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Environment and viruses' cooperation in altering cells epigenome to drive oncogenic transformation

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**Environment and viruses' cooperation
in altering cells epigenome to drive
oncogenic transformation**

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LIST OF ABBREVIATIONS

- AFB1 : Aflatoxin B1
- Aza : 5-aza-2'-deoxycytidine
- BZFL1 : BamHI Z fragment leftward open reading frame 1
- ChIP : Chromatin immunoprecipitation
- DNA : Deoxyribonucleic acid
- DNMT : DNA (cytosine-5)-methyltransferase
- DMSO : DiMethyl SulfOxide
- eBL : endemic Burkitt's Lymphoma
- EBNAS : EBV Nuclear Antigens
- EBV : Epstein Barr Virus
- HBV : Hepatitis B virus
- HCC : Hepatocellular carcinoma
- HHV-4 : Human Herpes Virus 4
- HL : Hodgkin's Lymphoma
- KDM2B : Lysine Demethylase 2B
- LMP1 : Latent Membrane Protein-1
- LCL : Lymphoblastoid Cell Lines
- NHL : Non Hodgkin's Lymphoma
- NPC : NasoPharyngeal Carcinoma
- PCR : Polymerase chain reaction
- RNA : Ribonucleic acid
- RT-qPCR : Quantitative reverse transcription PCR
- sBL : sporadic Burkitt's Lymphoma
- TGFBI : Transforming growth factor, beta-induced

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Abstract (French)

Plus de 90 % de la population adulte dans le monde est infectée par le virus de la famille des herpesviridae, appartenant à la sous-famille des gammaherpesvirinae : Epstein-Barr virus (EBV), mais généralement sans effet grave sur la santé. Malgré son omniprésence, l'EBV est également connu pour être associé à des malignités, parmi lesquelles le lymphome de Burkitt endémique (eBL), le cancer pédiatrique le plus fréquent en Afrique subsaharienne. EBV est associé à cette malignité depuis plus de 50 ans, mais le mécanisme exact par lequel le virus contribue au processus pathogène du eBL n'a toujours pas été élucidé. La géolocalisation de l'eBL suggère l'implication de facteurs environnementaux, tels que la malaria, dans le développement de cette pathologie. Notre étude précédente (Hernandez-Vargas et al. 2017) a montré que le contaminant alimentaire l'aflatoxine B1 (AFB1) et EBV coopèrent dans le processus de transformation des cellules B. Cependant, le mécanisme exact de leur interaction reste flou. On sait que l'épigénome est une interface entre l'environnement et l'ADN cellulaire et que de plus en plus de preuves indiquent que différents virus se défendent en utilisant des mécanismes épigénétiques pour déréguler le programme d'expression génique de la cellule hôte et moduler leur cycle de vie. Ces altérations épigénétiques pourraient être utilisées comme biomarqueurs d'exposition, pour évaluer les risques de cancer ou servir au diagnostic. Nous nous sommes demandé si les modifications épigénétiques induites dans les cellules B par une exposition précoce à l'AFB1 pouvaient jouer un rôle dans la transformation médiée par EBV et dans le développement du lymphome.

Dans une précédente étude, en analysant les modifications de méthylation de l'ADN dans des lignées cellulaires de lymphome de Burkitt endémique par rapport à des lignées sporadiques, nous avons identifié plusieurs positions génomiques méthylées différenciellement à proximité de gènes ayant un rôle potentiel dans le cancer, et parmi eux se trouvait le gène de la lysine (K)-

specific demethylase 2B (KDM2B). KDM2B code pour une histone H3 déméthylase dont l'implication dans certains troubles hématologiques a déjà été démontré ainsi que son rôle dans la différenciation des cellules hématopoïétiques. Cependant, le rôle de KDM2B dans le développement du lymphome médié par le virus Epstein-Barr n'a pas encore été étudié. Nous avons précédemment découvert que le gène KDM2B est méthylé de manière différentielle dans les lignées cellulaires dérivées des eBL par rapport aux cellules de la forme sporadique n'impliquant pas EBV (sBL). Cependant, le rôle de KDM2B dans le développement du lymphome de Burkitt endémique n'a pas été étudié auparavant. Il a été démontré que les virus oncogènes détournent l'épigénome de la cellule hôte afin de continuer leur cycle cellulaire et favoriser le processus de transformation en perturbant l'organisation de la chromatine de la cellule hôte. Ici, nous avons cherché à savoir si EBV modifie les niveaux de KDM2B pour permettre son cycle de vie et promouvoir la transformation des cellules B. Nous montrons que l'infection des cellules B par EBV entraîne une régulation négative des niveaux d'expression de KDM2B. Nous montrons également que LMP1, une des principales protéines de transformation d'EBV, induit un recrutement accru de DNMT1 sur le gène KDM2B et augmente sa méthylation. En modifiant les niveaux de KDM2B et grâce à une immunoprécipitation de chromatine (ChIP) des cellules B infectées par EBV, nous montrons que KDM2B est recruté sur les promoteurs du gène d'EBV et inhibe leur expression. En outre, la surexpression de KDM2B dans les cellules B immortalisées a entraîné une altération des niveaux d'expression de certains gènes liés à la différenciation. Nos données montrent que EBV dérégule l'expression de KDM2B par un mécanisme épigénétique et fournissent la preuve du rôle de KDM2B dans la régulation de l'expression des gènes viraux et de la cellule hôte, ce qui justifierait des recherches supplémentaires pour évaluer le rôle de KDM2B dans le processus de lymphomagenèse médiée par EBV.

Ensuite, pour comprendre si les modifications épigénétiques induites par une exposition à l'AFB1 de cellules B pourraient jouer un rôle dans la transformation médiée par EBV et ainsi que dans le développement du BL, nous avons effectué une analyse du méthylome entier d'un panel de lignées cellulaires dérivées de BL par rapport à des cellules B primaires et avons identifié des gènes méthylés différemment dans le lymphome. Notre analyse *in silico* a révélé que certains de ces gènes, tels que le transforming growth factor, beta-induced (TGFBI), étaient également méthylés différemment dans les globules blancs de nourrissons exposés à des niveaux élevés d'aflatoxine B1 *in utero*. Nous montrons que le promoteur du gène de TGFBI, souvent altéré dans les cancers, est significativement méthylé dans des lignées cellulaires dérivées de BL ainsi que dans des échantillons humains de BL. De plus, en utilisant des cellules B traitées avec de l'AFB1 et/ou infectées par l'EBV, nous avons observé que les deux agents induisent la méthylation du promoteur de TGFBI et réduisent l'expression de TGFBI. Nous avons également démontré que la protéine virale LMP1 active la voie NF- κ B pour induire le recrutement de DNMT1 sur le promoteur de TGFBI. L'AFB1 semble partager la même voie que LMP1 et avoir un effet synergique avec EBV dans la méthylation et la régulation de TGFBI. Enfin, nous avons constaté que le pattern de méthylation du promoteur de TGFBI, identifié dans des cellules B transformées, est maintenu dans l'ADN extracellulaire. Nos données montrent que le promoteur de TGFBI est méthylé dans la BL aux mêmes positions que dans l'ADN des enfants exposés aux aflatoxines, ce qui suggère que l'exposition précoce à la toxine pourrait modifier les niveaux de méthylation des gènes associés aux BL et jouer un rôle causal dans son développement. De plus, les signatures épigénétiques du promoteur de TGFBI, détectables dans l'ADN exosomal des cellules B transformées, pourraient être potentiellement utilisées comme un biomarqueur non invasif de la lymphomagenèse.

Abstract (English)

More than 90% of the adult population worldwide is infected by the gammaherpesvirus Epstein-Barr virus (EBV), usually without effect on health. Despite its ubiquity, EBV is also known to be associated with malignancies, including the endemic Burkitt Lymphoma (eBL), the most common pediatric cancer in Sub-Saharan Africa. EBV has been associated with this malignancy since more than 50 years, but the exact mechanism by which the virus contributes to the eBL pathogenic process is still not fully understood. The geolocation of the eBL suggests the involvement of environmental factors, such as malaria, in the development of this pathology. Our previous study (H.Vargas et al. 2017) showed that the dietary contaminant aflatoxin B1 (AFB1) and EBV cooperate in the process of B-cell transformation. However, the exact mechanism of interaction remains unclear.

It is known that the epigenome is an interface between the environment and the cellular DNA and increasing evidences indicates that different viruses defend themselves by using epigenetic mechanisms to deregulate the host cell gene expression program and modulate their life cycle. Here we asked whether epigenetic modifications induced in B-cells by early-life exposure to AFB1 could play a role in EBV-mediated oncogenic transformation and the development of BL. In a previous study, by analyzing the DNA methylation changes in endemic compared to sporadic Burkitt lymphoma cell lines, we identified several differentially methylated genomic positions in the proximity of genes with a potential role in cancer, among which the histone modifier lysine (K)-specific demethylase 2B (KDM2B) gene was the top hit. KDM2B encodes a histone H3 demethylase, an enzyme known to play a role in the differentiation of hematopoietic cells as well as in some hematological disorders. However, whether KDM2B plays a role in the development of Epstein-Barr virus-mediated lymphoma has not been investigated. We have previously found that the KDM2B gene is differentially methylated in cell lines derived from EBV-associated endemic Burkitt lymphoma compared with that in

EBV-negative sporadic Burkitt lymphoma-derived cells. Because oncogenic viruses have been proposed to be able to hijack the host cell epigenome to promote the transformation process by perturbing cell chromatin organization, this thesis project aimed to investigate whether EBV alters KDM2B levels to enable its life cycle and promote B-cell transformation. We first showed that infection of B-cells with EBV leads to the down-regulation of KDM2B levels. We then found that LMP1, one of the main EBV transforming proteins, induces increased DNMT1 recruitment to the KDM2B gene and augments DNA methylation levels at its promoter. By altering KDM2B levels and performing chromatin immunoprecipitation in EBV-infected B-cells, we demonstrate that KDM2B is recruited to the promoter of EBV genes and inhibits their expression. Furthermore, forced KDM2B expression in immortalized B-cells led to altered mRNA levels of some differentiation-related genes. Our data show that EBV deregulates KDM2B levels through an epigenetic mechanism and provide evidence for a role of KDM2B in regulating virus and host cell gene expression, warranting further investigations to assess the role of KDM2B in the process of EBV-mediated lymphomagenesis.

To further investigate whether epigenetic modifications induced in B-cells by early-life exposure to AFB1 could play a role in EBV-mediated transformation and in the development of BL, we performed a whole methylome analysis of a panel of BL-derived cell lines and primary B-cells and identified genes differentially methylated in BL. Our *in-silico* analysis revealed that some of these genes, such as TGFBI, were also differentially methylated in white blood cells of infants exposed to high levels of aflatoxin B1 *in utero*. We showed that the promoter of TGFBI gene, whose expression is often deregulated in cancer, is significantly hyper-methylated in BL-derived cell lines as well as in human BL samples. In addition, B-cells treated with AFB1 and/or infected with EBV exhibited an increased TGFBI promoter methylation and reduced gene expression. We also demonstrate that the viral protein LMP1 activates the NF- κ B pathway to induce the recruitment of DNMT1 to the TGFBI promoter.

Concerning the effect on TGFBI, the AFB1 appears to target the same pathway as LMP1 and to have a synergistic effect with EBV in affecting DNA methylation-mediated regulation of TGFBI. Finally, we found that the pattern of methylation of the TGFBI promoter, identified in transformed B-cells, can be detected in extracellular (exosomal) DNA. Our data show that the promoter of TGFBI is methylated in BL at the same positions as in the DNA of children exposed to aflatoxins, suggesting that early life exposure to the toxin could alter the methylation levels of genes associated to BL and may play a causative role in its development. Besides, epigenetic signatures of the TGFBI promoter, detectable in exosomal DNA of transformed B-cells, could be used as a non-invasive biomarker of lymphomagenesis. Finally, this work identified epigenetic biomarkers of the oncogenic viruses and aflatoxin (each alone or in viral–aflatoxin combinations) and deciphered whether altered epigenetic pathways underlie the observed increased predisposition to infections and specific human malignancies.

INTRODUCTION

I. Viruses and Cancer

a. History

The concept around viruses and cancer started in 1842 with Domenico Rigoni-Stern, introducing the notion of a transmissible agent which may play a role in some types of human cancer¹. We then had to wait almost 60 years to identify the first human virus by isolation of the yellow fever virus². Shortly afterwards, in 1911, Peyton Rous reported that a cell-free filtrate agent from extracts of a chicken tumour could induce sarcoma in healthy chicken (Rous sarcoma virus)^{2,3}. This discovery opened the door to the new field of tumour virology, demonstrating that some cancers could have a viral origin, the finding that led to the discovery of oncogenes. Fifty-three years after Rous's discovery, M.A. Epstein, B.G. Achong, and Y.M. Barr identified the Epstein-Barr virus (EBV) particles by electron microscopy in derived cell lines from African patients with Burkitt's Lymphoma^{4,5}. Since then, seven different viruses have been identified in the aetiology of human cancers: (Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papillomavirus (HPV), human T-cell lymphotropic virus (HTLV), hepatitis C virus (HCV), Kaposi's sarcoma virus (KSHV), and Merkel cell polyomavirus(MCV)). These 7 viruses have been found to cause 10–15% of human cancers worldwide⁶. It is interesting to notice that human cancer inducing viruses represent a large panel of the different types of viruses : small double-stranded DNA viruses (such as HPV and MCV), positive-stranded RNA viruses (such as HCV), both large double-stranded DNA viruses (such as EBV and KSHV), DNA viruses with retroviral features (such as HBV) and complex exogenous retroviruses (such as HTLV-I) (Table 1). Concerning the Human Immunodeficiency Virus (HIV), it is known to cause the acquired immune deficiency syndrome (AIDS), which increase the risk to develop cancer, including some linked to other viruses. But it doesn't appear to cause cancers directly⁷.

Table 1. Viruses involved in human cancer (from Yuan Chang et al. 2017¹)

Viruses	Virus discovered	Method of discovery	Virus classification	Malignancy	Other disease
Epstein-Barr Virus (EBV)	1964	Cell culture and electromicroscopy	<i>Herpesviridae</i> dsDNA	Burkitt's lymphoma Diffuse large B-cell lymphoma Hodgkin lymphoma Undifferentiated nasopharyngeal ca. Gastric adenocarcinoma Leiomyosarcoma Post-transplant lymphoproliferative ds.	Infectious mononucleosis X-linked lymphoproliferative Syndrome/(Duncan syndrome)
Hepatitis B Virus (HBV)	1965	Serologic screening	<i>Hepadnaviridae</i> dsDNA-RT	Hepatocellular carcinoma	Hepatitis Cirrhosis
Human T-Lymphotropic Virus-1 (HTLV-1)	1980	Cell culture, RT assay and electromicroscopy	<i>Retroviridae</i> ssRNA-RT, positive strand	Adult T-cell leukaemia (ATL)	HTLV-1 myelopathy/tropical spastic paraparesis (HAM/TSP)
Human Genital Papillomavirus (HPV)	1983	DNA hybridation	<i>Papillomaviridae</i> dsDNA	Cervical carcinoma Squamous cell head and neck ca. Squamous cell anal cancer Vulvar cancer	
Hepatitis C Virus (HCV)	1989	cDNA library screening	<i>Flaviviridae</i> ssRNA-RT, positive strand	Hepatocellular carcinoma Rare lymphomas?	Hepatitis Cirrhosis
Kaposi Sarcoma Herpesvirus (KSHV/HHV8)	1994	Representational difference analysis (RDA)	<i>Herpesviridae</i> dsDNA	Kaposi's sarcoma Primary effusion lymphoma Multicentric Castlemans ds.	
Merkel Cell Polyomavirus (MCV)	2008	Digital Transcriptome Subtraction (DTS)	<i>Polyomaviridae</i> dsDNA	Merkel cell carcinoma	

Cancers, having viral aetiology or not, are biological accidents, as all the seven types of viruses cause infection but the majority of infected persons do not develop cancers¹. The development of a tumour does not increase the virus transmissibility or enhances the virus replication; on the contrary tumours cause the death of the host and therefore the end of the viral replication. Most of the viruses establish persistent infections and only a small portion of infected people develop a tumour. So why after viral infection some individuals develop cancer? From viral infection to cancer, there is a complex path requiring the generation of mutations in the host cell genome induced by other non-infectious factors, such as age, genetic susceptibility, environmental factors and the status of the immune system.

Viruses like EBV and KSHV establish latency in cellular reservoirs that are maintained during the lifetime of the person. Occasional reactivation of the virus leads, through the activation of the lytic cycle, to cell lysis, and therefore, the release of new virions and the infection of neighbouring cells⁸. During this first phase, the production of the viral proteins induces a response of the host immune system, that in turn eliminate the infected cells. The other defence mechanism against the virus adopted by the infected cell is to inhibit the cell cycle progression and triggers apoptosis (Figure 1). To bypass the host defences, the expression of oncoproteins that induce the restart of the cell cycle and inhibit the apoptosis. The virus enters then in the second phase, called “latency”, that allows the virus to escape the immune system^{9,6}.

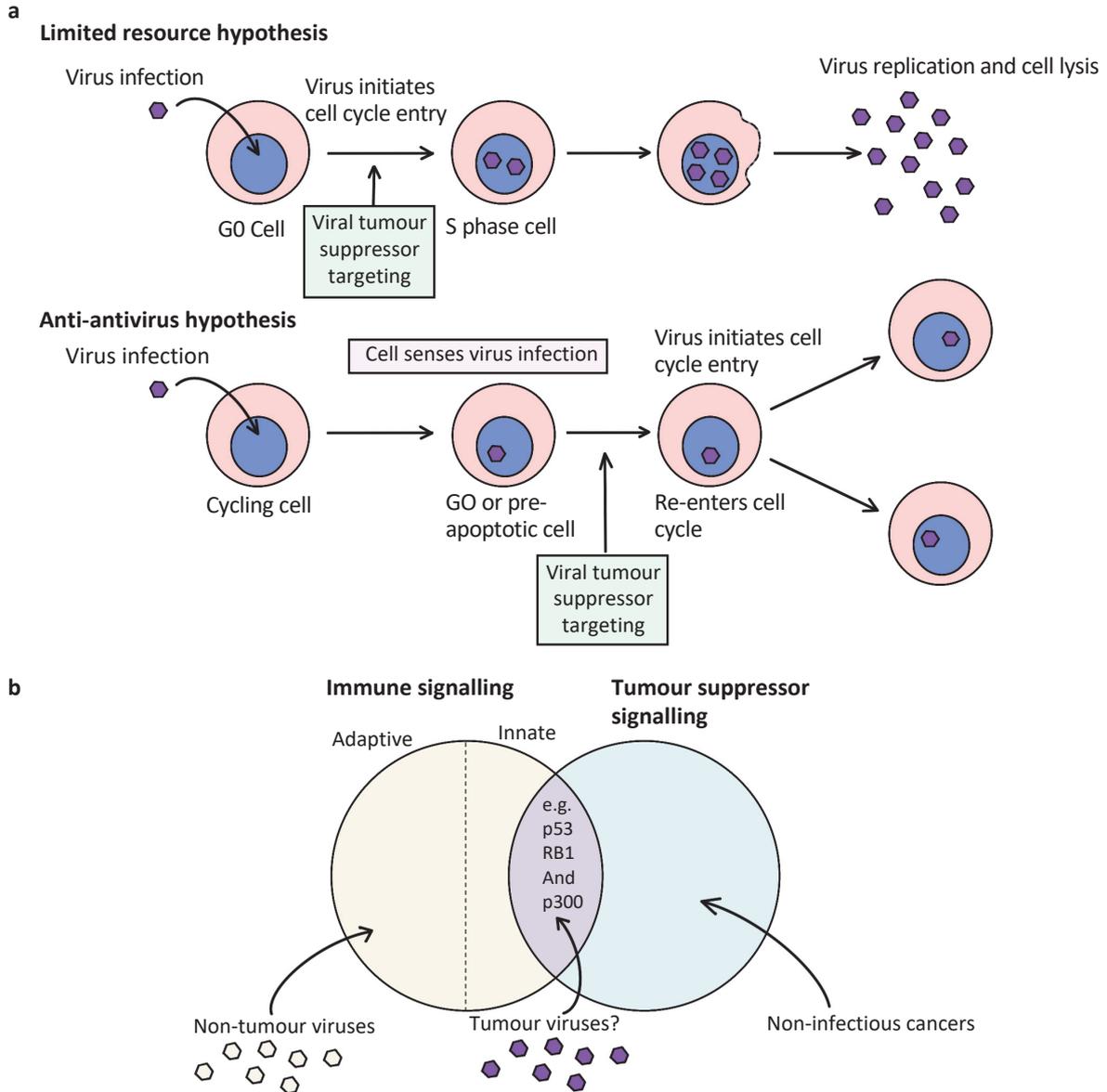


Figure 1. Two views for the origins of viral oncoproteins (from Patrick S. Moore and Yuan Chang 2010 ⁹): “(a) The tumour virus proteins target RB1 and p53 (two tumour suppressors) to drive a quiescent G0 cell into S phase of the cell cycle, allowing viral access to the nucleotide pools and replication machinery that are needed for replication and transmission. Viral tumorigenesis is a by-product of the molecular parasitism by viruses to promote their own replication. Cells respond to virus infection by activating RB1 and p53 to inhibit virus replication as part of the innate immune response. To survive, tumour viruses have evolved the means to inactivate these and other immune signalling pathways that place the cell at risk for cancerous transformation. This view holds that many tumour suppressor proteins have dual functions in preventing cancer formation and virus infection. (b) An illustration of the overlap between intracellular innate immune and tumour suppressor signalling. Under typical circumstances, viruses do not cause cancers except in the settings of immunosuppression and/or complementing host cell mutations. Non-tumorigenic viruses, which constitute the overwhelming majority of viruses, target many of the same innate immune and tumour suppressor pathways as tumour viruses but do so in ways that do not place the host at risk for carcinogenesis. Apart from p53, RB1 and p300, additional proteins are likely to have both tumour suppressor and innate immune functions.”

b. EBV

In 1958, Denis Burkitt, described for the first time a paediatric cancer of the maxilla in Uganda: the Burkitt's Lymphoma (BL) ¹⁰. Burkitt and Wright also noticed that the lymphoma had a specific geographical distribution, suggesting the involvement of an infectious agent¹¹. Subsequently, Anthony Epstein and his student Yvonne Barr and Denis Burkitt collaborated to search eventual viral particles in lymphoma biopsies. It was in 1964 that the trio identified viral particles in derived cell lines from Burkitt's lymphoma human samples using electron microscopy⁵, giving the new virus the name "Epstein-Barr virus (EBV)".

It is now known that EBV infects more than 90% of the adult population worldwide. Paradoxically, while most of the infections do not cause disease in the individual, the EBV is known to be linked to some malignancies, such as the Nasopharyngeal Cancer (NPC) and the BL¹².

i. Taxonomy and Epidemiology

EBV, also known as Human Herpesvirus 4 (HHV-4), is the first human tumour virus to have been identified, in 1964. EBV, belong to the gamma-herpesviruses subfamily, and it is the prototype of the Lymphocryptovirus genus¹². Viruses belonging to the Lymphocryptovirus genus can induce the B-cells transformation, and they are characterized by a long replicative cycle, a latency mainly localized in B lymphocytes and an important oncogenic potential¹³.

EBV is detected everywhere in the world and the transmission is generally made through contact with contaminated saliva. However, other transmissions modes have been identified, such as blood contact and organ transplant. In most of the cases, the primary infection happens during childhood in an asymptomatic way. The virus is detectable for all the lifetime in the circulating memory B-cells during latency and in the saliva during the lytic cycle . Nevertheless, EBV is involved in different human cancers of lymphoid or epithelial origins, such as Burkitt

lymphoma (BL), Hodgkin and non-Hodgkin lymphomas, nasopharyngeal carcinoma and a subset of gastric carcinoma.

