYRRIIEEGSGEVLAVSGPTATFAFVEELVADTYLARWDAFPREGLSFYAFNA
AKTTLGRWLVTVYAETNRYPWAAAGQGQPTAADIKAMAVELVEHSGGGAGGGEGEESGGG-
LFHRPESLSSVVASLPLARRRAVEILGVYAEASGGQTPPVAAVPVLAFDAARLRLLEPSGALFYDYVY
EALLWDQTYGVPDSVIEAFLAGMAAEMEALAARVQEAAGSRASFSPAAIEQVATVLLSAGLNETVAG
DYAMMLASVPRVSRSRWRWLEATAALLESLSGFALHFFRLLPTASPTSRFARVARAAYLRAEAEAVDR
RARRTSGPSTPAAAPAATAVGVGAAADPWDAVTPLRIFIVPPPAAEYEQVAGDLSSELLRSLLWVRYS
RLWQAPAPAPALPCKPPLLPGEQGRRQWTAAVAAAPRTDVEAYCRSLRAGQTARADPAYVHSPFFPA
AFIEFQIWPALRRVLSNELPKTRSLAALRWLVSFGSDLALPSPELTRARRPLELIYATVWEIYDGAPPMP
GESPQAVGLRPLNLEGEGKAGDAGAEGAEDEEGGGPWGLSSHDAVLRIMDAVREVSGIISETISASER
AAEAPPLAWPTSLFSLLFTLRYSTTAESLGLATRRFLVSGETLSEDISRLTGAAWRLCSRPLLYDAETGR
VQIPLATEEEEEAVVAVKEKSVSSSPRHYSTDLQTLKSVVEGIQDVCRDAAARWALATADTATLRRRL
LVPALRESRGIADHPLWAHTSEPLRPDLEELNERVEHALELGYSLTGALRRSVAYRFRDYTFARLFQPP
AIDAERAEAIVRRDARPPPVFIPAPRRLPQGGADTPPPLSMDDILYLGKSICKALVDVLDHHPAAPETT
PIKTYTPAMDLNPEQITVTPRSPSVLAAFARTARVQTHHLVPALTDDSPSPVGQTPPPFRILPAKKLAAI
LLGNGRNASKRRASRDLSPPPHGRWRAVLDSSPFSFSSSDFSDQDEGEGGEADLRGVPGGGG--
EGAYEEDRERPSDIDTAARAQKVETSCPRRRSPRTTPSPSRRASGGGGPDRGEAEAHTYPPYLSAAAA
ASRVRPRTRRGATRRPPRPTAEDE*

EBV Akata BOLF1

MASAMESDSSGGSGGADAQPPLAEVDGGLARVTRQLLLSGDDPAARLRALMPLELGIFGLGDLAQP
VLVRDFLNTLTLMSGHAYPAAVLRHHAYYLLRAASFSRRSFGLGHLEAALDVLASSLPPTTASPATDD
PLDGSRLIAETRALAAAYRRIIEEGSGEVLAVSGPTATFAFVEELVADTYLARWDAFPREGLSFYAFNA
AKTTLGRWLVTVYAETNRYPWAAAGQGQPTAADIKAMAVELVEHSGGGAGGGEGEESGGGGLFHR
PESLSSVVASLPLARRRAVEILGVYAEASGGQTPPVAAVPVLAFDAARLRLLEPSGALFYDYVYEALL
WDQTYGVPDSVIEAFLAGMAAEMEALAARVQEAAGSRASFSPAAIEQVATVLLSAGLNETVAGDYA
MMLASVPRVSRSRWRWLEATAALLESLSGFALHFFRLLPTASPTSRFARVARAAYLRAEAEAVDRRAR
RTSGPSTPAAAPAATAVGVGAAADPWDAVTPLRIFIVPPPAAEYEQVAGDLSSELLRSLLWVRYSRLWQ
APAPAPALPCKPPLLPGEQGRRQWTAAVAAAPRTDVEAYCRSLRAGQTARADPAYVHSPFFPAAFIEFQ
IWPALRRVLSNELPKTRSLAALRWLVSFGSDLALPSPELTRARRPLELIYATVWEIYDGAPPMPGESPQ
AVGLRPLNLEGEGKAGDAGAEGAEDEEGGGPWGLSSHDAVLRIMDAVREVSGIISETISASERAAEAP
PLAWPTSLFSLLFTLRYSTTAESLGLATRRFLVSGETLSEDISRLTGAAWRLCSRPLLYDAETGGVQIPL
ATEEEEEAVVAVKEKSVSYSPRHYSTDLQTLKSVVEGIQDVCRDAAARWALATADTATLRRRLLVPAL
RESRGIADHPLWAHTSEPLRPDLEELNERVEHALELGYSLTGALRRSVAYRFRDYTFARLFQPPAIDAE
RAEAIVRRDARPPPVFTPAPRRLLQGGADTPPPLSMDDILYLGKRICKALVDVLDHHPAAPETTPIKTY
TPAMDLNPEQITVTPRSPSVLAAFARTARVQTHHLVPALTDDSPSPVGQTPPPFRILPAKKLAAILLGNG
RNASKRRASRDLSPPPHGRWRAVLDSSPFSFSSSDFSDQDEGEGGEADLRGVPGGGGGGEGAYEEER
ERPSDIDTAARAQKVETSCPRRRSPRTTPSPSRRASGGGGPDRGEAEAHTCPPYLSAAAAASRVRPRTR
RGATRRPPRPTTEDE*