Two different EBV types can be found in human: EBV-1 and EBV-2 (or EBV-A and -B). The main differences between these two EBV types are in the sequence of the EBV nuclear antigens (EBNAs) and also in their immortalization capacity *in vitro*¹².

EBV mainly targets B-cells and the infection starts when the virus is in contact with the cell. The viral glycoprotein gp350/220 binds to the cellular receptor CD21 also called CR2 (Human complement Receptor 2) which in turn stimulates the endocytosis^{15,16}. Following this first contact, other viral glycoproteins play a role in inducing the viral endocytosis; once the viral DNA is inside the B-cell it circularizes, and it remains latent in the nucleus. The virus can also infect epithelial cells, where it has been associated with several human cancers, including the nasopharyngeal carcinoma (NPC) and gastric cancers¹².

ii. Structure

1. General structure

The structure of EBV includes 4 major components (Figure 2):

- A double-stranded linear DNA (172 kb) which encodes more than 85 genes. Most of them are translated into proteins, however, some latent genes are non-translated (EBV-encoded RNAs: EBERs)
- The icosahedral capsid composed of 162 sub-units.
- The Tegument, between the capsid and lipid envelope. It is composed of proteins (viral and cellular) and it is involved in the stages of maturation of the viral particle and also in the early stages of infection¹⁷.
- The envelope, originating from the cytoplasmic and nuclear membranes of the host. It includes the glycoproteins involved in the first step of the infection¹⁸.

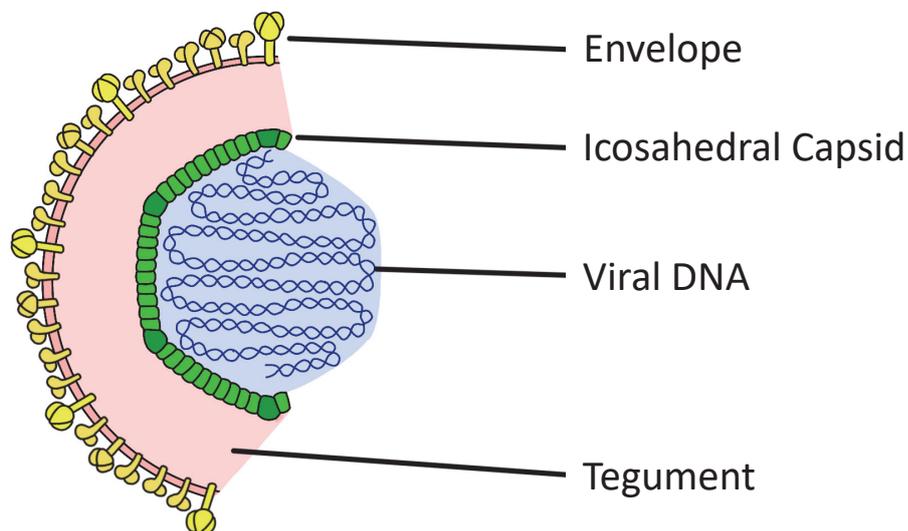


Figure 2. **Structure of EBV virion** (adapted from Chen *et al.*2012)

2. Structure of the viral genome

The viral genome of EBV consists of 172 kb with two repeated sequences at the two extremities (TR, Terminal Repeat)¹⁹. The EBV open reading frames (ORFs) are divided into latent and lytic genes, and most of them are translated¹² (Figure 3).

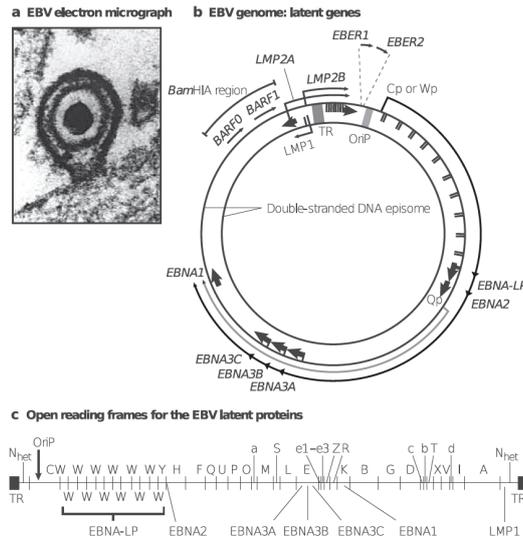


Figure 3. **The Epstein-Barr virus genome** (from IARC Monographs volume 100B 2009¹²): “(a) Electron micrograph of the Epstein-Barr virus (EBV) virion (b) Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome (c) Location of open reading frames of the EBV latent proteins on the Bam HI restriction-endonuclease map of the prototype B95.8 genome reprinted by permission from Macmillan Publishers Ltd: Young & Rickinson, Nature Reviews, 4:757, copyright (2004) “

iii. EBV life cycle:

- Viral latency: it consists of inhibiting the pathways that induce the lytic cycle to establish a long-term persistence. A parallel between the maturation of the B-cells and the EBV infection can be done, as shown in Figure 4 (adaptation Thorley-Lawson 2015).

During the primary infection, EBV enters through the tonsillar crypts, transits the epithelium and infect naive B-cells. Here the viral DNA circularize and expresses a set of latent genes which cause the cell to become active and proliferate and, in a process called “grow program” or Latency III. During this program, the nine latent proteins are expressed: the nuclear antigens (EBV nuclear antigens: EBNA_s) and the latent membrane proteins (LMP_s) (Table 2). To be activated, a B-cell requires signals through the B-cell antigen receptor from the T-helper cells. The virus uses the two latent proteins LMP1 and LMP2A: LMP1 mimic the CD40 activation from the T cell and LMP2 mimic BCR activation from the antigen (Figure 4). The virus drives the B-cell into becoming an activated blast without external signalling and the activated B-cell migrate into the follicle to initiate a germinal centre (GC). At this site, the virus enters in the latency II, also called the “Default program”, where only 3 viral proteins are expressed: EBNA1, LMP1 and LMP2A. The infected GC B-cells leaves the GC to enter in the memory compartment as a resting, long-lived, memory B-cell corresponding to the latency program: Latency 0 (Table 2). Occasionally these cells can divide in the periphery. The virus does not control the proliferation, the cycle is controlled by the normal memory homeostatic mechanisms. Here the virus expresses the protein EBNA1 which allow the virus to replicate its genome with the cell (EBNA1 only program: Latency I)^{20,21,22,12} (Table 2).

In vitro, when EBV infects B-Cells from the peripheral blood, the cells are activated to grow and proliferate as lymphoblastoid cell lines (LCLs). The LCLs does not produce viral particles, they are latently infected and the EBV genome (a circular episome in the nucleus) is maintained

as the cells divide in S phase by the host cell DNA polymerase with about 10 copies per cells. The LCLs are known as Latency III.

The viral genes functions expressed during latencies are summarised in Table 3²³. EBV can induce long-term persistence, however, it has the possibility to be replicative and infect new hosts. The reactivation involves a similar mechanism to the memory B-cells activation. A signal induces the differentiation of B-cell into an antibody-secreting plasma cell which, in turn, induces the reactivation of the virus²².

- Lytic cycle: it consists of the replication of the viral DNA, the expression of proteins used during the lytic cycle by using the enzymatic machinery of the virus and the cell. The cycle terminates with the death of the cell and the release of the new viral particles²⁴. During the lytic cycle, two Immediate Early (IE) genes are expressed and they activate early genes (E) involved in the viral DNA replication. Finally, the expression of late genes (L) induces the production of structural proteins and the formation of virions²⁵. Once the capsid is assembled in the nucleus, the viral DNA is packaged into the capsid. The nucleocapsid binds the internal nuclear membrane to go in the perinuclear space and be de-enveloped by the fusion with the external nuclear membrane. The nucleocapsid is then delivered into the cytoplasm where it acquires a layer of tegument proteins. The nucleocapsid-tegument bind an intracellular compartment derived from cis-Golgi to find its final envelopment and it became a mature virion, released by fusing with the plasma membrane²⁶.

Table 2. **EBV transcription programmes and diseases in normal B-cells** (Table adapted from Thorley-Lawson 2001 and IARC monographs 100B)

EBV Cycle	Program	Fonction	Gene Expression	Malignancies	Site <i>in vivo</i>
Lytic	Lytic Program	Replication of the virus in plasma and epithelial cell	All lytic genes	Lymphoid cancer	Epithelial Cells Tonsil Naïve B cells
	Latency III Grow program	Activation of B cell to become a proliferating lymphoblast	EBNA1/2/3A/3B/3C/LP LMP1/2A/2B, RNA and miRNA	Lymphomas associated to immunocompromised patient	Tonsil Naïve B cells
Latence	Latency II Default program	Differentiation of activated B cell into memory	EBNA1, LMP1/2A, RNA and miRNA	Hodgkin disease Nasopharyngeal carcinoma Peripheral T/NK lymphoma	Tonsil GC B cells
	Latency I EBNA1 only program	Allow virus in latency program cell to divide	EBNA1, RNA and miRNA	Burkitt Lymphoma Gastric carcinoma	Periphery dividing memory B
	Latency 0 Latency program	Allow lifetime persistence	RNA and miRNA	-	Periphery resting memory B

Table 3. **Summary of Epstein–Barr virus genes expressed in latent infections** (from Paul J. Farrell 201823)

Gene	Action
Required for transformation of adult peripheral B-cells to LCLs	
EBNA1	Binds to the plasmid origin of replication to allow viral genome replication by cell DNA polymerase
EBNA2	A transcription factor that induces cell and EBV genes to promote cell growth
EBNA3A and EBNA3C	Repress BIM and p16; prevent differentiation into plasma cell
LMP1	Constitutively induces NF-κB and is analogous to CD40; prevents apoptosis
BHLF1 or BALF1	BCL-2 family proteins that prevent apoptosis
BHRF1 miRNAs	Regulate PTEN, p27, and BHRF1
Also essential for transformation of germinal center B-cells to LCLs	
LMP2A	Signals via BCR and PI3-kinase pathways; prevents apoptosis
Also essential for transformation of cord (naïve) B-cells to LCLs	
EBNA-LP	Cooperates with EBNA2 and overcomes cell innate response to viral genome
Not required for transformation of purified B-cells	
EBER1 and EBER2	Functional RNAs that may affect innate responses and gene expression
BART miRNAs	Mediate evasion of T and NK cells during infection of B cells in peripheral blood lymphocytes
EBNA3B	Acts as a tumor suppressor gene in a mouse model of DLBCL

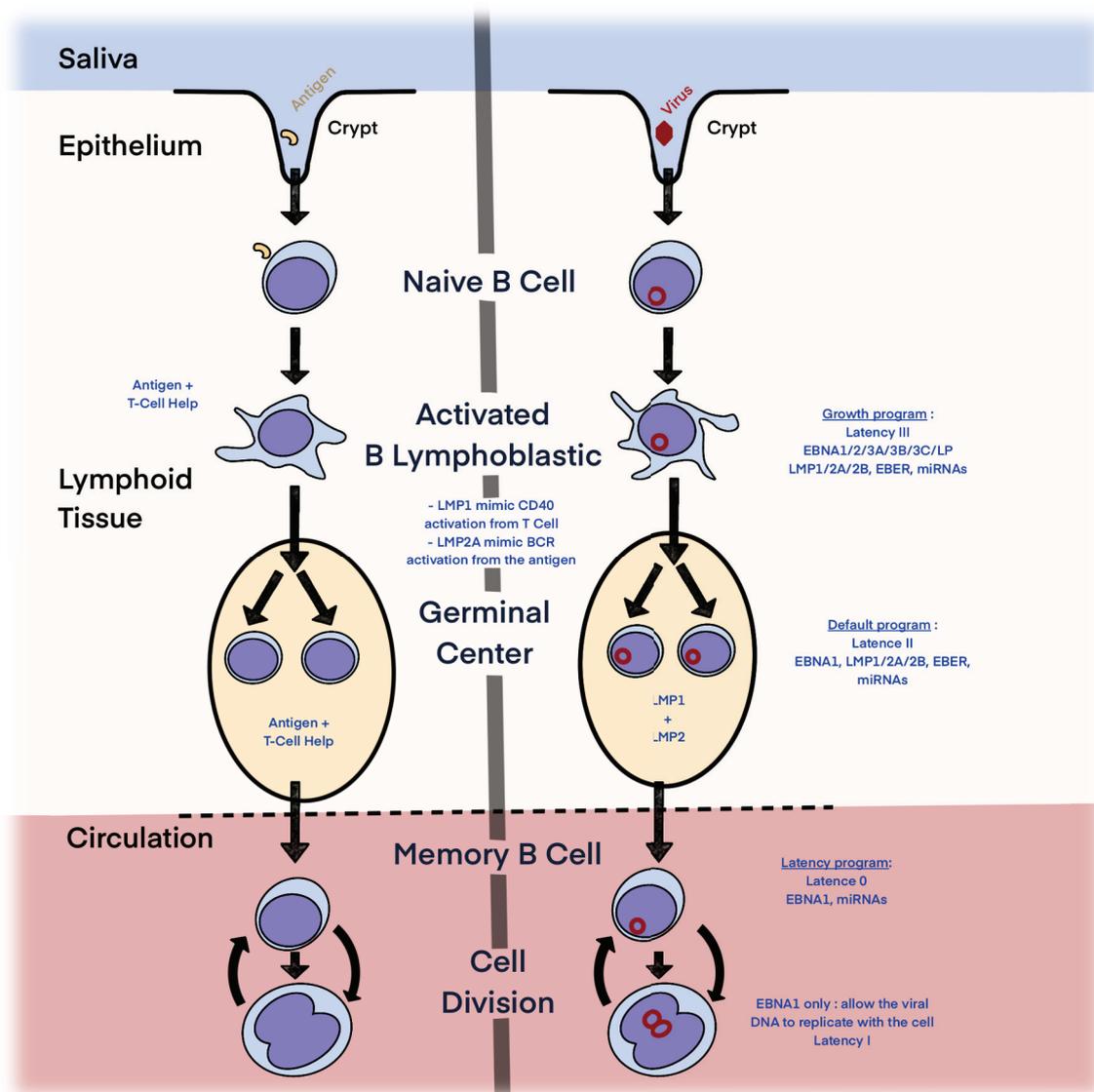


Figure 4. Model of how EBV uses normal B-cell biology to establish and maintain persistent infection in memory B-cells (Adapted from Thorley-Lawson 2015).

iv. EBV-associated malignancies:

It is well established that EBV infects more than 90% of the population without any consequences on health. However the virus may be involved in different diseases, benign or malign (Figure 5).

Given the ubiquitous infection of EBV in the population, the EBV-associated malignancies are suspected to be linked to a reactivation of the virus caused by the interaction with a cofactor.

Benign:

- Hairy leukoplakia:

It is a dysfunction of the epithelial cell from the buccal mucous membrane associated with EBV. It is often a mark of an immunocompromised patient. The disease occurs in almost 50% of patients with untreated HIV infection^{27,28}.

- Infectious mononucleosis (IM) :

Often the primary EBV infection occurs during adolescence or early adult life are manifested as infectious mononucleosis, a self-limiting lymphoproliferative disease. Infectious mononucleosis is usually acquired from a transfer of saliva¹².

Malign (Figure 5):

- Hodgkin's Disease (HD):

Hodgkin's disease is characterized by the presence of Reed-Sternberg cells. Only in 40-60% of HD cases, EBV is present, however IM is predisposing for both EBV-positive and EBV-negative HD²¹. In HD, EBV is in a Latency II (Default program).

- Burkitt's Lymphoma (BL):

There are three different subtypes of Burkitt's Lymphoma:

- the sporadic BL (sBL), associated with EBV for 15-20% of the cases;
- the endemic BL (eBL) associated with EBV in almost 100% of the cases;
- the immunodeficiency-associated BL often associated with an HIV infection.

The eBL has a specific geographical distribution, mainly affecting children in equatorial Africa and New Guinea. This geographical distribution suggests the implication of some environmental factors.¹² The virus is in Latency I (EBNA1 only program).

- Immunoblastic lymphomas:

B-cells tumours that express the growth program, appearing in immunocompromised individuals that are unable to have an effective cytotoxic T-lymphocyte response to these cells. Immunosuppression can have a iatrogenic, environmental or genetic origin²¹.

The virus is in Latency III (Growth program).

- Nasopharyngeal carcinoma:

It is somewhat surprising that despite its B lymphotropism, EBV is involved in the development of a carcinoma (notably NPC). Considering that 20% of all cancers in China and Taiwan are NPC this cancer type represents an important world health problem²². NPC is a cancer of epithelial cells in the oropharynx, with a frequent invasive and metastatic evolution. More than 98% of NPC cases are associated with EBV²⁹. EBV is in latency II (Default program), and the expression of LMP1 plays an important role in the transformation of the infected cells³⁰.

- Gastric cancers:

EBV is involved in 5-10% of worldwide gastric carcinomas. As for NPC, EBV is present in almost all cells and it is monoclonal. EBV is found in Latency I (EBNA1 only program)³¹.

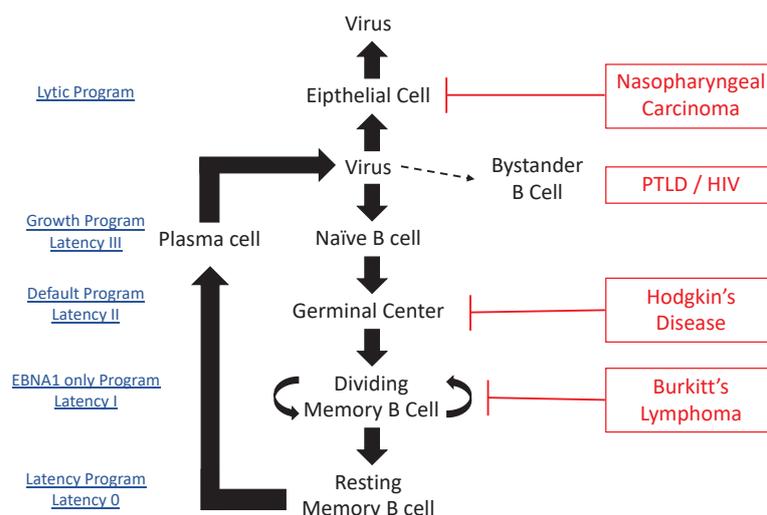


Figure 5. **The origin of EBV-positive diseases:** this figure shows the course of the virus in healthy carriers in black. To the right are shown the EBV-associated diseases and their proposed origin and to the left are listed the viral transcription programs expressed in the tumour and at the equivalent stage of infection. (Adapted from Thorley-Lawson 2005/2015)

II. Cancer

Cancer is a disease that has affected humanity from prehistoric times¹ until now. It is the second leading cause of death in developed countries and the third leading cause of death for adults in developing countries². Cancer is considered as a heterogeneous group of diseases characterised by uncontrolled growth of the cells. Generally, cancers are classified depending on the type of cells or the organ from which they originate. All human tissues are susceptible to this abnormal hyper-proliferation^{3,34}, therefore more than 100 different types of cancers have been identified so far.

Cancer risk factors can be categories in two groups: the individual risk related to genetics and the environmental factors. Initially, cancer was thought to be originated only by heritable (genetic) causes, however, only 5 – 10 % of all cancers are related to highly penetrant inherited mutations³³. Consequently, 90 to 95% of cancers are linked to human lifestyle and environmental factors. This idea was first proposed in 1950 during a symposium organised by The World Health Organization (WHO). During the symposium, scientific evidences showed that people who migrated from one country to another, develop types of cancer common in their adopted countries, rather than the ones from their original countries. It was the first indication that most of the cancers are due to environmental exposures and not to inherited genetic factors. This symposium also contributed to the creation of the International Agency for Research on Cancer (IARC) in 1965 with the mission to investigate, with a multidisciplinary approach, the causes of human cancers².

As shown in Figure 6, infections are an important risk factor and in developing countries the fraction of malignancies linked to infections is even higher. For example, almost 30% of cancers can be attributed to infections in sub-Saharan Africa and most of them are related to viruses³⁶.

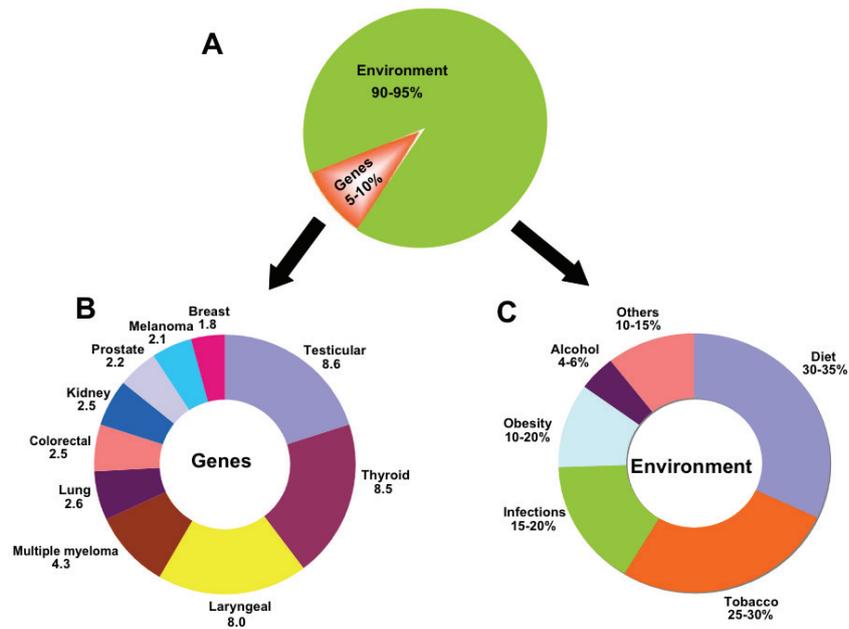


Figure 6. **The role of genes and environment in the development of cancer** (from Anand P. et al. 2008): " (a) The percentage contribution of genetic and environmental factors to cancer. The contribution of genetic factors and environmental factors towards cancer risk is 5–10% and 90–95% respectively. (b) Family risk ratios for selected cancers. The numbers represent familial risk ratios, defined as the risk to a given type of relative of an affected individual divided by the population prevalence. The data shown here is taken from a study conducted in Utah to determine the frequency of cancer in the first-degree relatives (parents + siblings + offspring). The familial risk ratios were assessed as the ratio of the observed number of cancer cases among the first degree relatives divided by the expected number derived from the control relatives, based on the years of birth (cohort) of the case relatives. In essence, this provides an age-adjusted risk ratio to first-degree relatives of cases compared with the general population. (c) Percentage contribution of each environmental factor. The percentages represented here indicate the attributable-fraction of cancer deaths due to the specified environmental risk factor."³⁷

a. Epigenetic and Cancer

For a long time, we have considered cancer as a primarily genetic disease, with inherited or spontaneous mutations that induce abnormal cellular functions. Now, it is well accepted that epigenetic changes, stable and mitotically heritable changes in gene expression that do not involve changes in DNA sequence itself, could play an important role in cancer development and progression³⁸.

To understand the role of epigenetic in cancer, we have to first understand how it works.

The epigenetic define all the heritable changes in gene expression that are not caused by a change in the DNA sequence³⁹. Epigenetic modifications are reversible events, in contrast with

other genetic modifications in DNA, like point mutations. However, similarly to genetic modifications, epigenetic modifications can be influenced by environmental factors⁴⁰.

Epigenetic mechanisms are indispensable for mammalian cells, as they are involved in different processes, from the embryonic development to initiation and maintenance of differentiation, without forgetting the X chromosome inactivation and the silencing of transposable elements^{41,42}. Moreover, thanks to the epigenetic modifications, stem cells can generate different cell types from the same DNA sequence.

Epigenetic modifications can be grouped into three different categories: chromatin modifications, RNA-interference and DNA methylation (Figure 7).

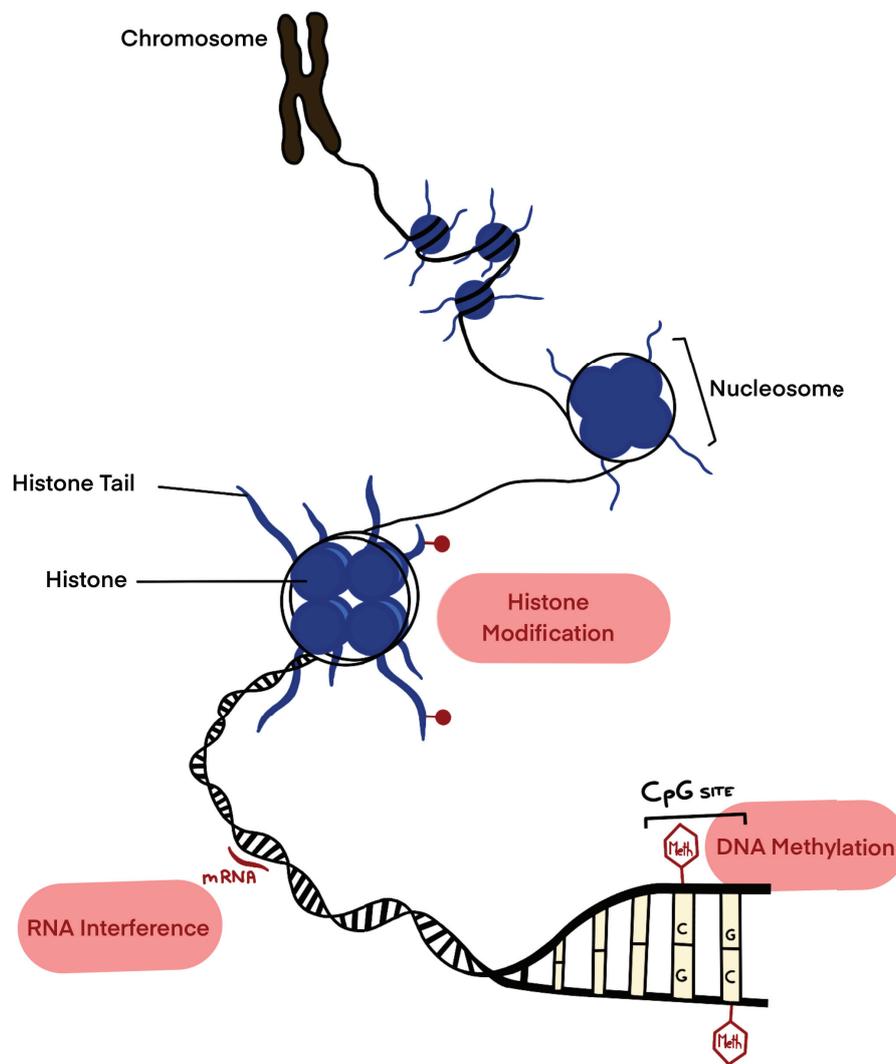


Figure 7. **Overview of the different epigenetic mechanisms:** three different mechanisms of epigenetic regulation with the histone modification, RNA-mediated gene silencing and the DNA methylation (adapted from Sawan, Vaissiere et al. 2008).

- **Histone modifications.** Inside the nucleus, the chromatin is found in two different forms: the heterochromatin and the euchromatin. The heterochromatin is the condensed form, and in this state, the DNA is not accessible. The heterochromatin can be found mainly during mitosis and meiosis. The euchromatin is the looser decondensed form, which allows the access of the cellular machineries to the DNA.

The chromatin is composed of nucleosomes, composed of octamer of histone proteins and two turns of genomic DNA (each 147 base pairs). The amino-terminal portion of the histone tail is conserved between various species and is subject to various post-translational modifications. These modifications affect the DNA-nucleosome and nucleosome-nucleosome interaction which modify the accessibility of the DNA leading to variation in gene expression.

- **RNA Interference:** The microRNAs (miRNAs) are non-coding RNAs, and they are able to regulate the expression of a lot of genes by targeting them. Different genes are targeted by miRNAs, such as genes involved in cell proliferation, maintenance and differentiation. It has been shown that some miRNAs are indispensable for the development of some mammalian organs⁴³.

- **DNA Methylation:** DNA methylation is the epigenetic mechanism that has been studied the most in relation to gene regulation. It refers to covalent modification of the DNA by attachment of a methyl group to a cytosine base in a CpG sequence by a DNA methyltransferase. When a cytosine is followed by a guanine residue, they form a CpG and the methyl group can be added at the position 5 on the cytosine⁴⁴ (Figure 8). Some regions, called CpG islands, are rich in CpG sites; they are often found close to gene promoters and typically remain methylated for genes that are expressed. In fact the DNA methylation of a promoter region is often associated with gene silencing⁴⁵. Moreover, approximately 60% of transcriptional start site coincide with CpG islands. For these reasons, DNA methylation is considered as a mechanism of transcriptional regulation and it is involved in diverse cellular function such as X chromosome inactivation,

cell differentiation, tissue-specific gene expression and others⁴². Consistent with the role of DNA methylation in cell differentiation dynamic regulation of methylation patterns have been shown to be critical for embryonic development^{46,47}.

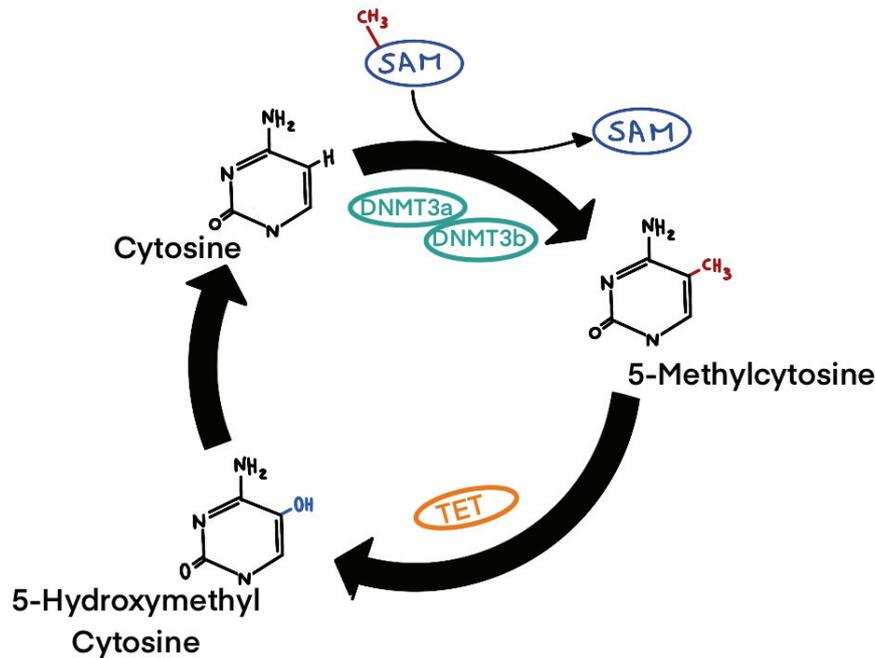


Figure 8. **Cytosine methylation/demethylation process** (Adapted from Genvan Institute VIDEO 2015 “DNA Methylation and Cancer”)

There are three enzymes, called DNA methyltransferases (DNMTs), responsible for the DNA methylation. Among these three DNMTs, two are involved in the *de novo* methylation of CpGs: DNMT3a and DNMT3b, whereas DNMT1 has a high affinity for semi-methylated DNA and is considered as a maintenance enzyme, able to restore the methylation pattern after DNA replication⁴⁸ (Figure 9).

The DNA methylation is a dynamic and reversible mechanism, which implies the existence of a demethylation process. The demethylation can occur in two ways: either by a passive demethylation *via* the absence of activity of DNMT1 after the DNA replication, which dilutes the CpGs methylation from the cell after several divisions. The second way is through the Ten-Eleven Translocation (TET) proteins which can actively demethylate CpG sites by converting

the 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) through oxidation. The TET family is composed by three different proteins: TET1-3⁴⁸.

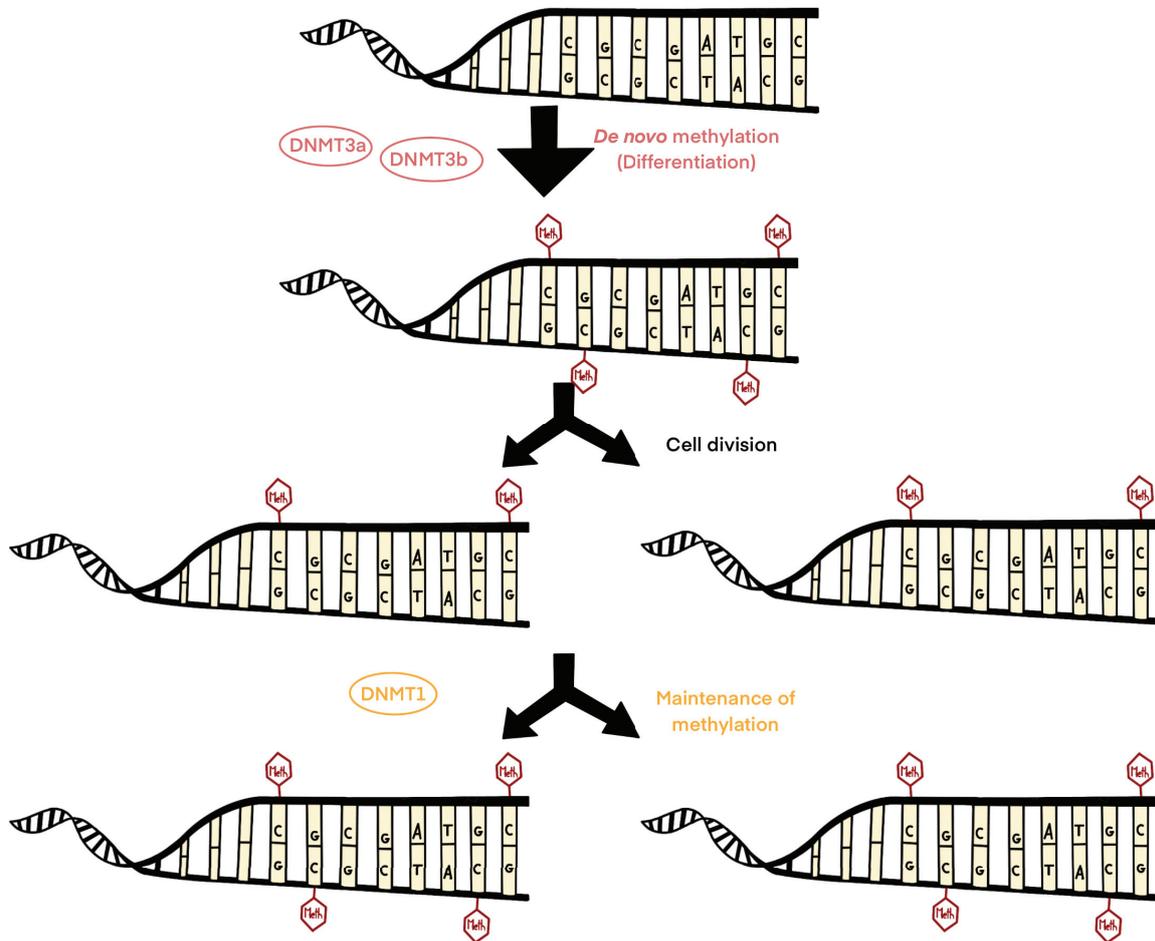


Figure 9. **De novo methylation and maintenance of DNA methylation:** De novo methylation of the unmethylated DNA (on top) by DNMT3a and DNMT3b, here represent the cell differentiation. Going down, after a cell division there is the maintenance of methylation by DNMT1 by symmetry but does not methylate unmodified CpGs.

b. Role of epigenetic regulation in Epstein-Barr Virus associated cancer:

At the present time, the implication of EBV in cancer is well-known even if all of the mechanisms are not well understood. More and more studies are focused on the involvement of the epigenetic regulation in EBV associated cancers since, as previously mentioned, the epigenetic modifications lead to an up or down-regulation of genes, and they play a role in the development of human cancers. Several studies showed how EBV can hijack epigenetic machineries to work to its advantage. In his review, Martin J Allday shows that EBV, through EBNA3C and EBNA3A, interacts with the cellular polycomb protein system to avoid the activation of the cellular defence mechanism called Oncogenic Stress Response⁴⁹. Our previous work is another example of how EBV deregulate gene expression through epigenetic modification. We showed that KDM2B is epigenetically silenced in EBV + BL cell lines ⁵⁰.

c. Lymphomas:

Lymphomas are the most common lymphoid malignancies; they originate from transformed lymphocytes. Lymphomas can be divided in two group : Hodgkin Lymphomas (HL) and Non-Hodgkin Lymphomas (NHL), with higher incidence for the NHL at 80 to 85% of all the lymphomas against 10 to 15% for the HL⁵¹. The HL and the NHL have different behaviours and treatments response. For this reason, it is important to find hallmarks corresponding to the type of lymphoma the patient is facing to choose the appropriate treatment. The epigenetic deregulation plays an important role in lymphoid malignancies. There are more and more articles in the literature talking about epigenetic variation/signatures corresponding to lymphoma's type and genetic modification of some epigenetic genes^{51,52,53,54,55,56,57}.

d. Hodgkin Lymphomas (HL)

The lymphoma is a type of cancer that originates in white blood cells (lymphocytes). Thomas at King (1798 – 1866) described the HL for the first time in 1832, however, it is from 1865 that the disease took the name of Hodgkin lymphoma⁵⁸.

The HLs are characterized by the presence of Reed-Sternberg cells, giant multinucleated cells, in an inflammatory background. The age range is between 20 and 34 years, and the HL has a good response to treatment. HL is divided into two subtypes based on morphology and immunochemistry: classical Hodgkin Lymphomas and the nodular lymphocyte predominant Hodgkin Lymphomas⁵⁹.

e. Non-Hodgkin Lymphomas (NHL)

The other type of lymphomas is the NHL. The NHLs are a heterogeneous group of malignancies including more than 50 different diseases derived from B-cells, T-cells or Natural Killer cells. Approximately 4% of cancers cases for children aged between 0 to 14 years are due to NHL⁶⁰. In 2018 it was the cause of 2,6% of all cancers deaths (248 700 deaths) and it is the 13th most common cancer type worldwide³³.

So far, the pathologic diagnosis of lymphoma is based on the World Health Organization classification of lymphoid neoplasms, which includes 4 main categories: precursor B and T-cells neoplasms, mature B-cell neoplasms, mature T/NK-cell neoplasms and immunodeficiency-associated lymphoproliferative disorders, see Table 4⁶¹. As for the HLs, a reliable diagnosis is critical to define the appropriate treatment. Several studies are focusing on different subjects to improve the current classification. One of the subjects is the epigenetic, a heritable change that can modulate the gene expression and the chromatin organization. Some studies identified aberrant gene methylation, as a specific marker of some NHL subtypes (for example in the studies of Hernandez-Vargas H *et al.* 2017⁶²).

Table 4. Lymphoma is based on the World Health Organization classification of lymphoid neoplasms (from Non-Hodgkin Lymphoma: Diagnosis and Treatment Stephen M. Ansel⁶¹)

Lymphomas based on the world Health Organization classification of lymphoid neoplasms
Precursor B- and T-cell neoplasms
Precursor B-cell lymphoblastic leukemia/lymphoma
Precursor T-cell lymphoblastic leukemia/lymphoma
Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
Lymphoplasmacytic lymphoma
Plasma cell neoplasms
Splenic marginal zone lymphoma
Extranodal marginal zone lymphoma
Nodal marginal zone lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Primary mediastinal large B-cell lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Hodgkin lymphoma
Mature T- and natural killerecell neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Adult T-cell leukemia/lymphoma
Extranodal natural killere/T-cell lymphoma, nasal type
Enteropathy-type T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitislike T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30. T-cell lymphoproliferative disorders
Peripheral T-cell lymphoma, not otherwise specified
Angioimmunoblastic T-cell lymphoma
Anaplastic large-cell lymphoma, anaplastic lymphoma kinasepositive
Anaplastic large-cell lymphoma, anaplastic lymphoma kinaseegative
Immunodeficiency-associated lymphoproliferative disorders
Lymphoproliferative diseases associated with primary immune disorders
Lymphomas associated with human immunodeficiency virus infection
Posttransplant lymphoproliferative disorders
Data from WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues

i. Burkitt's Lymphoma

Among the NHL there is the Burkitt Lymphoma (BL). The BL is an aggressive rapidly growing B-cell NHL. There are three different subtypes of Burkitt's Lymphoma: sporadic, endemic and immunodeficiency associated. In all three BLs there is a chromosome translocation of the MYC proto-oncogene on chromosome 8. Most frequently MYC is translocated into an immunoglobulin (Ig) heavy chain locus on chromosome 14. Although it is also possible to find him translocated into the Ig light chain loci, the κ locus on chromosome 2 or the λ locus on chromosome 22. The result of all of these translocations is the same with an MYC protein over-expression which drive to cell proliferation.

For each subtype of BL different epidemiology, risk factors and clinical presentations/conditions are involved^{63,64,23}.

1. Sporadic

The Sporadic variant (sBL) of the BL is principally found/observed in developed countries (outside Africa/in western countries) and rarely associated with EBV infection. The sporadic BL is more common as paediatric cancer (30% of paediatric lymphomas)^{65,33}.

2. Endemic

The endemic variant (eBL) is mainly found in Africa (equatorial Africa and Papua New Guinea) and it is associated with EBV infection in > 90% of the cases. The geographical localisation coincides with the presence of *plasmodium falciparum* (malaria) (Figure 10)^{66,64}.

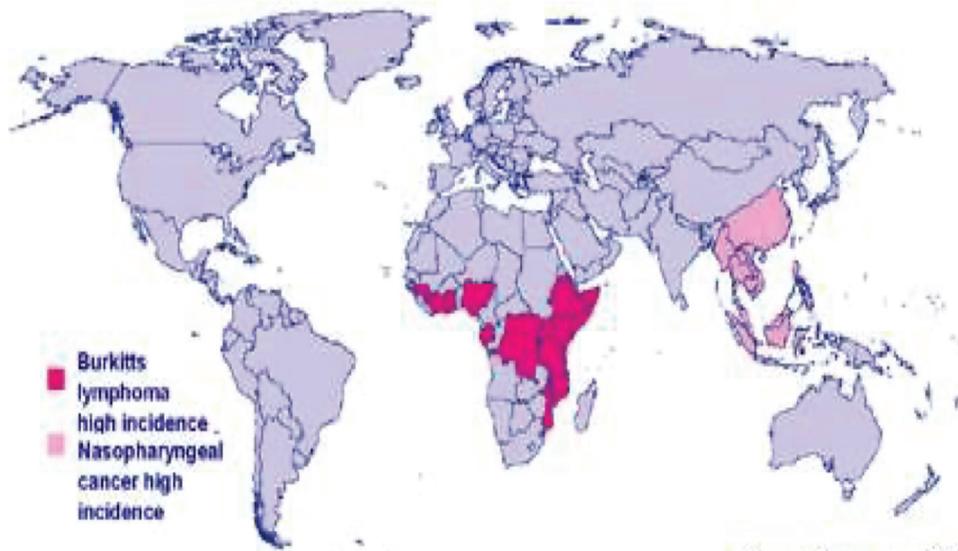


Figure 10. Epstein-Barr virus-associated Burkitt's Lymphoma & Nasopharyngeal Carcinoma (from Okano, M 1988)

But even if the hypothesis of the implication of malarian infections to contribute to the development of eBL is well accepted, its mechanism and interaction with EBV are not well understood. Malarian infections are not the only potential environmental factor involved in the specific geographical distribution of the eBL. For example the carcinogenic dietary contaminants, the aflatoxins, which are predominant in this area⁶⁷, are known to cooperate with viruses in the development of diseases⁶⁸.

News studies, like the one of Vargas et al. 2017⁶⁹, are identifying epigenetic hallmarks of the eBL which can help to understand the mechanism and interaction with EBV in the eBL^{65,33}.

3. Immunodeficiency related

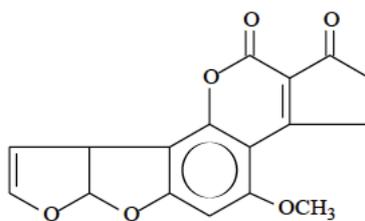
The immunodeficiency associated Burkitt lymphoma is most commonly found in patient with HIV infections. EBV is identified in only 30 – 50 % of immunodeficiency-related Burkitt lymphomas^{33,65}.

III. Environmental factors

As previously described, cancer is not mainly induced by heritable mutations³³. Identifying environmental factors that are carcinogenic to human and their mechanisms of actions is important, as it can help to develop strategies for cancer prevention and treatment. Around half of all newly diagnosed cancer cases in Europe are potentially avoidable⁷⁰. As defined in the Dictionary of environmental and sustainable Development (Gilpin, A 1996. Dictionary of environmental and sustainable Development John Wiley and Son. 247p): “An environmental factor is any factor, abiotic (temperature, amount of sunlight, pH...) or biotic (include the availability of food organisms and the presence of biological specificity, competitors, predators and parasites), that influence living organisms”.

a. Aflatoxin

Dietary contaminants are part of the environmental factors concerning people all over the world. One of the well-known contaminants is the aflatoxin. The aflatoxin is a mycotoxin produced mainly by two fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, occurring naturally in improperly stored products. It has been first identified in 1961 in animal feed responsible for the deaths of 100 000 turkeys in the United Kingdom. Since the 1970s, it is classified as carcinogenic to human (group 1) in Monographs volume 56 (IARC – WHO)⁷¹. Aflatoxin has been often studied as a human carcinogen. Aflatoxin contamination is mostly detected in tropical region, nevertheless, the movement of agricultural commodities around the globe increases the presence of aflatoxins also in the temperate zone of the world. There are 14 different aflatoxins produces in nature, AFB1, AFB2, AFG1 and AFG2 are the one that concern the most the economy and the public health because of their incidence and toxicities. In particular, AFB1 (Figure 11) is the most studied one, as it is known to have the highest carcinogenic effect.



B₁: C₁₇H₁₂O

Mol. wt: 312.3

Figure 11. **Aflatoxin B1** (from IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS 2002)

b. AFB1 and cancer

It has been shown that an exposure to the AFB1 and HBV is the main risk for the development of chronic liver disease and hepatocellular carcinoma (HCC). Moreover, AFB1 favours transformation of hepatocytes by inducing a specific mutation at codon 249 of TP53 (p.R249S) (Chemin I et al. 2012) ^{72,68}.

In countries with a high incidence of HBV, HCC is often associated with exposure to aflatoxin. The HBV isn't the only virus with which the AFB1 interacts. Accardi et al. showed that AFB1 can cooperate with EBV for the *in vivo* and *in vitro* transformation of B-cells⁷³.

Recent studies have shown that the AFB1 can have an effect on the methylome. Vargas et al. showed that an exposure to the aflatoxin *in utero* from contaminated food consumption of the mother results in differential DNA methylation patterns in infants. However, the mechanism underlying the synergy between AFB1 and EBV in the eBL development has not yet been investigated.