EBV M81 BOLF1

MASAMESDSSGGSGGADAQPPLAEVDGGLARVTRQLLLSGDDPAARLRALMPLELGIFGLGDLAQP
VLVRDFLNTLTLMSGHAYPAAVLRHHAYYLLRAASFSRRSFGLGHLEAALDVLASSLPPTTASPATDD
PLDGSRLIAETRALAAAYRRIIEEGSGEVLAVSGPTATFAFVEELVADTYLARWDAFPREGLSFYAFNA
AKTTLGRWLVTVYAETNRYPWAAAGQGQPTAADIKAMAVELVEHSGGGAGGGEGEESGGGGLFHR
PESLSSVVASLPLARRRAVEILGVYAEASGGQTPPVAAVPVLAFDAARLRLLEPSGALFYDYVYEALL
WDQTYGVPDSVIEAFLAGMAAEMEALAARVQEAAGSRASFSPAAIEQVATVLLSAGLNETVAGDYA
MMLASVPRVSRSRWRWLEATAALLESLSGFALHFFRLLPTASPTSRFARVARAAYLRAEAEAVDRRAR
RTSGPSTPAAAPAATAVGVGAAADPWDAVTPLRIFIVPPPAAEYEQVAGDLSSELLRSLLWVRYSRLW
QAPAPAPALPCKPPLLPGEQGRRQWTAAVAAAPRTDVEAYCRSLRAGQTARADPAYVHSPFFPAAFIE
FQIWPALRRVLSNELPKTRSLAALRWLVSFGSDLALPSPELTRARRPLELIYATVWEIYDGAPPMPGES
PQAVGLRPLNLEGEGKAGDAGAEGAEDEEGGGPWGLSSHDAVLRIMDAVREVSGIISETISASERAAE
APPLAWPTSLFSLLFTLRYSTTAESLGLATRRFLVSGETLSEDISRLTGAAWRLCSRPLLYDAETGGVQI
PLATEEEEEAVVAVKEKSVSYSPRHYSTDLQTLKSVVEGIQDVCRDAAARWALATADTATLRRRLLVP
ALRESRGIADHPLWAHTSEPLRPDLEELNERVEHALELGYSLTGALRRSVAYRFRDYTFARLFQPPAID
AERAEAIVRRDARPPPVFTPAPRRLLQGGADTPPPLSMDDILYLGKRICKALVDVLDHHPAAPETTPIK
TYTPAMDLNPEQITVTPRSPSVLAAFARTARVQTHHLVPALTDDSPSPVGQTPPPFRILPAKKLAAILLG
NGRNASKRRASRDLSPPPHGRWRAVLDSSPFSFSSSDFSDQDEGEGGEADLRGVPGGGGGGEGAYEE
ERERPSDIDTAARAQKVETSCPRRRSPRTTPSPSRRASGGGGPDRGEAEAHTCPPYLSAAAAASRVRP
RTRRGATRRPPRPTTEDE*

EBV BOLF1 amino acid sequences alignment

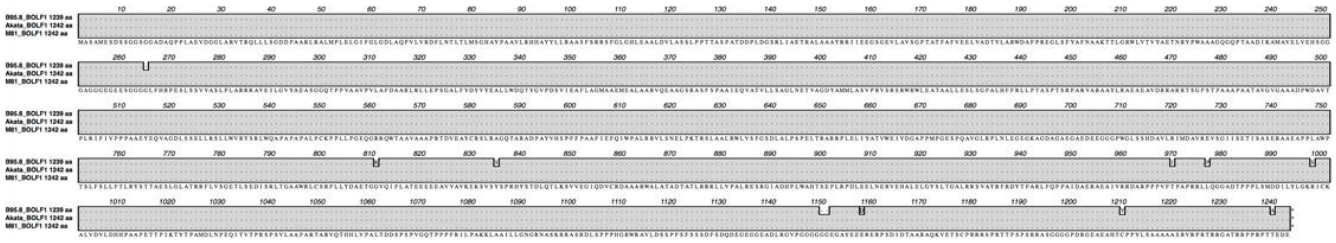


Figure 1: BOLF1 amino acid sequences and BOLF1 protein sequences alignment in EBV B95.8, M81 and Akata strains. The amino acid sequences from three EBV strains (above) were aligned by using ClustalW2 on the BOLF1 protein sequences from EBV Akata, M81 and B95.8 strains. This alignment (below) shows multiple polymorphisms between the EBV B95.8 strain and the EBV M81 and Akata strains.

10. List of figures

11. List of tables

Acknowledgements

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