IV. AIMS OF THE THESIS

Previous epidemiological and experimental studies have strongly implicated biological agents in various human malignancies. IARC has classified several viral and bacterial agents (such as EBV) as a Group 1 carcinogen in humans (IARC-Monograph, 2012). Different biological agents (directly and/or the inflammatory process that they trigger) were proposed to promote the development of several cancer types by inducing deregulation of the epigenome (Herceg et al., 2018, Herceg & Paliwal, 2011, Lambert, Paliwal et al., 2011). However, the exact mechanisms by which these agents promote carcinogenesis are still poorly understood. The picture is further complicated by the fact that some of the cancers (such as EBV-associated malignancies) appear to be associated with a geographical localisation or populations, underlying the involvement of environmental co-factors in their pathogenesis. Although several studies have conducted DNA methylation analysis in normal tissue and tumours associated with viruses and other biological agents using candidate gene or genome-wide approaches, these studies tend to be limited in sample size and DNA methylome coverage. Furthermore, the associations identified through molecular epidemiology studies have not been followed up by mechanistic studies aimed at providing biological plausibility. To address these limitations and take advantage of our strong expertise in epigenomics and mechanisms of carcinogenesis associated with infectious agents, this thesis aimed to provide insights into epigenetic mechanisms underlying the cooperation between EBV and AFB1 in the development of BL.

Specific aims of this thesis are:

- To investigate whether epigenetic regulator KDM2B plays a role in Burkitt's Lymphoma development and gain better understanding of epigenetic mechanisms by which EBV can promote this process.
- Understand the interaction of the Aflatoxin B1 with the virus in promoting oncogenic transformation and cancer development.
- Characterize epigenetic biomarkers associated to an increased risk of cancers development.

RESULTS

Interplay between the Epigenetic Enzyme Lysine (K)-Specific Demethylase 2B and Epstein-Barr Virus Infection

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ABSTRACT The histone modifier lysine (K)-specific demethylase 2B (KDM2B) plays a role in the differentiation of hematopoietic cells, and its expression appears to be deregulated in certain cancers of hematological and lymphoid origins. We have previously found that the *KDM2B* gene is differentially methylated in cell lines derived from Epstein-Barr virus (EBV)-associated endemic Burkitt lymphoma (eBL) compared with that in EBV-negative sporadic Burkitt lymphoma-derived cells. However, whether KDM2B plays a role in eBL development has not been previously investigated. Oncogenic viruses have been shown to hijack the host cell epigenome to complete their life cycle and to promote the transformation process by perturbing cell chromatin organization. Here, we investigated whether EBV alters KDM2B levels to enable its life cycle and promote B-cell transformation. We show that infection of B cells with EBV leads to downregulation of KDM2B levels. We also show that LMP1, one of the main EBV transforming proteins, induces increased DNMT1 recruitment to the *KDM2B* gene and augments its methylation. By altering KDM2B levels and performing chromatin immunoprecipitation in EBV-infected B cells, we show that KDM2B is recruited to the EBV gene promoters and inhibits their expression. Furthermore, forced KDM2B expression in immortalized B cells led to altered mRNA levels of some differentiation-related genes. Our data show that EBV deregulates KDM2B levels through an epigenetic mechanism and provide evidence for a role of KDM2B in regulating virus and host cell gene expression, warranting further investigations to assess the role of KDM2B in the process of EBV-mediated lymphomagenesis.

IMPORTANCE In Africa, Epstein-Barr virus infection is associated with endemic Burkitt lymphoma, a pediatric cancer. The molecular events leading to its development are poorly understood compared with those leading to sporadic Burkitt lymphoma. In a previous study, by analyzing the DNA methylation changes in endemic compared with sporadic Burkitt lymphoma cell lines, we identified several differential methylated genomic positions in the proximity of genes with a potential role in cancer, and among them was the *KDM2B* gene. *KDM2B* encodes a histone H3 demethylase already shown to be involved in some hematological disorders. However, whether KDM2B plays a role in the development of Epstein-Barr virus-mediated lymphoma has not been investigated before. In this study, we show that Epstein-Barr virus deregulates KDM2B expression and describe the underlying mechanisms. We

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also reveal a role of the demethylase in controlling viral and B-cell gene expression, thus highlighting a novel interaction between the virus and the cellular epigenome.

KEYWORDS Burkitt lymphomas, EBV, epigenetic, KDM2B

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects more than 95% of the adult population worldwide. After infection, EBV establishes a lifelong latency, often with no adverse health consequences. Despite its ubiquity, EBV infection is also associated with many human cancer types, among which is endemic Burkitt lymphoma (eBL), the most common childhood cancer in equatorial Africa (1). Although this malignancy was associated with EBV infection more than 50 years ago, the exact mechanism by which the virus contributes to the eBL pathogenic process is still not fully understood.

Many studies have highlighted a key role of epigenetic deregulations in cell transformation and cancer development. Increasing evidence indicates that different viruses may abrogate cellular defense systems by hijacking epigenetic mechanisms to deregulate the host cell gene expression program and modulate their own life cycle (2, 3). Our recent study of the methylome profiles of sporadic Burkitt lymphoma (sBL)- versus eBL-derived cell lines revealed an EBV infection-specific pattern of methylation, with aberrant methylation being detected in genes with a known role in lymphomagenesis, such as *ID3*, which is often found to be mutated in sBL (4). We therefore hypothesized that a virus-driven mechanism is responsible for modifying the epigenome of B cells to facilitate the lymphomagenic process, circumventing the need for mutations in lymphoma driver genes. Among the genes differentially methylated in eBL compared with sBL, we identified the lysine (K)-specific demethylase 2B (*KDM2B*) gene, which encodes a histone H3 demethylase known to target specific sites, such as trimethylated lysine 4 (H3K4me3) and dimethylated lysine 36 (H3K36me2). *KDM2B* sets the stage for DNA methylation and gene silencing by recruiting polycomb-1 proteins to unmethylated CpG regions (5) and plays a key role in somatic cell reprogramming (6). It also represses the transcription of rRNA genes, thus inhibiting cell growth and proliferation (7). *KDM2B* has been identified as a putative tumor suppressor by retroviral insertion analysis in mice (8). Low levels of *KDM2B* expression have been found in aggressive brain tumors, suggesting its potential role in cancer development. Moreover, *KDM2B* is involved in hematopoietic cell development and plays opposite roles in tumors of hematopoietic and lymphoid origins (9). Although high levels of *KDM2B* expression have been observed in different hematological malignancies, its depletion from hematopoietic cells has been reported to activate the cell cycle and reduce the activity of interferon and lymphoid-specific transcription factors, thereby contributing to myeloid transformation (9). However, whether *KDM2B* affects the EBV life cycle has not been determined, and its role in eBL has not been assessed. Here, using *in vitro* EBV infection models, we aimed to assess whether EBV can alter the expression of *KDM2B* by inducing methylation of its gene. Finally, we investigated how this event affects EBV infection and B-cell homeostasis. Overall, our data highlight a novel cross talk between EBV and the cellular epigenome and identify *KDM2B* to be a master regulator of EBV gene expression, in addition to B-cell gene expression, suggesting a role for EBV-mediated *KDM2B* deregulation in the lymphomagenic process.

(This article was submitted to an online preprint archive [10].)

RESULTS

***KDM2B* is epigenetically silenced in EBV(+) BL-derived cell lines.** Our previous comparative analysis of the whole-genome methylation profiles of a set of EBV-positive [EBV(+)] and EBV-negative [EBV(-)] Burkitt lymphoma (BL)-derived cell lines (4) led to the identification of two CpGs (CpG15695155 and CpG21423404) flanking a CpG island named CpG127 (Fig. 1A) in an intragenic putative regulatory region of *KDM2B* (as shown by the accumulation of the H3K27 acetylation [H3K27Ac] marker) (Fig. 1A). CpG15695155 and CpG21423404 were highly methylated in EBV(+) BL-derived cells

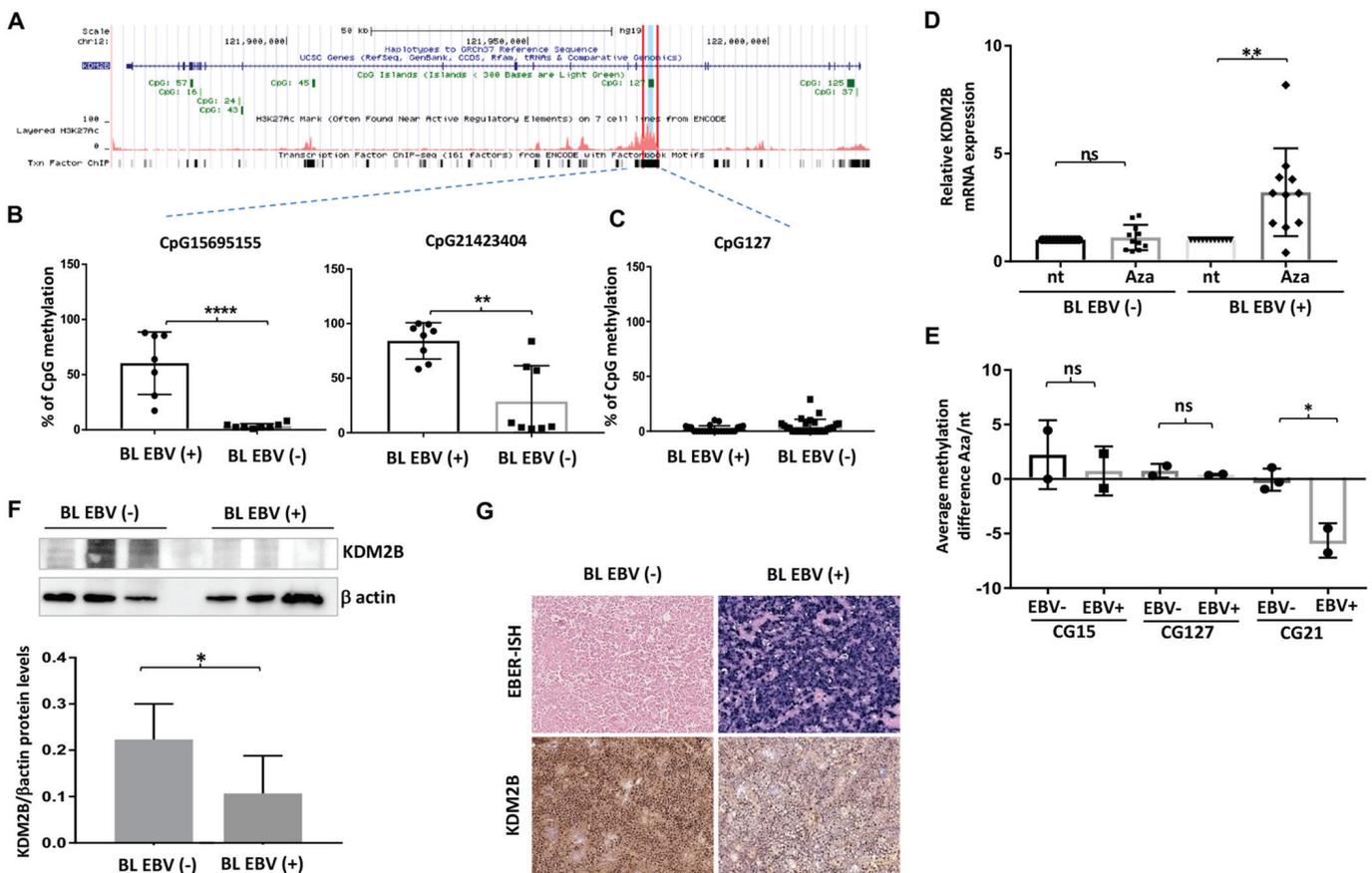


FIG 1 The *KDM2B* gene is methylated and silenced in EBV(+) BL cell lines and specimens. (A) Schematic diagram of the *KDM2B* gene (modified from the UCSC Genome Browser). Red lines show CpG15695155 and CpG21423404, and CpG island 127 is in light blue. ChIP data (obtained with the lymphoma cell line GM12878) for the distribution of the H3K27Ac marker within the selected region are also shown. ChIP, chromatin immunoprecipitation. (B) The histograms show the average percentage of methylation measured by pyrosequencing of CpG15695155 and CpG21423404 in the DNA of 10 EBV(+) and 9 EBV(-) BL cell lines (****, $P < 0.0001$; **, $P < 0.01$). (C) The histogram shows the average percentage of methylation at 17 positions within CpG island 127 measured in 2 EBV(+) and 2 EBV(-) BL cell lines. The difference between the two groups was not significant. (D) Three EBV(+) and 3 EBV(-) BL cell lines were cultured in the presence of dimethyl sulfoxide (DMSO; nontreated [nt]) or 5-aza-2'-deoxycytidine (Aza; 10 μ M) for 48 h. The *KDM2B* mRNA expression level was evaluated by RT-qPCR. The pooled results of 4 independent experiments are presented in the histogram (**, $P < 0.01$; ns, not significant). *KDM2B* mRNA levels in Aza-treated cells were measured relative to the levels in DMSO-treated control cells. (E) Aza-treated EBV(-) and EBV(+) BL cells from 2 independent experiments were processed for DNA extraction and analyzed by pyrosequencing for the methylation level at CpG15695155 (CG15) and CpG21423404 (CG21) as well as for the average methylation at 17 positions within CpG island 127 (CG127). The average difference in the percentage of methylation between Aza- and DMSO-treated cells is indicated in the histogram (*, $P < 0.05$; ns, not significant). (F) Three EBV(+) and 3 EBV(-) BL cell lines were cultured and analyzed by immunoblotting for *KDM2B* expression levels. The histogram shows the average *KDM2B* expression levels normalized to the β -actin signal, measured in 4 independent experiments by Image Lab software (Bio-Rad) in EBV(+) versus EBV(-) BL cells (*, $P < 0.05$). (G) *KDM2B* levels in EBV(+) and EBV(-) BL samples were analyzed by immunohistochemistry. The same samples were analyzed for EBER expression by ISH for EBER (EBER-ISH) as described in Materials and Methods. The images shown are representative of the *KDM2B* staining obtained in 11 EBV(+) and 11 EBV(-) BL specimens.

compared with EBV(-) BL-derived cells. Here, to validate these data we performed direct pyrosequencing on DNA extracted from 10 EBV(+) BL-derived cell lines and 9 EBV(-) BL-derived cell lines (Table 1). The samples for which the pyrosequencing gave results technically suitable for analysis are displayed in the histogram in Fig. 1B. Pyrosequencing analysis confirmed that the *KDM2B* gene is hypermethylated at CpG15695155 and CpG21423404 in EBV(+) BL cell lines compared with EBV(-) BL cell lines (Fig. 1B). In contrast, we did not observe high methylation levels or differences between EBV(+) and EBV(-) BL cell lines when analyzing 17 positions within the CpG island 127 (Fig. 1C). Next, we assessed whether the high DNA methylation level of the *KDM2B* gene would affect its expression level. Treatment of 3 EBV(-) BL and 3 EBV(+) BL cell lines with the demethylating agent 5-aza-2'-deoxycytidine (Aza) for 48 h led to a significant rescue of *KDM2B* expression in EBV(+) BL cells, whereas this treatment had no noticeable effect on *KDM2B* mRNA expression in EBV(-) BL cells (Fig. 1D). Pyrosequencing analysis of DNA from EBV(+) and EBV(-) BL cell lines exposed to Aza or to

TABLE 1 Description of BL-derived cell lines used in the present study^a

BL case identifier	Diagnosis	EBV infection	Cytogenetic information	Clinical data	Ethnic origin
BL103	BL	EBV(-)	t(8;14)		Caucasian
BL70	BL	EBV(-)	t(8;14)	Dec	Caucasian
BL56	BL	EBV(-)	t(8;14)	Dec	
BL58	BL	EBV(-)	t(8;14)	Dec	Caucasian
BL53	BL	EBV(-)	t(8;14)	Dec	Caucasian
BL102	BL	EBV(-)	t(8;22)	Dec	Caucasian
BL104	BL	EBV(-)	t(8;22)		Caucasian
BL2	BL	EBV(-)	t(8;22)	Dec	Caucasian
BL41	BL	EBV(-)	t(8;14)		Caucasian
BL110	BL	EBV(+)	t(8;14)		Caucasian
BL135	BL	EBV(+)	t(8;14)		African
BL65	BL	EBV(+)	t(8;14)		African
BL116	BL	EBV(+)	t(8;14)		African
BL60	BL	EBV(+)	t(8;22)	Dec	African
BL79	BL	EBV(+)	t(8;14)		African
BL112	BL	EBV(+)	t(8;14)		Caucasian
I100		EBV(+)			
I373		EBV(+)			
I176B		EBV(+)			

^aThe cells were obtained from the IARC Biobank. Abbreviations: BL, Burkitt lymphoma; EBV, Epstein-Barr virus; Dec, deceased.

dimethyl sulfoxide (DMSO) for 48 h revealed a moderate but significant reduction in the methylation level at CpG21423404 in Aza-exposed EBV(+) BL cells, whereas methylation at all the other positions analyzed remained unchanged (Fig. 1E). We then determined whether the different methylation patterns observed in EBV(+) and EBV(-) BL cells affected KDM2B protein expression. We analyzed KDM2B protein expression in 3 EBV(+) and 3 EBV(-) BL-derived cell lines and observed a significantly lower expression level of KDM2B protein in EBV-infected cells (Fig. 1F). Moreover, we analyzed 11 EBV(+) BL and 11 EBV(-) BL samples (Table 2) by immunohistochemistry for the KDM2B protein expression level; 8 of the 11 EBV(+) BL samples showed weak KDM2B staining, and 10 of the 11 EBV(-) BL samples showed a strong signal for KDM2B immunohistochemistry, suggesting that EBV infection induces reduced expression of

TABLE 2 BL case main features^a

BL subtype	Age (yr)	Sex	Site of biopsy	Detection of the following:			
				EBER	MYC by FISH B.A.	MYC-IGH by FISH	MYC-IGK or IGL by FISH
sBL	40	F	Inguinal lymph node	-	+	n.p.	n.p.
sBL	18	M	Ileum	-	+	n.p.	n.p.
sBL	38	F	Lymph node	-	+	n.p.	n.p.
sBL	40	F	Bone marrow	-	+	n.p.	n.p.
sBL	20	M	Lymph node	-	-	-	n.p.
sBL	15	M	Lymph node	-	+	n.p.	n.p.
sBL	25	F	Ileum	-	+	n.p.	n.p.
sBL	45	M	Stomach	-	+	+	n.p.
sBL	16	M	Abdomen mass	-	+	n.p.	n.p.
sBL	20	M	Lymph node	-	-	-	n.p.
sBL	14	M	Lymph node	+	+	n.p.	n.p.
eBL	9	F	Abdomen mass	+	+	n.p.	n.p.
eBL	5	F	Soft tissues	+	+	n.p.	n.p.
eBL	6	F	Maxilla	+	+	n.p.	n.p.
eBL	10	F	Ileum	+	+	n.p.	n.p.
eBL	3	F	Lymph node	+	+	n.p.	n.p.
eBL	4	F	Lymph node	+	+	+	n.p.
eBL	10	M	Maxilla	+	+	-	+ (IGL), - (IGK)
eBL	7	M	Abdomen	-	+	n.p.	n.p.
eBL	12	F	Maxilla	-	+	n.p.	n.p.
eBL	3	M	Stomach	-	+	n.p.	n.p.
eBL	5	M	Ileum	-	+	n.p.	n.p.

^aAbbreviations: F, female; M, male; n.p., not performed; EBER, Epstein-Barr virus-encoded small RNA; B.A., break apart; eBL, endemic Burkitt lymphoma; sBL, sporadic Burkitt lymphoma, FISH, fluorescence *in situ* hybridization.

the KDM2B protein *in vivo* (Fig. 1G). Of note, the EBV(+) samples with stronger KDM2B staining had fewer Epstein-Barr virus-encoded small RNA (EBER)-expressing cells, as determined by EBER *in situ* hybridization (ISH) (data not shown). In conclusion, these data show that two specific CpG sites in the regulatory region of *KDM2B* are hypermethylated in EBV(+) BL cell lines compared with EBV(-) BL cell lines, confirming our previous whole-genome methylation profiling data (4). Moreover, KDM2B expression also appears to be reduced in eBL specimens, which is probably mediated by DNA methylation at CpG21423404. These data suggest that EBV may regulate KDM2B expression by inducing the methylation of a specific position within a regulatory region of its gene.

EBV infection of B cells downregulates KDM2B expression. To assess the ability of EBV to deregulate KDM2B expression, primary B cells isolated from 3 independent donors were infected with EBV. Cells were collected at different times after infection and analyzed for the expression level of KDM2B mRNA by reverse transcription-quantitative PCR (RT-qPCR). It appeared that soon after infection, the expression level of KDM2B was drastically reduced (Fig. 2A). This downregulation was also seen at the protein level (Fig. 2B). The reduced expression of KDM2B was maintained during the immortalization process and stayed low in lymphoblastoid cell lines (LCL). This result suggests that EBV infection plays a role in the regulation of KDM2B mRNA expression. To exclude the possibility that this result could be due to the activation of the primary B cells independently of EBV infection, as a consequence of the engagement of the membrane B-cell receptors by the virus, we stimulated primary B cells from 2 independent healthy donors with CD40 ligand (CD40L) and interleukin 4 (IL-4) with or without EBV. Analysis of the EBNA1 expression by RT-qPCR showed that the infection worked efficiently (Fig. 2C). Both treatment with CD40L and IL-4 and infection with EBV similarly activated the B cells, as shown by the strong induction of expression of CCL22 (Fig. 2D), a cytokine that is known to be produced during primary B-cell activation and EBV infection (11). Importantly, significant downregulation of KDM2B mRNA expression was observed only when the cells were infected with EBV (Fig. 2E). Moreover, infecting the primary B cells with UV-inactivated EBV led to reduced downregulation of KDM2B compared with that in the cells infected with the untreated virus (Fig. 2F), further indicating that this event is independent of B-cell activation and requires active expression of the viral genes. To assess whether downregulation of KDM2B depends on differences between the proliferation status of the cells, we measured KDM2B expression in Akata2000 cells, an EBV(+) BL-derived cell line, and in Akata31 cells, which were derived by expansion of a clone of Akata2000 cells that had lost the virus. Analysis of the viral DNA in both cell lines confirmed that Akata31 cells had few or no EBV genomes compared with Akata2000 cells (Fig. 2G). Interestingly, Akata2000 cells displayed very low levels of KDM2B mRNA compared with Akata31 cells, indicating that KDM2B expression varies according to the amount of virus (Fig. 2G) and independently of the cell type. Similarly, reactivation of EBV by exposing Raji cells to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (NaB) treatment led to reduced expression of KDM2B (Fig. 2H). Finally, at 48 h after EBV infection, KDM2B mRNA and protein expression levels were reduced in RPMI-8226 (RPMI) cells (Fig. 2I), confirming the results obtained in EBV-infected primary B cells and further proving that downregulation of KDM2B expression is a direct effect of the virus and not a side effect of the activation of the cells or of the immortalization process.

To determine whether the reduced expression of KDM2B in EBV-immortalized cells could be due to an increase in DNA methylation, LCL were treated with the demethylating agent Aza and analyzed by RT-qPCR for the KDM2B expression level. Aza-treated LCL showed increased expression of KDM2B mRNA compared with their untreated counterparts, indicating that downregulation of KDM2B in EBV-infected cells is mediated by DNA methylation (Fig. 3A). Because incorporation of Aza into DNA impedes its methylation by DNA methyltransferases (DNMTs) (12), we hypothesized that EBV could contribute to KDM2B silencing by increasing the recruitment of DNMT1 to the *KDM2B*

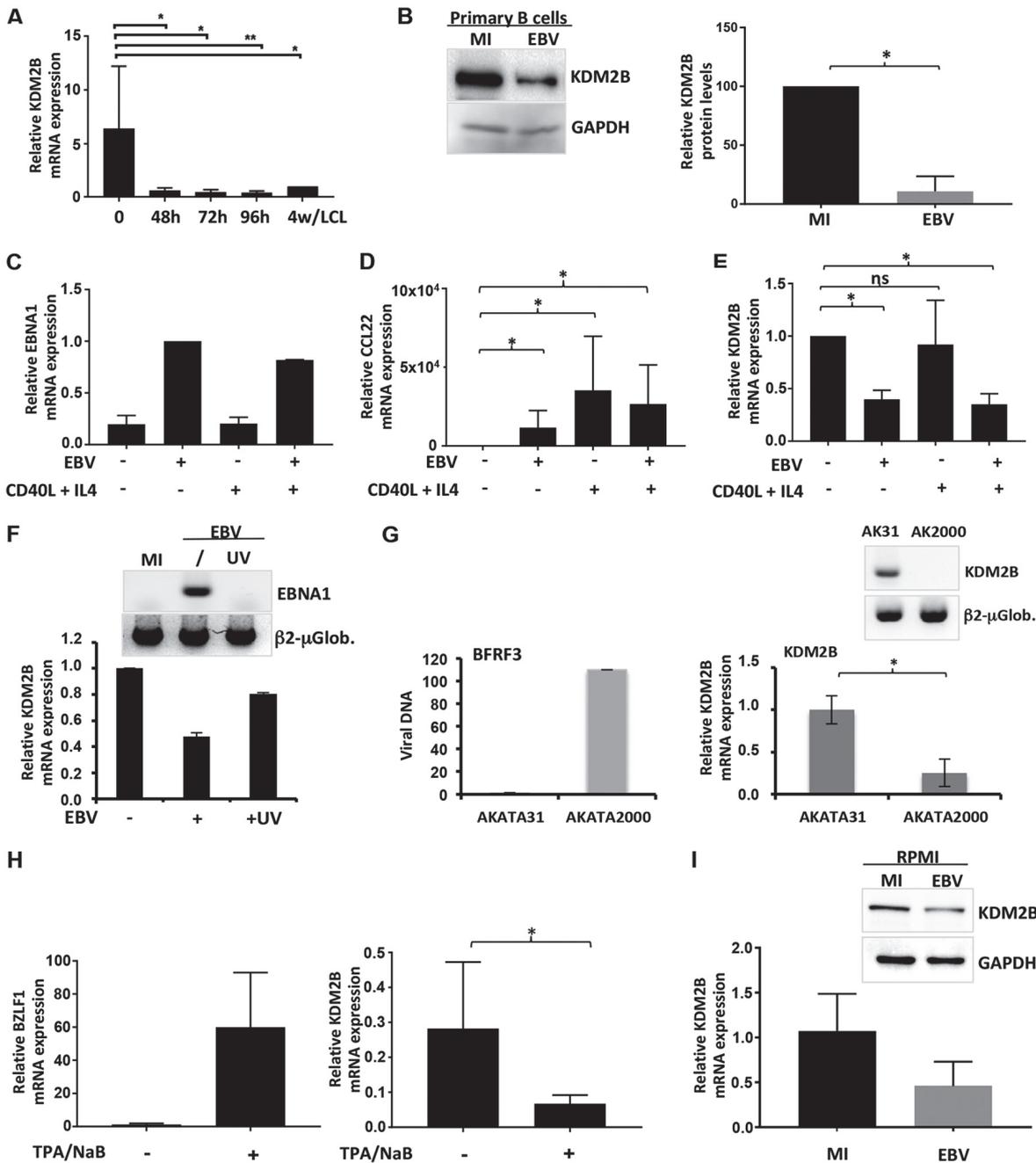


FIG 2 EBV-dependent silencing of KDM2B expression *in vitro*. (A) Primary B cells from 3 independent donors were infected with EBV and collected to make dry pellets at the indicated time points. Some of the infected cells were left in culture for 4 weeks (4w) to generate LCL. Cell pellets were processed and analyzed for the expression level of KDM2B mRNA by RT-qPCR. The difference between the levels of KDM2B in primary B cells (time point 0) and in EBV-infected cells was significant (*, $P < 0.05$; **, $P < 0.01$). The levels of KDM2B were measured relative to its levels in LCL (which was used as the calibrator, for which the value was 1). (B) KDM2B protein expression levels in primary B cells mock infected (MI) or infected with EBV for 48 h were analyzed by Western blotting (left). The KDM2B protein signal was normalized to the levels of GAPDH. The histogram (right) shows the average from 3 independent experiments. (C to E) Primary B cells from 2 independent healthy donors were activated by treating them for 24 h with CD40L and IL-4, as described in Materials and Methods, and/or infected with EBV for 48 h. Cells were then processed and analyzed for the expression levels of EBNA1 (C), CCL22 (D), and KDM2B (E) mRNA by RT-qPCR. The results shown in the histogram are the average from 2 independent experiments (*, $P < 0.05$; ns, not significant). EBNA1 mRNA levels were measured relative to its levels in EBV-infected untreated cells. CCL22 and KDM2B mRNA levels were measured relative to their levels in mock-infected untreated cells. (F) Primary B cells were infected with 2 aliquots of the same EBV batch, one of which was UV inactivated before infection. At 48 h after infection, cells were collected and EBNA1 and KDM2B expression levels were assessed by RT-PCR and qPCR, respectively. The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells. (G) Akata2000 and Akata31 cells were collected, processed for DNA/RNA extraction, and analyzed for the presence of the EBV genome by PCR (left) as well as for KDM2B expression levels by RT-qPCR (lower right) or RT-PCR (upper right). Viral DNA (left) indicates the relative amount of the *BFRF3* gene present in Akata2000 versus Akata31 cells that was analyzed by real-time PCR and normalized to the amount of GAPDH in the extracted DNA. The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells (*, $P < 0.05$). (H)

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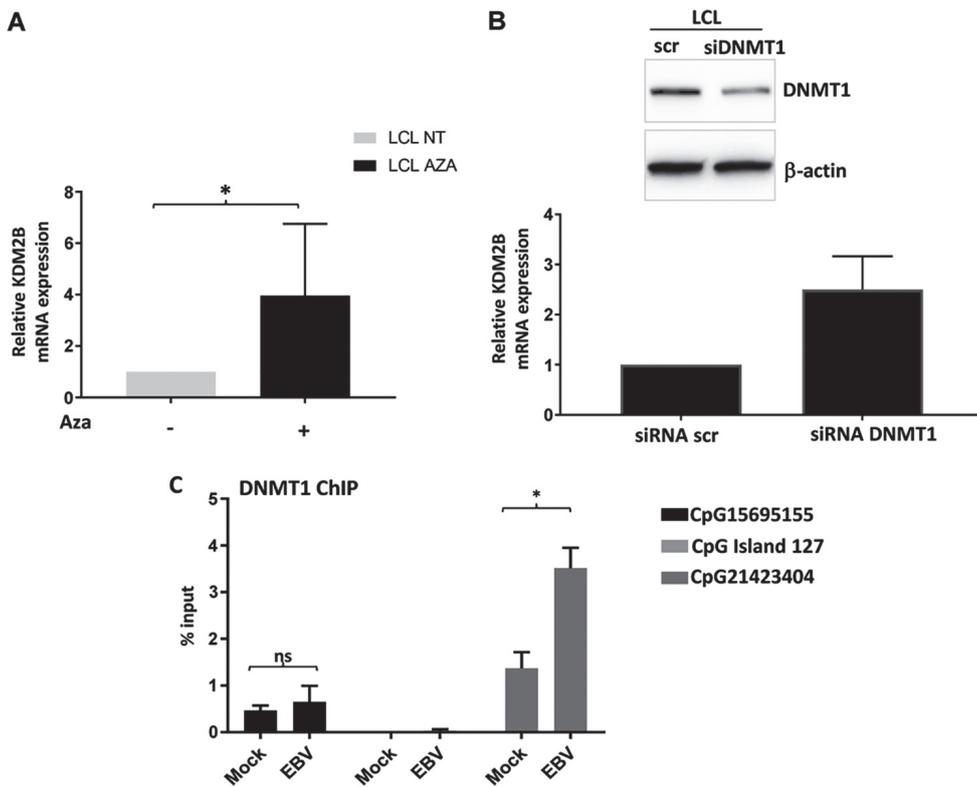


FIG 3 Methylation-dependent silencing of KDM2B expression. (A) LCL were cultured for 96 h in the presence of DMSO (nontreated [NT]) or 5-aza-2'-deoxycytidine (Aza). Cells were then collected, and the KDM2B expression levels were analyzed by RT-qPCR. The histogram shows the average KDM2B mRNA levels measured in 3 independent experiments (*, $P < 0.05$). KDM2B mRNA levels in Aza-treated LCL were measured relative to its levels in DMSO-treated cells. (B) LCL were transfected with stabilized siRNA targeting DNMT1 (siDNMT1) in two independent experiments. At 4 days after transfection, cells were collected and analyzed for the protein levels of DNMT1 (top) and the mRNA levels of KDM2B (bottom). KDM2B mRNA levels in DNMT1-targeted siRNA-treated cells were measured relative to its levels in cells treated with scrambled (scr) siRNA. (C) RPMI cells infected with EBV or mock infected were fixed and processed for ChIP with a DNMT1 antibody or an IgG antibody as a negative control. The eluted DNA was analyzed by qPCR with primers flanking CpG15695155, CpG21423404, and CpG island 127 (primer sequences are described in Table 3) (*, $P < 0.05$; ns, not significant).

gene. Indeed, depletion of DNMT1 in LCL by transfection of DNMT1 small interfering RNA (siRNA) led to an increased KDM2B mRNA level (Fig. 3B). Moreover, chromatin immunoprecipitation (ChIP) experiments showed increased recruitment of DNMT1 to the CpG site CpG21423404 of the *KDM2B* gene in EBV-infected RPMI cells (Fig. 3C).

Taken together, these results indicate that infection of B cells with EBV leads to reduced expression of KDM2B, mediated by the recruitment of DNMT1 to its gene promoter.

The oncogenic viral protein LMP1 induces the silencing of KDM2B. Next, we assessed whether LMP1, the main EBV oncoprotein, plays a role in the deregulation of KDM2B expression. To do this, we generated RPMI cells stably expressing LMP1 (RPMI-LMP1 cells). As a negative control, the cells were transduced with the empty retroviral vector (pLNSX) (Fig. 4A). As revealed by RT-qPCR analysis, the expression level of KDM2B was reduced in the RPMI cells expressing LMP1 (Fig. 4A). Thus, LMP1 appears to play a role in the EBV-mediated downregulation of KDM2B expression. In addition, treating RPMI-LMP1 cells with Aza led to a rescue of KDM2B mRNA levels (Fig. 4B). In

FIG 2 Legend (Continued)

Raji cells untreated or treated with TPA (50 ng/ml)-NaB (3 mM) for 48 h were analyzed for expression levels of BZLF1 and KDM2B mRNA by RT-qPCR (*, $P < 0.05$). (I) RPMI cells untreated or infected with EBV were collected, processed for RNA and protein extraction, and analyzed for the levels of the KDM2B protein (Western blotting, top) and mRNA (RT-qPCR, bottom). The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells.

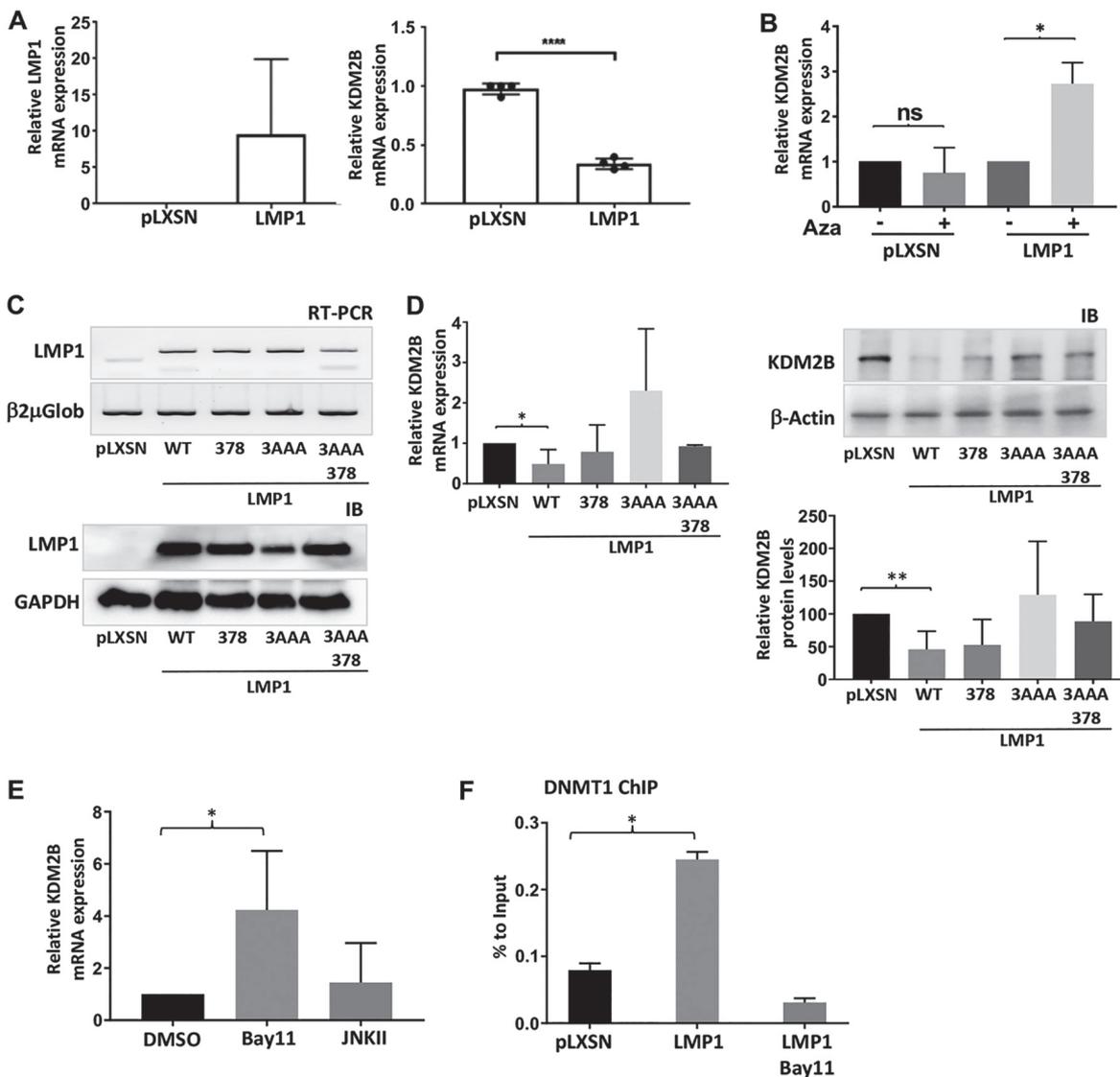


FIG 4 LMP1 mediates downregulation of KDM2B. (A) RPMI cells were stably transduced with pLXSN or with pLXSN-LMP1 (LMP1) in four independent transduction experiments. Cells were then collected, and the expression levels of LMP1 (left) and KDM2B (right) were analyzed by RT-qPCR (****, $P < 0.0001$). (B) RPMI-pLXSN or RPMI-LMP1 cells were cultured in the presence of Aza (+) or DMSO (-) for 48 h, and the KDM2B mRNA expression level was analyzed by RT-qPCR. The histogram shows the average from 2 independent experiments (*, $P < 0.05$; ns, not significant). (C and D) Louckes cells were stably transduced with pLXSN, pLXSN-LMP1 (LMP1), or pLXSN-LMP1 mutants (3AAA, 378, and 3AAA/378). Cells were collected and processed for RNA and protein analysis. mRNA expression and protein levels of LMP1 were detected by RT-PCR (C, top) and immunoblotting (C, bottom). KDM2B mRNA and protein levels were also shown by RT-qPCR and immunoblotting, respectively (D, left and right). In panel D, the difference between KDM2B mRNA and protein levels in Louckes cells with pLXSN and in Louckes cells stably expressing wild-type LMP1 was significant (*, $P < 0.05$; **, $P < 0.01$). The levels of KDM2B mRNA in cells expressing wild-type (WT) LMP1 and LMP1 mutants were measured relative to its level in cells expressing pLXSN. (E) RPMI-LMP1 cells were treated for 2 h with BAY11-7082 (Bay11) (10 μ M) or with JNK inhibitor II (JNKII) (10 μ M) in three independent experiments. KDM2B mRNA levels were analyzed by RT-qPCR (*, $P < 0.05$). (F) RPMI pLXSN and RPMI-LMP1 cells, the latter of which were untreated or treated with BAY11-7082 (10 μ M) for 2 h, were fixed to perform ChIP with DNMT1 or IgG antibodies. The eluted DNA was analyzed by qPCR with primers designed to surround CpG21423404. The histogram shows the average percentage of recruitment of DNMT1 in 4 independent experiments (*, $P < 0.05$).

contrast, no change in KDM2B mRNA expression was observed in the control cells after treatment with Aza (Fig. 4B).

LMP1 activates both the NF- κ B and Jun N-terminal protein kinase (JNK) pathways through its CTAR1 and CTAR2 domains, respectively. Therefore, to gain insights into the mechanism by which LMP1 deregulates KDM2B expression, we generated RPMI and Louckes cells expressing LMP1 mutants harboring mutations in CTAR1 (3AAA mutant), CTAR2 (378 mutant), or both (3AAA/378 double mutant) and therefore having a

hampered ability to activate the NF- κ B pathway, the JNK pathway, or both (13). After selection, cells were analyzed for LMP1 expression by RT-qPCR and immunoblotting. All the cells generated expressed similar levels of the LMP1 transcript and protein (Fig. 4C), with the exception of the LMP1 3AAA mutant, which appeared to be present at lower protein levels than the wild-type LMP1 and the other LMP1 mutated molecules. This is probably due to a reduced protein stability of the LMP1 3AAA mutant caused by the mutation in the CTAR1 in the context of a wild-type CTAR2 domain; however, the 3AAA/378 double mutant and the 378 mutant showed mRNA and protein levels similar to those of wild-type LMP1. We then compared the ability of the different LMP1 mutants to deregulate KDM2B expression. Whereas wild-type LMP1 efficiently downregulated KDM2B mRNA and protein expression (Fig. 4D), both the 3AAA mutant and the double mutant were unable to downregulate KDM2B mRNA expression, and the 378 mutant partially maintained the ability to inhibit KDM2B expression (Fig. 4D), indicating that the CTAR1 domain and, in part, the CTAR2 domain play a role in the LMP1-mediated regulation process of KDM2B expression. The same results were observed in RPMI cells stably expressing LMP1 and its CTAR mutants (data not shown). To further evaluate the impact of the NF- κ B and JNK pathways on LMP1-mediated KDM2B deregulation, we treated RPMI-LMP1 cells with specific chemical inhibitors: BAY11-7082 and JNK inhibitor II, respectively. Whereas the JNK inhibitor had no effect, treating RPMI-LMP1 cells with the NF- κ B inhibitor BAY11-7082 led to a rescue of KDM2B mRNA expression (Fig. 4E).

Previous studies showed that LMP1 activates and induces the recruitment of DNMT proteins to the promoters of cancer-related genes (14–16). In line with these findings, our ChIP experiments showed an increase in the recruitment of DNMT1 to the *KDM2B* gene at the CpG21423404 position in LMP1-expressing cells compared with control cells (Fig. 4F). Treating RPMI-LMP1 cells with BAY11-7082 significantly reduced the amount of DNMT1 recruited to the *KDM2B* gene. Taken together, these data indicate that EBV induces silencing of KDM2B expression mainly via the ability of its main transforming protein, LMP1, to activate the NF- κ B signaling pathway.

LMP1 is not the only EBV protein able to induce downregulation of KDM2B expression. LMP1 is only rarely expressed in EBV(+) BL samples. Therefore, our observation that KDM2B is downregulated and silenced in EBV(+) BL cell lines and specimens led us to investigate whether EBV genes other than LMP1 could play a role in this event. Infection of primary B cells with a recombinant EBV lacking the *LMP1* gene (EBV Δ LMP1) still led to decreased expression of KDM2B mRNA (Fig. 5A). Downregulation of KDM2B occurred at 12 h after infection of primary B cells with EBV; as expected at this early time point, we could not yet detect LMP1 expression, and only the EBNA5 were efficiently expressed (Fig. 5B). Moreover, when comparing KDM2B mRNA expression levels in different EBV(+) BL-derived cell lines, in an LCL, and in primary B cells, we observed a reduced level of KDM2B transcript in all the BL cells, independently of whether they were in latency phase I or III (Fig. 5C). As expected, although BL cells in phase I expressed EBNA1 but had little or no LMP1 expression, BL cells in phase III and the LCL efficiently expressed both genes (Fig. 5C). Taken together, these experiments indicate that KDM2B deregulation can also occur in the absence of LMP1 expression; therefore, other EBV proteins could be involved in this event in BL cells. Transient transfection of Louckes and RPMI cells with different constructs expressing a panel of EBV genes (Fig. 5D and E) indicated that, in addition to LMP1, different latent viral proteins could downregulate KDM2B mRNA. To rule out the possibility that this result was the consequence of the activation of the NF- κ B pathway as a side effect of the transfection of the cells, we checked the levels of I κ B α phosphorylation. As expected, based on its known function (17), among the analyzed EBV proteins, LMP1 was the most efficient in inducing NF- κ B activation (Fig. 5F), indicating that the other viral proteins may use other mechanisms to downregulate KDM2B mRNA expression. MicroRNA-146-5p (miRNA-146-5p), which is known to target KDM2B expression, has been reported to deregulate KDM2B mRNA levels during human papillomavirus (HPV) infection (18). A previous study showed that EBNA2 induces the expression of miRNA-

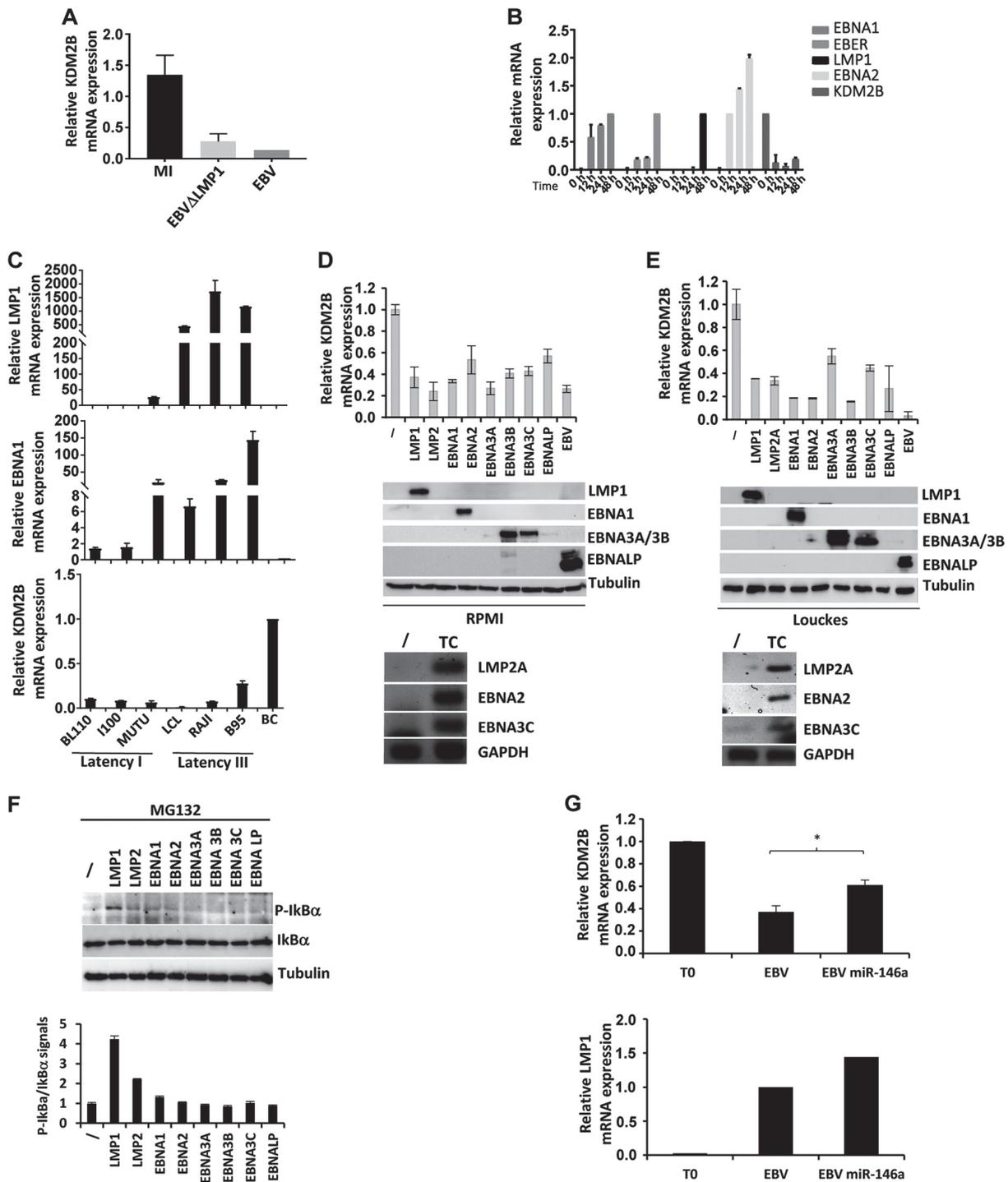


FIG 5 EBV also uses LMP1-independent mechanisms to downregulate KDM2B. (A) B cells from two donors were infected with EBV or EBVΔLMP1 or mock infected (MI) and collected at 48 h after infection. Reverse-transcribed RNA samples were analyzed by qPCR for the KDM2B expression level. (B) B cells from two donors were infected with EBV and collected at 12, 24, and 48 h after infection. Cells were processed for RNA extraction and analyzed by qPCR for the expression levels of EBNA1, EBNA2, EBER, LMP1, and KDM2B transcripts. The expression levels of viral gene transcripts were measured relative to their levels in B cells collected 12 h (EBNA2) or 48 h (EBNA1, EBER, LMP1) postinfection. The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells. (C) Different EBV(+) cell lines in latency phase I (BL110, I100, MUTU) or in latency phase III (LCL, Raji, B95) and primary B cells (BC) were collected, processed for RNA extraction and reverse transcription, and analyzed by qPCR for LMP1 (top), EBNA1 (middle), and KDM2B (bottom) mRNA levels. (D and E) RPMI (D) and Louckes (E) cells were transiently transfected with different constructs carrying individual EBV genes (TC) in three independent experiments. At 48 h after transfection, cells were processed for RNA and protein extraction and analyzed for the KDM2B expression level by RT-qPCR analysis. The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells. Western blotting (LMP1, EBNA1, EBNA3A/3B, and EBNA3C) or RT-PCR analysis (LMP2A, EBNA2, and EBNA3C) was performed to measure the expression of the different viral latent proteins. (F) RPMI cells were transfected with different EBV gene-carrying constructs. At 44 h after transfection, cells were exposed to the proteasome inhibitor MG132 for 4 h and then collected, processed for total protein extraction, and analyzed for the indicated proteins by immunoblotting. The histogram

(Continued on next page)

146-5p (19). Therefore, we tested whether miRNA-146-5p could contribute to KDM2B downregulation during EBV infection. To do this, we treated primary B cells with a miRNA-146-p5-specific inhibitor before EBV infection. As shown in Fig. 5G, this treatment partially rescued KDM2B expression in the miRNA-146-p5 inhibitor-treated cells. These results show that EBV may be able to reduce intracellular levels of KDM2B by a redundancy of mechanisms, further indicating that this event could be important for the virus.

KDM2B regulates expression of viral genes in EBV-infected B cells. To evaluate the biological relevance of EBV-mediated KDM2B downregulation, we hypothesized that the epigenetic enzyme could regulate EBV transcription, similar to what was observed for other histone modifiers and chromatin-interacting proteins (e.g., EZH2, CTCF, KMT5B) (20–22). To assess whether ectopic expression of KDM2B in B cells could have an impact on EBV infection, we transfected increasing concentrations of a KDM2B construct into Louckes cells, an EBV(–) BL-derived cell line. One day after transfection, cells were infected with EBV-green fluorescent protein (GFP) and monitored for the infection efficiency 24 h later. Figure 6A shows efficient overexpression of ectopic KDM2B at both the protein and mRNA levels at 24 h after transfection. The infection efficiency of Louckes cells transfected with the KDM2B expression vector or the control empty vector (pCDNA) was indistinguishable, as revealed by TaqMan PCR, showing that the increased KDM2B expression level did not alter the viral genome copy number per cell (Fig. 6B). In contrast, the GFP mean fluorescence intensity (MFI) decreased in the presence of enhanced KDM2B expression (Fig. 6C), indicating that KDM2B could affect viral gene expression. Indeed, RT-qPCR analysis of the expression levels of different EBV transcripts (LMP1, EBNA1, and BZLF1) at 24 h after infection showed a significant and dose-dependent reduction in their mRNA levels in the presence of an increasing amount of KDM2B (Fig. 6D). Taken together, these data suggest that KDM2B plays a role in regulating EBV gene expression.

The results presented above, obtained by overexpressing KDM2B, could be due to a generalized chromatin demethylation as a consequence of high intracellular levels of the demethylase. Therefore, next we depleted endogenous KDM2B in Louckes cells by transfecting an siRNA targeting KDM2B mRNA. RT-qPCR and Western blot analysis showed that KDM2B was efficiently downregulated 24 h after transfection with the specific siRNA (Fig. 6E). Cells were then infected with EBV-GFP and monitored for the infection efficiency, as described above. At 24 h after infection, neither the percentage of GFP-positive cells nor the genome copy number had changed significantly between the control cells (cells transfected with scrambled siRNA [siSCR]) and the cells transfected with the siRNA directed against KDM2B (Fig. 6F and data not shown), indicating that the loss of KDM2B does not affect the efficiency of infection. However, efficient depletion of KDM2B by siRNA led to a significant increase in EBV transcripts 24 h after infection (Fig. 6G). This result indicates that a reduced intracellular level of KDM2B promotes the expression of the viral genes, further confirming the ability of KDM2B to repress EBV gene expression at early stages of infection.

KDM2B inhibits viral gene expression in latently infected EBV-immortalized B cells. To test whether the activity of KDM2B in regulating EBV gene expression is required for the maintenance of virus latency, similar to what has been reported for other epigenetic enzymes (20), we aimed to overexpress KDM2B ectopically and examine its impact on viral gene expression in EBV-immortalized B cells. LCL displayed detectable KDM2B mRNA and protein levels, although they were lower than those in primary B cells (Fig. 2A and B and data not shown). Therefore, KDM2B was overex-

FIG 5 Legend (Continued)

shows the average phosphorylated I κ B α (P-I κ B α) signal normalized to the levels of total I κ B α . (G) B cells from different donors were untreated or treated with a miRNA-146a-5p inhibitor for 24 h before infection with EBV. At 48 h after infection, cells were collected and processed for RNA extraction and reverse transcription. cDNA samples were analyzed by qPCR for LMP1 and KDM2B expression levels. The levels of KDM2B mRNA were measured relative to its levels in mock-infected cells. The histograms show the average expression levels measured in 2 independent experiments (*, $P < 0.05$). T0, time zero.

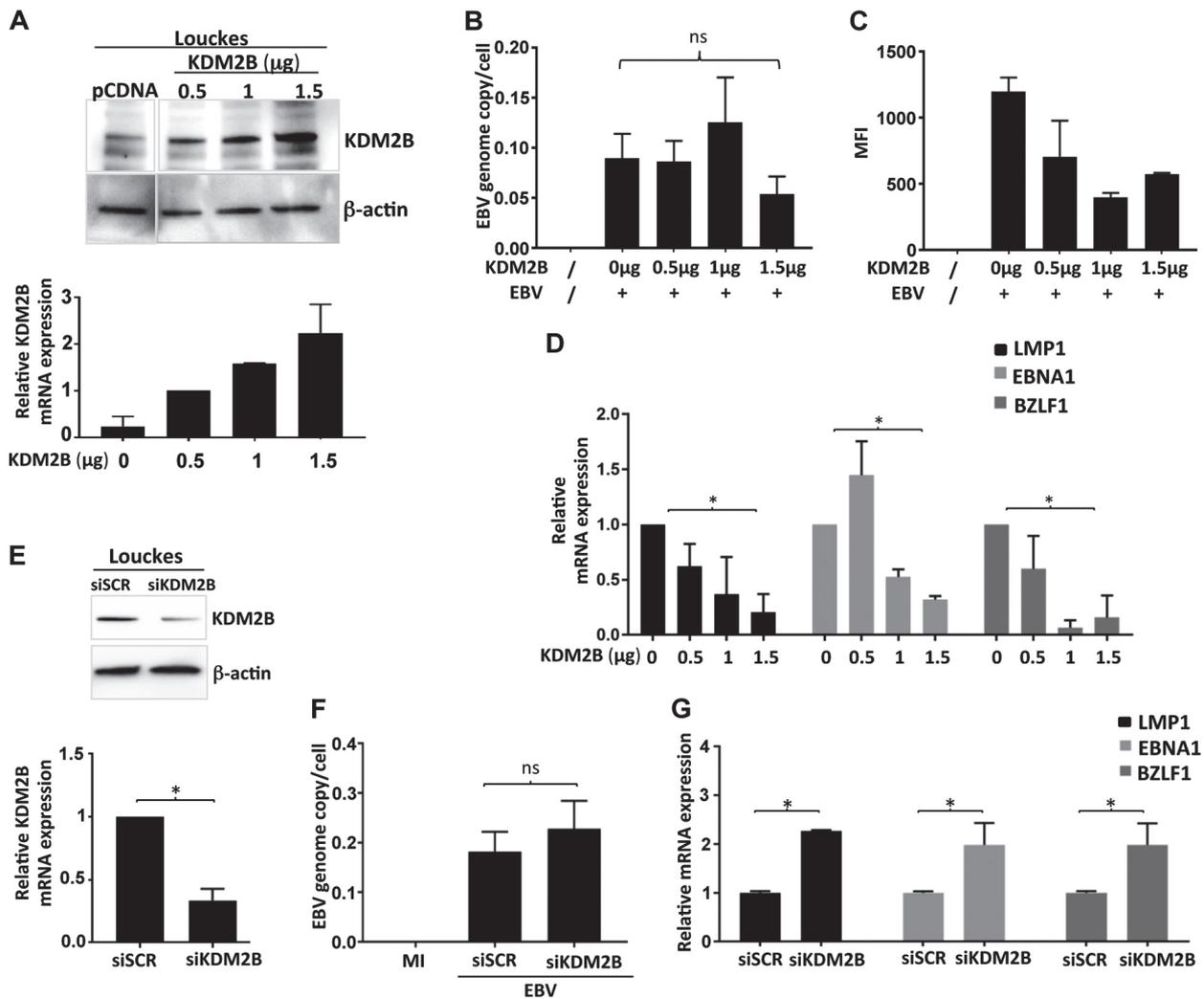


FIG 6 KDM2B deregulation alters EBV gene expression. (A) Louckes cells were transiently transfected with increasing concentrations of the KDM2B expression vector in three independent experiments, collected at 24 h after transfection, and processed for protein and RNA extraction to assess KDM2B levels by immunoblotting (top) or qPCR (bottom). (B) Cells were then infected with EBV, and at 24 h after infection they were collected and processed for FACS analysis and for RNA/DNA extraction. DNA samples were used to measure EBV genome copy number by TaqMan PCR (ns, not significant). (C) Live cells were analyzed by FACS to measure the GFP mean fluorescence intensity (MFI). (D) cDNA samples were analyzed for the expression level of EBV early and late genes by qPCR. The levels of EBV genes transcripts were normalized to the mRNA levels of the housekeeping gene β -globin and calculated relative to their levels in cells transfected with the empty vector (KDM2B, 0 μ g). The values shown in the histogram are the average from 3 independent experiments (*, $P < 0.05$). (E to G) Louckes cells were transfected with KDM2B siRNA and scrambled siRNA as a control in three independent experiments. (E) At 24 h after transfection, half of the cells were collected and analyzed for the expression levels of KDM2B protein (top) and mRNA (bottom), and half of the cells were infected with EBV. At 24 h after infection, cells were collected and processed for RNA/DNA extraction. The number of EBV genome copies per cell was determined by TaqMan PCR on the DNA template (MI, mock infected; Louckes cell DNA was also included as a negative control) (F), and qPCR of the cDNA samples enabled the assessment of the mRNA expression levels of different viral genes, calculated as explained in the legend to panel D (G). siSCR, scrambled siRNA; siKDM2B, siRNA targeting KDM2B mRNA.

pressed in LCL by ectopic expression of a KDM2B construct. At 48 h after transfection, LCL were collected and processed for total protein and DNA/RNA extraction. Western blotting and RT-qPCR showed substantial increases of KDM2B at the protein and mRNA levels, respectively (Fig. 7A). LCL overexpressing KDM2B carried a similar number of EBV genome copies compared with the same cells transfected with the control pCDNA vector (Fig. 7B), as revealed by TaqMan PCR analysis. We then analyzed the mRNA expression level of different EBV transcripts by RT-qPCR. Ectopic expression of KDM2B led to a significant reduction in the mRNA level of all the analyzed EBV genes (Fig. 7C). In contrast, depletion of KDM2B from LCL by transfecting KDM2B siRNA (Fig. 7D) led to a significant increase in the expression of the viral genes compared with the expression level of the same genes in LCL transfected with scrambled siRNA (Fig. 7F). Similar to

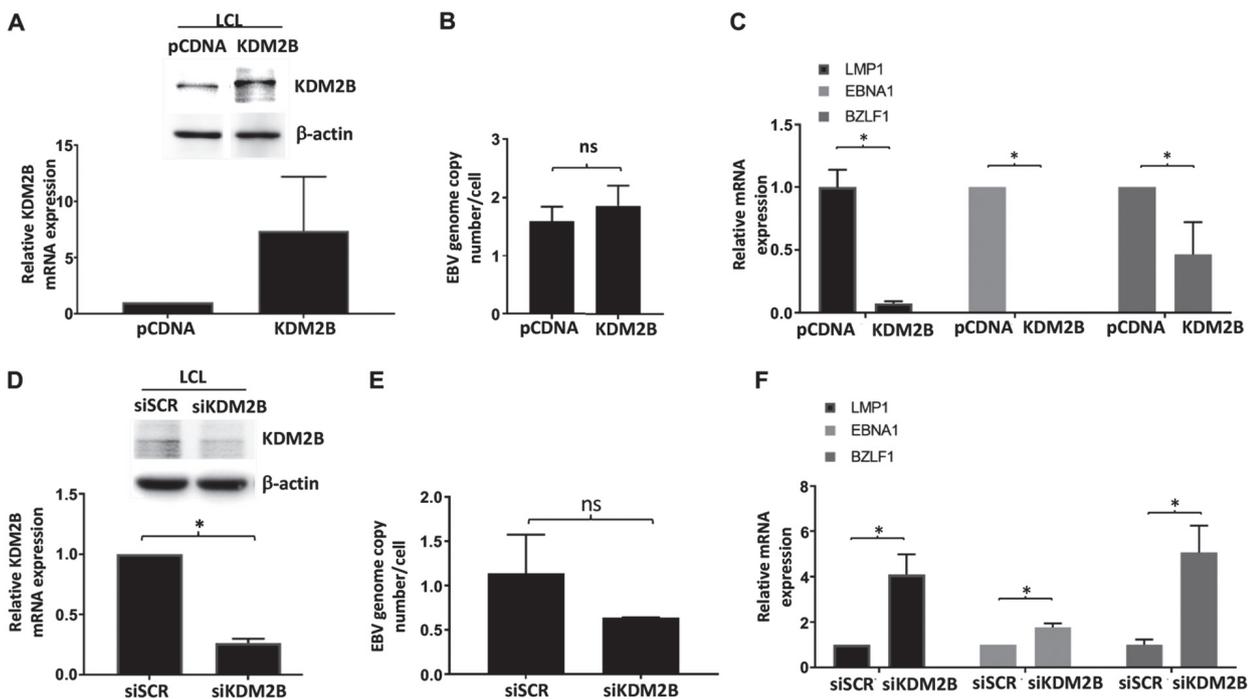


FIG 7 KDM2B regulates EBV gene expression in latently infected cells. (A to C) LCL were transfected with 1.5 μ g of pCDNA3-KDM2B or with pCDNA as a control in three independent experiments. Cells were collected at 24 h after transfection and processed for RNA/DNA and total protein extraction. (A) KDM2B mRNA and protein levels were shown by qPCR (bottom) and immunoblotting (top; the lines corresponding to conditions with 0.5 μ g and 1 μ g of KDM2B, originally present in the Western blot, are not shown because they were excluded from all the analyses). (B) DNA samples were analyzed by TaqMan PCR to assess the number of EBV genome copies per cell (ns, not significant). (C) The mRNA expression levels of EBV latent and early genes were assessed by qPCR (*, $P < 0.05$). The mRNA levels of viral genes in LCL overexpressing KDM2B were measured relative to their levels in pCDNA-transfected control cells and normalized on the mRNA levels of β -globin. (D to F) (D) LCL transfected in three independent experiments with KDM2B siRNA (siKDM2B) or scrambled siRNA (siSCR) as a control were collected at 48 h after transfection and analyzed for the levels of KDM2B protein (top) and transcript (bottom). (E) DNA samples were analyzed by TaqMan PCR to assess the number of EBV genome copies per cell. (F) The mRNA expression levels of EBV latent and early genes were assessed by qPCR (*, $P < 0.05$). Viral transcript levels in KDM2B siRNA-treated cells were measured relative to their levels in siSCR-treated cells and normalized to the mRNA levels of β -globin.

what we observed in EBV-infected Louckes cells, removal of KDM2B did not significantly affect the EBV genome copy number (Fig. 7E). Taken together, these data indicate that KDM2B controls viral gene expression in latently infected cells.

Next, we determined whether KDM2B could directly bind to EBV gene promoters to regulate their expression. To do this, purified primary B cells infected or mock infected with EBV for 48 h, as well as the corresponding LCL, were formaldehyde fixed and processed for KDM2B ChIP (Fig. 8A). ChIP analysis showed that KDM2B can be recruited to the EBV genome and, more precisely, to the Qp promoter at 48 h after infection. We also found KDM2B recruited to the Cp promoter and to a region of the BZLF1 promoter proximal to the start site (Zp 0) in EBV-immortalized B cells. However, we did not detect KDM2B recruitment when we analyzed a region of the BZLF1 promoter 600 bp upstream of the start site (Zp +600) in the same cells (Fig. 8A, top), indicating that KDM2B is specifically recruited to a specific region of the viral genome. As expected, we did not detect KDM2B recruitment to the promoter of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase); however, we observed efficient KDM2B binding to a sequence located downstream of the transcription start site of the ribosomal DNA (rDNA) repeated units 8 (H8) (Fig. 8A, bottom), which was previously reported to be targeted by the epigenetic enzyme (23). These data indicate that KDM2B is directly recruited to specific EBV promoter regions. To further assess the ability of KDM2B to be recruited to the EBV genome, B cells were untreated or EBV infected, collected, and processed for immunofluorescent *in situ* hybridization (immuno-FISH) experiments. EBV DNA molecules were detected by FISH with a labeled probe directed against the BamHI W EBV genomic repeated region; KDM2B was concomitantly de-

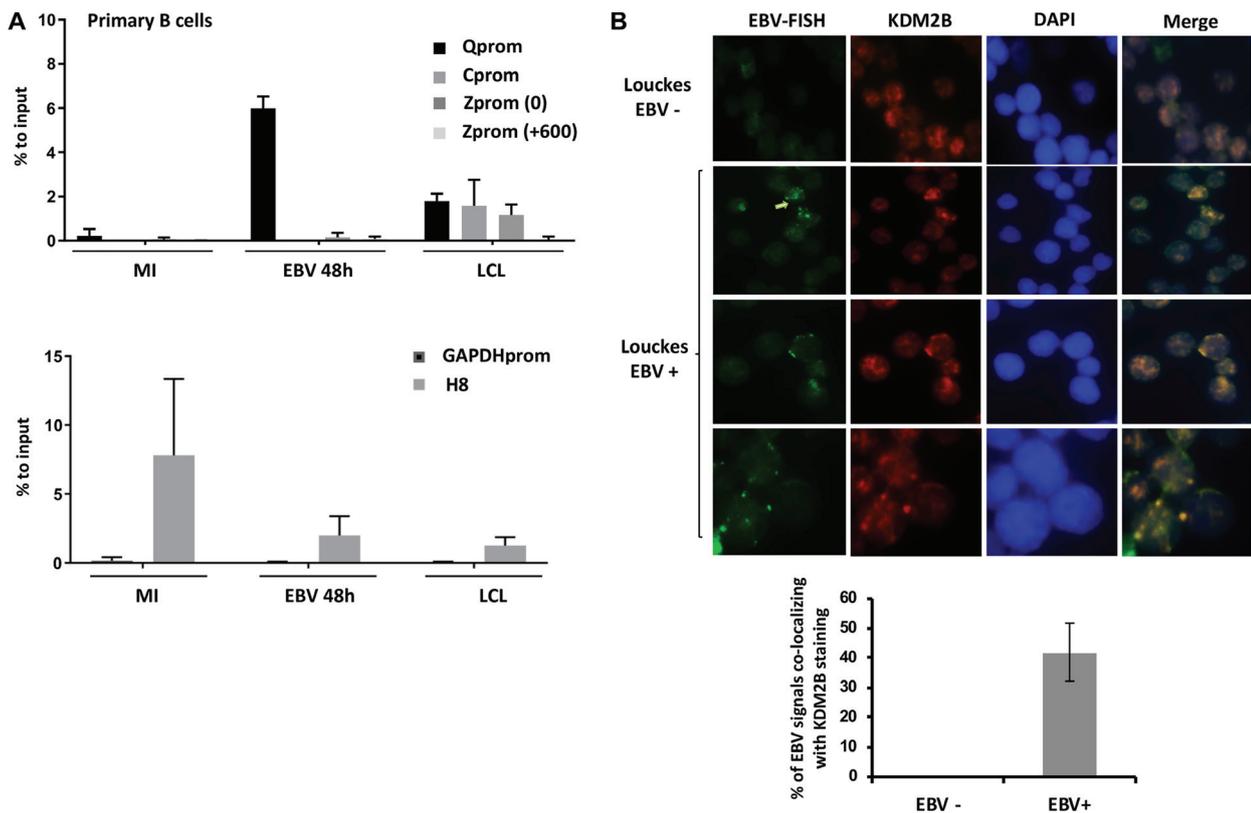


FIG 8 KDM2B is recruited to the EBV genome. (A) Primary B cells from three donors were infected with EBV for 48 h or left immortalized until they generated LCL. Mock-infected (MI) B cells, B cells infected with EBV for 48 h, and LCL were formaldehyde fixed and processed for ChIP using a KDM2B antibody and an IgG antibody. The eluted DNA was analyzed by qPCR with primers designed on different EBV gene promoters. As negative controls, the GAPDH promoter was also amplified, and the recruitment of KDM2B to its known cellular gene target, H8, was also assessed. Qprom, Cprom, and Zprom, Qp, Cp, and Zp EBV promoters, respectively. (B) Louckes cells untreated or infected with EBV (the B95-8 strain) were fixed on glass slides and processed for immuno-FISH. EBV DNA was detected by FISH, and KDM2B was detected by immunofluorescence. Overlapping EBV and KDM2B signals are shown in the merge fields as yellow dots. The histogram shows the percentage of merged dots, estimated by counting the number of EBV signals that colocalized with the KDM2B patches in at least 3 different fields from 3 independent stainings. DAPI, 4',6'-diamidino-2-phenylindole.

ected by immunofluorescence by using an anti-KDM2B antibody. In EBV-infected B cells, KDM2B patches partially overlapped or were found in close proximity to the viral DNA (on average, 40% of the green dots colocalized with the red dots) (Fig. 8B), suggesting that the epigenetic enzyme is recruited to or is close to the viral genome at early stages of infection. Taken together, our data demonstrate a role of KDM2B in controlling EBV gene expression.

Deregulation of KDM2B mRNA level in B cells alters their expression profile.

KDM2B has been shown to play a role in cell differentiation, cell growth, and the proliferation of hematopoietic cells (9). We therefore assessed whether altered KDM2B expression could also have an impact on the B-cell phenotype in our experimental models. Louckes cells and LCL overexpressing KDM2B did not show an altered proliferation ability, nor did they show an altered cell cycle or apoptotic profiles (Fig. 9A to D). Therefore, to gain further insights into the impact of KDM2B deregulation on B cells, we performed an analysis with an RNA expression chip array in Louckes cells expressing increasing levels of KDM2B (Fig. 9E). Cellular genes whose expression was significantly altered by the enhanced levels of KDM2B were identified by bioinformatics analysis (Fig. 9E). In line with the known function of KDM2B as a demethylase of H3K4me3, the set of genes altered by KDM2B was enriched in H3K4me1 (Fig. 9F). Among the genes that were significantly deregulated in the presence of altered KDM2B expression, some play a role in immunity (Fig. 9G). Our analysis revealed that KDM2B overexpression was associated with deregulated expression of genes involved in the tumor necrosis factor receptor 2 (TNFR2) pathway (Fig. 9G). Interestingly, this pathway is important for the

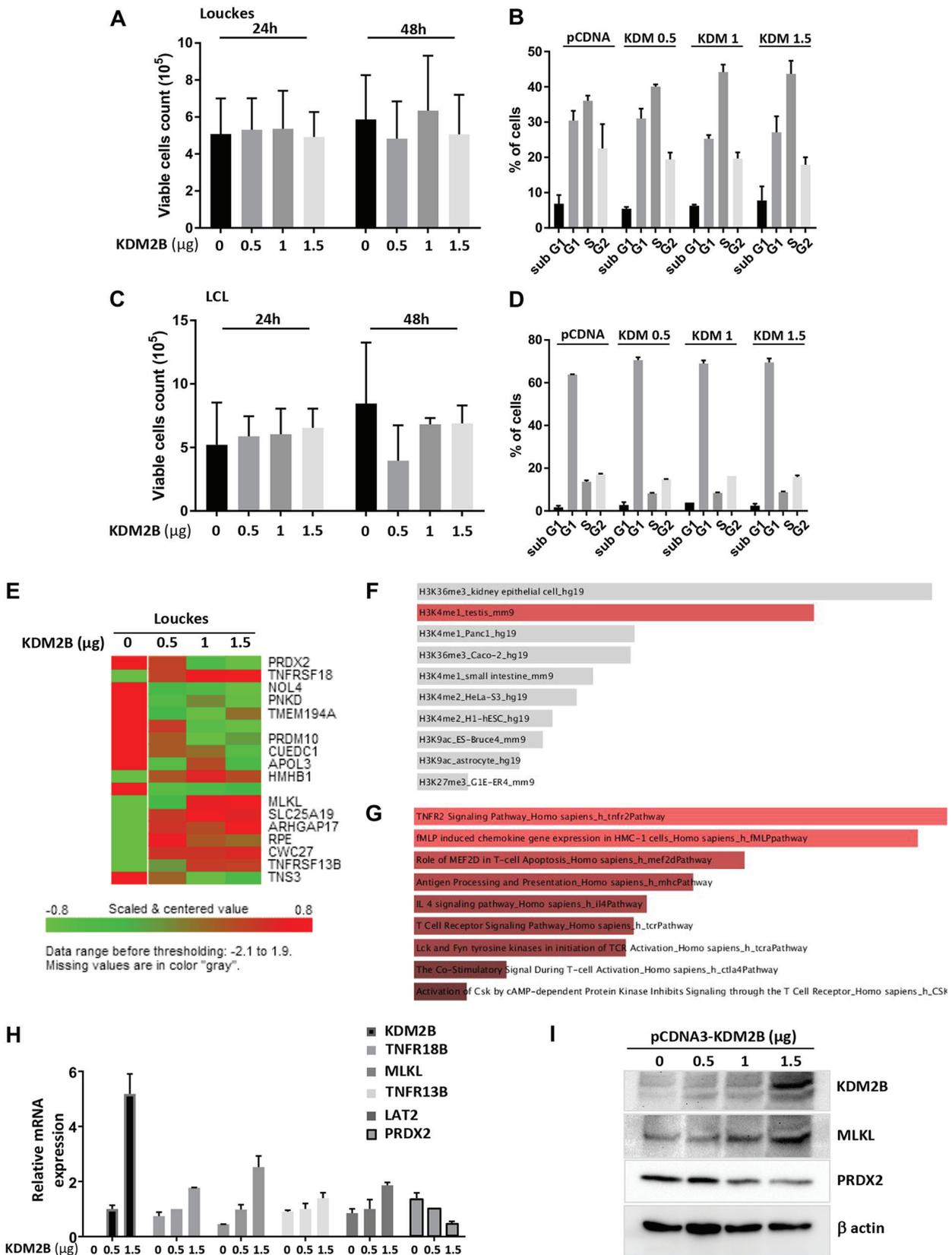


FIG 9 KDM2B deregulation alters cellular gene transcription. (A to D) Louckes cells (A and B) and LCL (C and D) were transfected with increasing concentrations of the KDM2B expression vector. (A and C) At 24 and 48 h after transfection, viable cells were counted. (B and D) Transfected cells were ethanol fixed and processed for cell cycle analysis by flow cytometry. (E) Louckes cells, generated as described in the legend to Fig. 5A, were collected and processed for RNA extraction and RNA expression profiling, as described in Materials and Methods. The differential expression analysis was conducted using BRB-ArrayTools software. (F and G) Genes differentially regulated in Louckes cells overexpressing KDM2B compared

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transition of B cells from germinal center B cells to resting memory B cells (24). Moreover, the TNFR2 pathway mediates specific tumor necrosis factor (TNF) effects and is an important mediator of the cell antiviral response (9). Deregulated expression of genes in this pathway was confirmed and validated by RT-qPCR (Fig. 9H) and by Western blot analysis for two of them (MLKL and PRDX2), which were, respectively, induced and downregulated during KDM2B overexpression in Louckes cells (Fig. 9I). Taken together, these data indicate that KDM2B plays a role in B-cell differentiation and in regulation of the TNF pathway, which is often altered during the lymphomagenic process.

DISCUSSION

In this study, we show that a regulatory region within the gene encoding the KDM2B protein is more methylated in EBV(+) BL than in EBV(−) BL, confirming data from our previous study, which aimed to characterize the whole epigenetic profile of a set of BL-derived cell lines of eBL or sBL origins. This region is found to be methylated in certain cancer-derived cell lines (ENCODE DNA methylation tracks, CpG methylation by Methyl 450K bead arrays from ENCODE/HAIB, UCSC Genome Browser). Furthermore, KDM2B levels appear to be deregulated in cancers of hematological origin (9). We therefore assessed whether increased methylation of the *KDM2B* gene in eBL is mediated by EBV to deregulate intracellular levels of the histone modifier and promote EBV-mediated lymphomagenesis. Indeed, KDMs have been shown to have altered expression in cancer (25). For instance, a KDM2B paralogue, KDM2A, behaves as a tumor suppressor in hematopoietic stem cells, in which it antagonizes mixed-lineage leukemia-associated leukemogenesis by erasing H3K36me2 markers (26). Similarly, altered levels of KDM2B could modify the chromatin structure and the pattern of expression of B cells and favor their transformation.

eBL specimens and derived cell lines showed low KDM2B protein expression, underscoring a potentially important role of KDM2B downregulation in the lymphomagenic process *in vivo*. Treating eBL cell lines with a chemical that blocks DNA methylation led to a rescue of the KDM2B mRNA level, indicating that DNA methylation contributes to the silencing of KDM2B expression in these cells. However, the same treatment left the level of the KDM2B transcript in EBV(−) sBL-derived cell lines unchanged. These events are similar to what we have previously described for another gene, *ID3* (4). *ID3*, which plays a key role in lymphomagenesis and which is often found mutated in the sBL variant (27, 28), was found to be silenced by hypermethylation in eBL-derived cell lines (4). EBV infection therefore plays a direct role in eBL pathogenesis by altering the cellular epigenome and deregulating the expression of genes with a key role in lymphomagenesis. Indeed, *in vitro* infection of primary B cells with EBV led to rapid downregulation of KDM2B expression. Low KDM2B mRNA levels at early stages of EBV infection of primary B cells were also observed in a data set from RNA expression profiling performed in an independent study (E. Manet, unpublished data). Our data also show that downregulation of KDM2B by EBV is not due to a specific B-cell activation status but is, rather, a direct result of infection by an actively transcribing virus. We also show that downregulation of KDM2B expression in EBV-infected cells is mediated by DNMT1 recruitment to its gene and by its DNA methylation. It has already been reported that EBV can alter DNMT1 activity through its viral proteins LMP1 and LMP2A (29). In particular, Tsai and colleagues showed that LMP1 activates DNMT1 activity (15) and that this event requires activation of the JNK/AP1 pathway.

Here, we show that cells stably expressing LMP1 display lower levels of KDM2B. The

FIG 9 Legend (Continued)

with their expression in Louckes cells transfected with an empty pCDNA vector were analyzed for their enrichment in specific pathways by using the Enrichr web tool. Enrichment results for the Epigenomics Roadmap HM ChIP sequencing and BioCarta 2016 databases (top and bottom, respectively) are shown. hESC, human embryonic stem cells; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; mhc, major histocompatibility complex; TCR, T-cell receptor; MEF2D, myocyte enhancer factor 2D. (H and I) Differential expression of a set of genes found to be deregulated in the expression profile array was validated by qPCR (H) and immunoblotting (I). The levels of the different transcripts were measured relative to their levels in Louckes cells transfected with 0.5 μ g of the KDM2B construct.

ability of LMP1 to downregulate KDM2B depends mostly on its NF- κ B activity, as shown by using an LMP1 molecule mutated on its NF- κ B-activating domain and by blocking the NF- κ B pathway via a specific chemical inhibitor. A LMP1 mutant lacking the JNK activation pathway also downregulates KDM2B, but to a lesser extent than wild-type LMP1. This indicates a partial contribution of that pathway to LMP1-mediated KDM2B downregulation. However, exposing the cells to a specific JNK inhibitor had no effect on the KDM2B mRNA expression level. Moreover, our ChIP experiments showed that expression of LMP1 in the EBV(-) RPMI cells is able to trigger DNMT1 recruitment to the *KDM2B* gene. The finding that the recruitment of DNMT1 to the *KDM2B* gene can be hampered by treating LMP1-RPMI cells with the I κ B α kinase inhibitor further confirms its dependence on the ability of LMP1 to induce the NF- κ B pathway. Recently published data showed that HPV16 E6/E7 transforming proteins inhibit the expression of miRNA-146-5p, known to target the *KDM2B* transcript, which results in an increase in the *KDM2B* expression level in HPV16-infected cells. In contrast to E6/E7, the EBV transforming protein LMP1 induces miRNA-146-5p via its ability to activate NF- κ B (18). EBNA2 also induces miRNA-146-5p (19). This event could contribute to the reduction in the *KDM2B* mRNA level in EBV-infected cells. Our data show that blocking miRNA-146-5p in primary B cells before infection leads to a partial rescue of *KDM2B* levels compared with those in untreated cells. It is therefore possible that the virus uses alternative mechanisms to target *KDM2B*: (i) increasing its DNMT1-mediated gene methylation associated with LMP1 protein expression and (ii) controlling *KDM2B* mRNA levels by a specific miRNA associated with the expression of LMP1 and/or EBNA2. Further studies are needed to assess the contributions of deregulated miRNA-146-5p levels to the events described here. Our data also indicate that proteins other than LMP1 and EBNA2 appear to be able to mediate downregulation of *KDM2B*. EBV may need to tightly control *KDM2B* levels and activity to regulate expression of its own genes as well as that of cellular genes. Indeed, a recent study from Gillman and colleagues showed that EBNA3C interacts with *KDM2B* by its TFGC motif (HD motif) (30). Mutation of the latter motif impairs the ability of EBNA3C to bind to *KDM2B* and repress its target genes (30).

Previous studies by us and others showed that EBV can modulate the level and the activity of different epigenetic enzymes (20, 21, 31), which in turn play a role in regulating viral gene expression. One example is EZH2, whose intracellular expression level is induced in B cells by EBV infection in an LMP1-dependent manner (32). EZH2, in turn, is recruited to the viral genome, where it participates in the establishment and maintenance of EBV latency by methylating H3K27 in proximity to the BZLF1 and BRFL1 promoters (22). Our observation that EBV infection alters *KDM2B* expression prompted us to assess whether the epigenetic enzyme could regulate EBV infection and/or the EBV life cycle. Altering *KDM2B* expression in B cells before EBV infection or in latently EBV-infected B cells led to the deregulated expression of all analyzed viral genes. This was consistent with the recruitment of *KDM2B* to their respective promoters, as observed by ChIP experiments. In line with its ability to demethylate the active chromatin marker and repress transcription, *KDM2B* depletion in EBV-infected B cells led to increased viral gene expression, whereas its forced ectopic expression had the opposite effect and caused a strong reduction in the levels of viral transcripts. Taken together, our data indicate that *KDM2B* plays a role in controlling EBV gene expression, for instance, during the establishment of latency. Recruitment of *KDM2B* to the viral episome during the first step of infection could be necessary for the repression of viral gene transcription, especially at the end of the pre-latent phase, when the EBV lytic genes are silenced to allow the virus to persist in resting peripheral B cells (32). In contrast, *KDM2B* could work as a host restriction factor; therefore, its downregulation during the early stages of EBV infection would allow efficient viral gene expression and replication during the pre-latent phase. Future studies will be needed to investigate the exact role of EBV-mediated *KDM2B* deregulation in EBV life cycle control.

It is known that in order to escape the immune surveillance of the host and establish a chronic infection, EBV has evolved different mechanisms to maintain B cells in a status

of long-lived circulating memory B cells and prevent them from differentiating into antibody-secreting plasma cells. A recent study showed that EBNA3A and EBNA3C block the terminal differentiation of memory B cells to plasma cells by epigenetically repressing the gene encoding BLIMP-1, a master regulator in B-cell differentiation (33). Notably, a previous study showed that KDM2B plays a key role in the differentiation of hematological stem cells (9). Therefore, EBV-mediated deregulation of KDM2B expression could contribute to the mechanism that prevents the cells from undergoing terminal differentiation. This hypothesis is supported by our data from an RNA profile analysis conducted on cells transfected with increasing levels of a KDM2B-expressing construct. Cells harboring high KDM2B levels showed a deregulated expression of genes enriched in the TNFR2 pathway, which is known to play a role in the differentiation from B cells to plasma cells (24). TNFR2 also regulates the interferon pathway, an important mediator of the antiviral response. Ablation of KDM2B in hematopoietic cells has previously been shown to downregulate the interferon response (9). The downregulation of the KDM2B expression level upon EBV infection could therefore contribute to the viral escape from immune system surveillance.

Taken together, our data show a novel interplay between EBV infection and the host epigenome. EBV alters KDM2B expression via an epigenetic mechanism involving LMP1 and other latent viral proteins. The histone modifier, in turn, plays a role in regulating the expression of viral and host cell genes. These data, in addition to the observed deregulated KDM2B levels in BL-derived cell lines, indicate that altered expression of this epigenetic enzyme could contribute to B-cell transformation. Future studies aimed at investigating the functional importance of *KDM2B* gene methylation and downregulated expression during the EBV-mediated lymphomagenic process are warranted.

MATERIALS AND METHODS

Case selection, immunophenotype, and FISH. We studied 22 morphologically and immunophenotypically typical BL cases (11 sporadic and 11 endemic). All cases were diagnosed according to the updated World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues (34). The cases were retrieved from the archives of Siena University Hospital (Siena, Italy; $n = 2$) and Nairobi University (Nairobi, Kenya; $n = 20$). Before enrolling the cases in this study, they were reevaluated by an expert hematopathologist (L.L.), and the diagnosis was confirmed by morphology on histological slides stained with hematoxylin and eosin (H&E) or Giemsa and by immunophenotyping. The main clinical features of our samples are summarized in Table 2. The study was conducted at the University of Siena in Italy according to the principles of the Helsinki declaration after approval of the local review board. All the procedures were carried out automatically on representative paraffin sections from each case by Bench Mark Ultra (Ventana, Monza, Italy) using extended antigen retrieval and with diaminobenzidine (DAB) as the chromogen.

Cell culture and treatment. Peripheral B cells were purified from blood samples using the RosetteSep human enrichment kit (catalog number 15064; Stem Cell Technologies). LCL were generated in this study by infection of primary B cells from different donors. The myeloma-derived RPMI-8226 cells (http://web.expasy.org/cellosaurus/CVCL_0014) and the BL-derived cell lines, including the BL EBV(-) Louckes cell line (http://web.expasy.org/cellosaurus/CVCL_8259), were obtained from the International Agency for Research on Cancer (IARC) Biobank. The Akata2000 and Akata31 cell lines (35) were also used in the present study. Primary and immortalized B cells were cultured in RPMI 1640 medium (Gibco, Invitrogen Life Technologies, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (PAA; Pasching, Austria) or in advanced RPMI 1640 medium (catalog number 12633012; Life Technologies). EBV (the B95-8 strain) particles produced by culturing HEK293^{EBVgfp} cells were used to infect B cells. EBV infection of B cells was performed either using a recombinant EBV-GFP genome or using the EBV mutant strain EBV Δ LMP1 lacking the entire LMP1. The percentage of GFP-positive cells was assessed by fluorescence-activated cell sorting (FACS; FACSCanto system; Becton, Dickinson) and spanned from 10% to 15% at 24 to 48 h postinfection in Louckes and RPMI cells and 60% to 80% when measured at 48 h postinfection in primary B cells. Analysis of the cell cycle and apoptosis (sub-G₁ phase) was performed by ethanol fixing the cells and staining their DNA with propidium iodide at a final concentration of 5 μ g/ml. Subsequently, cells were analyzed by FACS. Inactivation of EBV was performed with UV light (6×10 mJ). EBV reactivation in BL cells was done by treatment of the cells with TPA (50 ng/ml) in association with NaB (3 mM). Anti-miRNA treatment was done by the addition of Hsa-miR-146a-5p miRCURY LNA miRNA power inhibitor (250 nM; Qiagen) directly to the cell culture medium 24 h before infection with EBV. To induce their activation, primary B cells were seeded at a density of 0.5×10^6 cells in 6-well dishes and treated with 100 ng/ml recombinant human CD40 ligand (hCD40L; catalog number 6245-CL; R&D Systems) and 20 ng/ml of recombinant human IL-4 (R&D Systems) for 48 h. Cells were then collected and processed for further analysis. To block DNA methylation, cells were treated with 5-aza-2'-deoxycytidine ($\geq 97\%$; catalog number A3656; Sigma-Aldrich) at a final concentration of 10 μ M for 48

TABLE 3 Primers used for qPCR and ChIP-qPCR

Primer use and primer	Sequence	
	Forward	Reverse
qPCR		
LMP1	CCAGTCCAGTCACTCATAACG	CCTACATAAGCCTCTCACACT
EBNA1	GGTCGTGGACGTGGAGAAAA	GGTGGAGACCCGGATGATG
DNMT1	GAG GAA GCT GCT AAG GAC TAG TTC	ACT CCA CAA TTT GAT CAC TAA ATC
β 2 microglobulin	CTCACGTCATCCAGCAGAGA	CGGCAGGCATACTCATCTTT
Beta globin	GCATCTGACTCCTGAGGAGA	AGCACACACACCAGCACATT
CCL22	ACTGCACTCCTGGTTGTCCT	CGGCACAGATCTCCTTATCCC
PRDX2	GTG TCC TTC GCC AGA TCA CT	ACG TTG GGC TTA ATC GTG TC
Actin	CTG GGA GTG GGT GGA GGC	TCA ACT GGT CTC AAG TCA GTG
GAPDH	GCCAAAAGGGTCATCATC	TGCCAGTGAGCTTCCCGTTC
KDM2B	CCC AGC ATC TGA AGG AGA AG	GTT GGA GGA ATC AGC CAA AA
LAT2	ACTCCTCTCTCCTGCAGA	CGAGGATAGTAGGGGCAAGG
GNA15	AGAATCGCTTGAACCCAGGA	ATTTTGAACCTCTGGCCTCA
TNFRSF13B	AACTCGGGAAGGTACCAAGG	GAAGACTTGGCCGGACTTTG
TNFRSF18	CTCTTGAACCCGAGCATGG	ACTCGGAACAGCACTCCTC
LTA	ACTCCTCTCTCCTGCAGA	AGGAAGAGACGTTTCAGGTGG
MLKL	AGGTCTAGGCCACACTTGTG	TGCAGGTCATGGGCTTCTAA
BZLF1	AATGCCGGGCCAAGTTTAAGCA	TTGGGCACATCTGCTTCAACAGGA
BDLF1	CGCAGACATGCTCGATGTA	TAGTGGTGCCCCAGGTATG
EBER	CCCTAGTGGTTTCGGACACA	ACTTGCAAATGCTCTAGGCG
BDRF1	CGGAGTGGCTCAGTCAAGG	AGGTGGGCTGACACAGAC
ChIP-qPCR		
CpG island 127	TGACCTCTGCAGCTTCTCT	GATGATCTGCCGCCAACTT
Z prom (0)	TAGCCTCGAGGCCATGCATATTTCAACT	GCCAAGCTTCAAGGTGCAATGTTTAGTG
Z prom (+600)	AGGTATGTTCTGCCAAAGC	GTTTCATGGACAGGTCTGTG
H8 rDNA	AGTCGGGTTGCTTGGGAATGC	CCCTTACGGTACTTGTGACT
BZLF1 prom	GGAGAAGCACCTCAACCTG	CTCCTTACCGATTCTGGCTG
EBNA Cp	AGT TGG TGT AAA CAC GCC GT	TCCACCTCTAAGGTCCCACG
Globin prom	AGGACAGGTACGGCTGTCATC	TTTATGCCAGCCCTGGCTC
GAPDH prom	CGTGCCCAAGTTGAACCCAGG	AGGAGGAGCAGAGAGCGAAG
EBNA Qp	GGCTCACGAAGCGAGAC	GTCGTCACCCAAATTTCTGTC
KDM2B cg21423404	ACCTGACACACCTCAACTCC	TTGTGGTTTGGGAGAAAGGGT
KDM2B cg15695155	CTTGCCCTTCCCCTACTAGAG	CCCTCTTCCCAAACCATG

or 96 h. To inhibit the different pathways, cells were treated with the $\text{I}\kappa\text{B}\alpha$ kinase inhibitor BAY11-7082 (Calbiochem) at a final concentration of 10 μM or with the JNK inhibitor II SP600125 (catalog number 420119; VWR International), used at a final concentration of 10 μM . Cells were preincubated with the different inhibitors for 1.5 h and 2 h.

qPCR. Total RNA was extracted using an AllPrep DNA/RNA minikit (Qiagen). RNA reverse transcription to cDNA was carried out by the use of RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific), according to the manufacturer’s protocol. Quantitative PCR (qPCR) was performed using a MesaGreen qPCR MasterMix Plus for SYBR assay (Eurogentec). For each primer set, the qPCR was performed in duplicate and the mRNA levels obtained were normalized to the average mRNA levels of three housekeeping genes (β -globin, β -actin, and GAPDH) measured in the same samples or to the mRNA level of β_2 -microglobulin only. For each PCR, a sample in which the DNA template was replaced with PCR-grade water was included as a negative control. To measure the EBV genome copy number per cell, total DNA was extracted using an AllPrep DNA/RNA minikit (Qiagen) and measured by use of a NanoDrop spectrophotometer. Similar amounts of DNA were used as a template for TaqMan PCR, performed according to the protocol described by Accardi et al. (36). The PCR primer sequences are indicated in Table 3. All the primers used for the first time in the present study were assessed for their efficiency (90% to 110%).

miRNA-146-5p analysis. To analyze cellular miRNA, total RNA extracted by the AllPrep DNA/RNA minikit (Qiagen) was reverse transcribed using an miRCURY LNA miRNA PCR system (miRCURY LNA RT kit; catalog number 339340) according to the manufacturer’s protocol. The cDNA was then analyzed for the levels of miRNA-146-5p using a specific miRNA qPCR primer (catalog number 339306; Hsa-miR-146a-5p miRCURY LNA miRNA PCR assay) and a PCR kit (catalog number 339345; miRCURY LNA SYBR green PCR kits) on a Bio-Rad qPCR machine (CFX96 Touch real-time PCR).

KDM2B overexpression and gene expression silencing. The KDM2B coding region was cloned into a pCDNA3 vector in frame with a hemagglutinin (HA) tag at the N terminus. LCL (1×10^7) and Louckes cells (5×10^6) were transfected with increasing concentrations of HA-KDM2B pCDNA3 (0.5, 1.0, and 1.5 μg) or with the pCDNA3 vector as a control by electroporation using a Neon transfection system (10- μl tips; pulse voltage, 1,350 V; pulse width, 30 ms; pulse number, 1). At 24 h after transfection, the cells were collected and processed for RNA/DNA extraction. Gene silencing of KDM2B was performed

TABLE 4 Primers used for pyrosequencing

Pyrosequencing primers	Sequence		Sequencing primer
	Forward	Reverse ^a	
KDM2B cg15695155	GGAGTGGGGTAGAGTTGAA	CCTACATACTACTAAACCC	AGGTTTGGT GAGTTTTAGGTGG GGATGGGTAGTT AGGGAAGGAATG AGTGGAGATAATG GGGTGGTTGGGATAG
CpG.127	AAATACAACAACCTCCTACC	AAATACAACAACCTCCTACC	TTTGGTTGGTTTGT TTTTTTAAGTATAT TTAAGTTTTTTTTA GTAGGTGGTGATT GGTTATTAGAGT GTTGTTTATATG TTAATATAATGGT
KDM2B cg21423404	GATAAGTATAGGGAGGTTTGTGA GGGTGGAAATGGTTTTATAG	CTATAAAACCATTCCAACCC CCTCCCTAATAACTAAACTACA	GGATGGGTAGTT TGTTTTGGTTAT GTAGGTGGTGATT AGTGYTTTTTGTGA
KDM2B cg15695155	GAGTTTTAGGTGGTAYGGATG	GAYGGATAGGGAGGAGTTAGT	
KDM2B cg00031896	GTAAAGGAGGAAATTAGGATTA	TATGTTTTAAAGGAGTTGTATG	
KDM2B cg21423404	AGGAGGAGTTTAGAGTTATAGT	AGTTATTGTAGGGGTAGATTTTAG	
KDM2B cg12251659	GGAGGGAGTGGGAGGTAT	TTGGAGGGTYGAGTTGTAGG	

^aThe reverse primer was labeled with biotin at the 5' end.

using KDM2B (human) unique 27-mer siRNA duplexes (catalog number HSS150072; Thermo Fisher Scientific). LCL (1×10^7) and Louckes cells (5×10^6) were transfected with the siRNA (final concentration, 250 nM) by electroporation using the Neon transfection system (10- μ l tips; pulse voltage, 1,350 V; pulse width, 30 ms; pulse number, 1). At 48 h after transfection, the cells were collected and processed for RNA/DNA extraction. The levels of silencing were evaluated by qPCR using KDM2B-specific primers, indicated in Table 3.

Immunoblotting and antibodies. Whole-cell lysate extracts were obtained using lysis buffer, as previously described (37). The cell extracts were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for immunoblotting using standard techniques. The following antibodies were used for immunoblotting: KDM2B (catalog number 09-864 from Merck Millipore and catalog number ab5199 from Abcam), mouse monoclonal anti-human MLKL (catalog number SC-293201; clone 3B2; CliniSciences), LMP1 (S12 monoclonal antibody), β -actin (clone C4; MP Biomedicals), and GAPDH. Images were produced using a ChemiDoc XRS imaging system (Bio-Rad).

Immuno-FISH. Fifty thousand cells were resuspended in 5 μ l of phosphate-buffered saline (PBS). The cells were gently spread on a microscope glass slide, air dried, and fixed in 4% paraformaldehyde-PBS for 10 min at room temperature. Slides containing fixed cells were washed 3 times in PBS for 5 min, permeabilized with PBS-0.5% Triton X-100 (Sigma-Aldrich) for 15 min, and then washed twice with PBS-0.05% Tween. The slides were then soaked in methanol-0.3% H₂O₂ for 30 min and incubated for 1 h with antibody diluent (catalog number S3022; Dako) and then for 30 min with Image-iT FX signal enhancer. The slides were incubated overnight at 4°C with an anti-KDM2B antibody (catalog number ab5199; Abcam) diluted to a concentration of 1 μ g/ml, followed by incubation with a secondary antibody (anti-goat immunoglobulin; 5 μ l/ml; Elite kit; Vector). To amplify the signal, the slides were incubated for 30 min at 37°C with ABC kit reagents according to the manufacturer's protocol. EBV DNA staining by FISH was performed as previously described (36), using a biotinylated probe to the EBV DNA genomic region BWRP1 (A300P.0100 DS-Dish-Probes). The stained cells were visualized with a fluorescence microscope with an incubator (Nikon Eclipse).

Immunohistochemistry and ISH for EBER. Immunohistochemistry analysis for KDM2B (dilution, 1:200; catalog number ab5199; Abcam) was performed by an automated staining system (Ventana BenchMark Ultra; Roche Diagnostics, Monza, Italy) on formalin-fixed, paraffin-embedded 4- μ m-thick sections. An UltraView universal detection kit (Ventana) using a horseradish peroxidase multimer and DAB (as the chromogen) was used. ISH for EBER was carried out in each sample on 4- μ m-thick sections, as previously reported (38). A control slide, prepared from a paraffin-embedded tissue block containing a metastatic nasopharyngeal carcinoma in a lymph node, was used as a positive control.

Chromatin immunoprecipitation. ChIP was performed with Diagenode Shearing ChIP and OneDay ChIP kits according to the manufacturer's protocols. The following antibodies were used: KDM2B (catalog number ab5199; Abcam), DNMT1 (catalog number MAB0079; Abnova), and IgG (Diagenode). The eluted DNA was used as a template for qPCR with primers designed on the *KDM2B* gene. The primers used for quantitative ChIP are listed in Table 3. The value of binding obtained for each antibody was calibrated on the input sample and normalized to the values for IgG.

Bisulfite modification and pyrosequencing. Samples for pyrosequencing were processed as previously described (4, 39). The primers are indicated in Table 4.

Whole-genome expression analysis. Differential expression analysis was performed using human HT-12 expression BeadChips (Illumina) as previously described (4, 36). Probes with *P* values of <0.01, a false-discovery rate (FDR) of <0.05, and a fold change in expression of at least 1.5 were considered differentially expressed.

Statistical analysis. Statistical significance was determined by Student's *t* test. The *P* value of each experiment is indicated in the corresponding figure legend. Error bars in the graphs represent the standard deviation.

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R.A. designed, analyzed, and interpreted the experiments and wrote the first draft of the manuscript. H.G. and R.C.V.-A. contributed to the design, execution, and analysis of the experiments, in addition to revising the initial submission. R.A., H.G., Z.H., A.J., R.C.V.-A., L.M., L.L., F.M., H.H.-V., A.D., M.P.C., A.R., M.G.C., M.C.R.-M., C.C., G.D., C.S., M.A.M., F.L.C.-K., and E.M. contributed to the execution of the experiments, prepared the figures and tables, and reviewed the manuscript; in addition, they all contributed to revising the initial submission and the subsequent versions of the article.

We declare that we have no competing interests.

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article, and they do not necessarily represent the decisions, policy, or views of the International Agency for Research on Cancer/World Health Organization.

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Epigenetic modifications of the cancer-related gene TGFBI in B-cells exposed to Aflatoxin and infected with Epstein-Barr virus: potential role in the development of Burkitt's lymphoma

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• INTRODUCTION

Epstein-Barr virus (EBV) infection is associated with several diseases, including Burkitt's Lymphoma (BL), a paediatric cancer common in sub-Saharan Africa. The geolocation of the endemic form of BL suggests the involvement of environmental factors such as malaria in the development of this pathology. Our previous study showed that the dietary contaminant aflatoxin B1 (AFB1) and EBV cooperate in the process of B-cell transformation in different experimental models. The underneath mechanism remains obscure. It is well established that exposure to certain environmental factors can modify the cell DNA architecture and stably alter the expression of genes with a role in transformation. Here we aim to understand whether epigenetic modifications induced in B-cells by early-life exposure to AFB1 could stably modify the expression of lymphoma associated cancer genes and play a role in the development of BL. Moreover, we are interested in studying if these epigenetic alterations could be used as biomarkers of exposure or cancer risk.

• RESULTS

○ Identifying the epigenetic signatures of BL, EBV and AFB1

The mycotoxin Aflatoxin is known to cooperate with viruses in the development of malignancies, for example with HBV in the development of the hepatocellular carcinoma (HCC)⁶⁸. A previous study of our team showed that AFB1 stimulates infection and transformation of B-cells by EBV in *in vivo* and *in vitro* models⁷³. The geographical distribution in Sub Saharan Africa (Figure 10) of the eBL colocalise with the presence of AFB1. Furthermore a study from the Epigenetic group of IARC⁷⁴ showing an AFB1-specific epigenetic signature found in the blood of Gambian children exposed to AFB1 *in utero*, led us to the hypothesis that

an *in utero* and early life exposure to AFB1 associated to an EBV infection might lead to the development of a Burkitt Lymphoma through an epigenetic mechanism.

The results of our first study showed that EBV deregulates KDM2B levels through an epigenetic mechanism, providing evidence for a role of KDM2B in regulating virus and host cell gene expression and EBV-mediated lymphomagenesis. In the second study of the IARC Epigenetic group, we aimed to extend these findings by characterising additional genes that may be involved in EBV-mediated lymphomagenesis. To this end we investigated epigenetic (methylome) profiles of EBV positive BL (BL EBV(+)) cell lines and compared them to those of BL EBV(+) and (-) derived cell lines⁶⁹. We also compared the methylome of BL EBV (+) and (-) to primary B-cells, to obtain a BL associated epigenetic signature (Figure 12a). We next overlapped the three signatures (AFB1, BL EBV, BL) using a Venn diagram from which we obtained a list of cancer-related genes that were found differentially methylated in the blood DNA of Gambian children, in BL EBV(+) derived cell lines and EBV(-) derived cell lines (Figure 12b). Among those genes, we chose to focus on the Transforming growth factor β induced (TGFBI also known as Beta ig-h3/Big-H3).

TGFBI is an extracellular matrix protein that serves as a ligand recognition sequence for several integrins and plays a role in the cell-collagen interactions⁷⁵. As the name suggests, TGFBI is inducible by the transforming growth factor- β and has an effect on cell adhesion⁷⁶. TGFBI has been shown to act as a tumour suppressor gene and its deregulation was proposed to contribute to tumorigenesis^{77,78,79}. Recently, P-A Bissey *et al.* 2018 demonstrated the implication of TGFBI in the Nasopharyngeal Carcinoma (NPC) cisplatin resistance caused by the miRNA miR-449b targeting TGFBI, which alter the TGF β pathway⁸⁰. Additional motivation for focussing on TGFBI comes from the reports showing hypermethylation-mediated deregulation of the genes in several cancers (lung, breast, liver, pancreas and leukaemia)^{81,77,78,79}, although epigenetic deregulation of TGFBI has not been described yet in lymphomas.

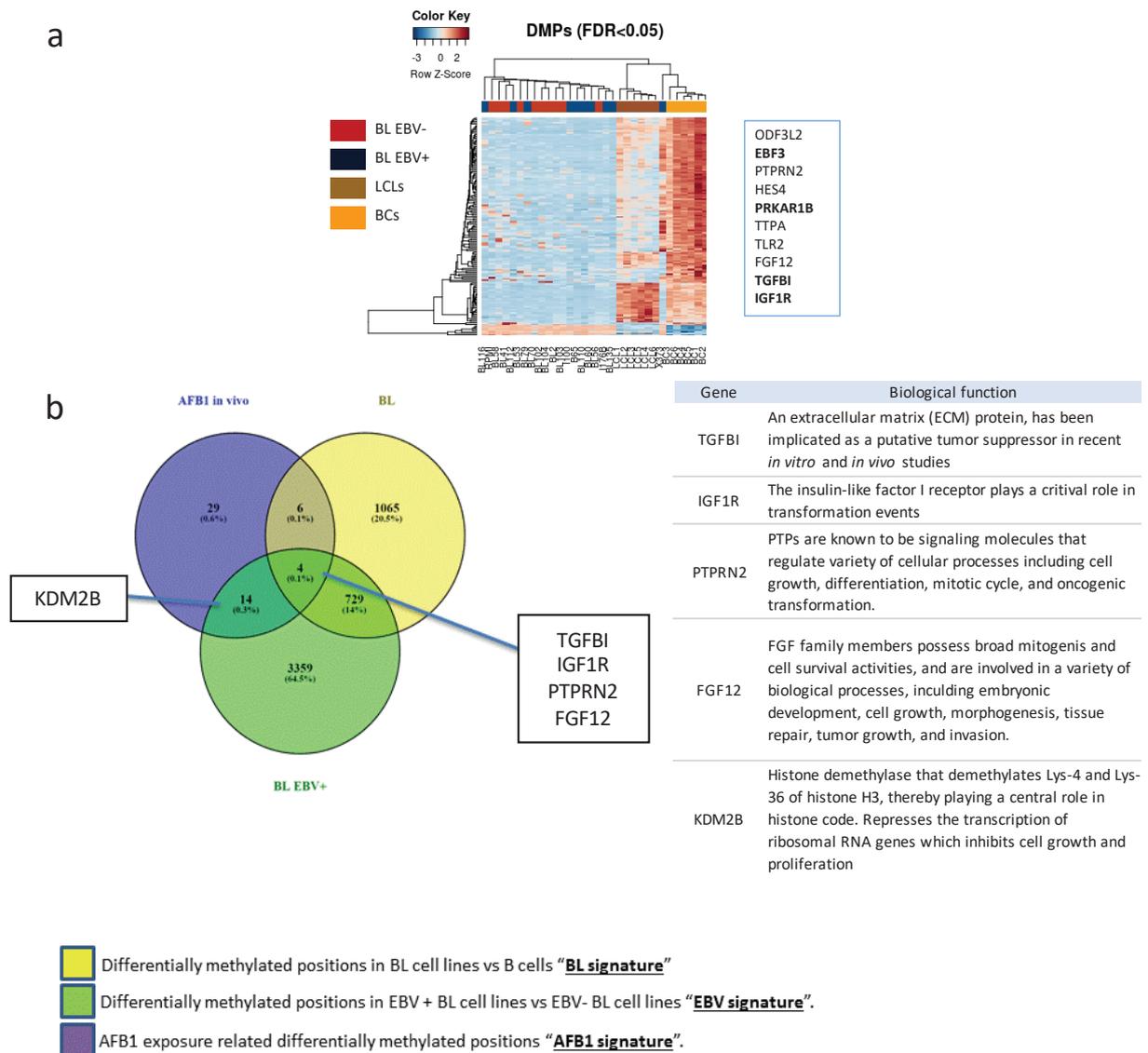


Figure 12. **Identifying the epigenetic signatures of BL, EBV and AFB1.** (a) Heat map of differentially methylated positions (DMPs) identified in the BL (EBV(+)) and EBV(-)) and the primary B-cells (b) Venn diagram showing the comparative analysis of genes in proximity of the deregulated CpGs in the different experimental conditions. Known biological functions of the overlapping genes are reported in the table on the right side.

- Hypermethylation of TGFBI promoter

Taking into account the previous studies on the methylation of TGFBI promoter in a different type of cancers^{77,78} and the epigenetic signatures identified in our study, three different CpGs have been selected for further analysis: the CpG CG00386408 (in this thesis referred as CG00), the CpG CG11482794 (CG11) and the CpG CG21034676 (CG21) (Figure 13a). First, we validated the results generated on the different arrays by pyrosequencing (sequencing technology considered as a "gold standard" for quantitative methylation analysis at single base resolution), using DNA samples from BL-derived cell lines and comparing the methylation levels of our CpGs of interest to those of primary B-cells. Our pyrosequencing analyses showed a significant higher level of methylation on the three CpGs (Figure 13b), confirming the results obtained by the arrays. The results are currently being confirmed *in vivo* by pyrosequencing using DNA of human BL samples and lymph nodes DNA as control. The validation on the human samples is still ongoing, however, the preliminary data showed a significantly higher level of methylation on the CpGs CG11 and CG00 and a slightly higher level on the CpGs CG20 in BLs compare to the controls the Lymph nodes (Figure 13c).

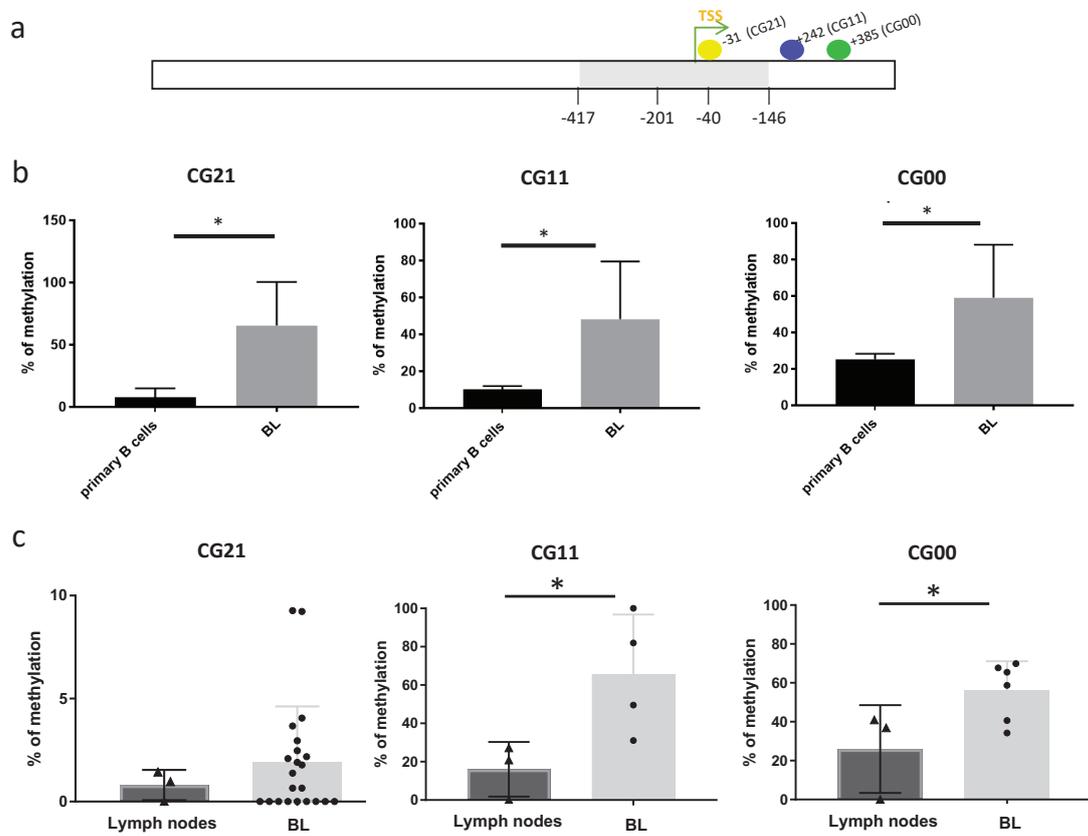


Figure 13. **Hypermethylation of TGFBI promoter.** (a) Representation of the TGFBI promoter featuring the three CpG of interest: CG00386408 (CG00), CG11482794 (CG11) and CG21034676 (CG21). The gray zone corresponds to the area found hypermethylated in several cancers. (b - c) Pyrosequencing analysis for the level of methylation of the CpGs CG21, CG11 and CG00 in DNA samples from primary human B-cells and BL derived cell lines (b) and from lympho-nods from healthy persons and 20 BL specimens (c).

- EBV infection leads to TGFBI silencing

To better understand the impact of EBV on TGFBI, we studied the effect of the virus in an *in vitro* model. We infected primary B-cells with EBV, and the virus immortalises them to become lymphoblastoid cell lines (LCLs). We collected the cells at three different time points to analyse them by qPCR: before the infection (B-cell), 48h after infection (B-cell + EBV) and after immortalisation (LCL). We observed that in presence of the virus there is a down-regulation of TGFBI, even during the early stages of the infection and this level is maintained after the B-cell immortalisation (Figure 14a). To rule out that the TGFBI down-regulation is due to the immortalisation process itself, we also infected a BL EBV(-) derived cell line, the Louckes, followed by the analysis of TGFBI expression. We found that in presence of the virus, TGFBI expression is down-regulated compared to the mock-infected cells (Figure 14b), confirming the EBV-specific effect observed in the primary B-cells. We then analysed the protein level of TGFBI in four EBV (-) and three EBV (+) cell lines: the TGFBI protein level was found significantly lower in the EBV positive cell lines compared to the EBV negative ones (Figure 14c). The immunostaining results (obtained in collaboration with Dr Lucia Mundo) revealed similar results using human samples. We compared BL EBV(+), BL EBV(-) and lymph nodes and we observed a noticeable decrease of TGFBI in the BL EBV(+) (Figure 14d). To assess whether an epigenetic mechanism is involved in the down-regulation of TGFBI we next aimed to block the methylation in EBV-infected cells followed by examining the impact on TGFBI expression. To this end, we treated the LCL for 96 hours with 5-aza-2'-deoxycytidine (Aza), an inhibitor of DNA methylation (LCL Aza), or DMSO (LCL NT), and analysed the expression of TGFBI by qPCR (Figure 14e). The results show that the treatment with Aza induces a rescue of TGFBI mRNA level. Because EBV could affect the activity of DNMT1 methyltransferase, the recruitment of DNMT1 on the TGFBI promoter has been measured by Chromatin Immunoprecipitation (ChIP). Our analysis revealed that Louckes cells infected with EBV

exhibited a marked recruitment of DNMT1 on our three CpGs of interest (Figure 15f). Together, these results show that EBV induce a negative regulation of TGFBI, *in vitro* and *in vivo*, and that this regulation may be mediated by an epigenetic mechanism involving the recruitment of DNMT1 on TGFBI promoter.

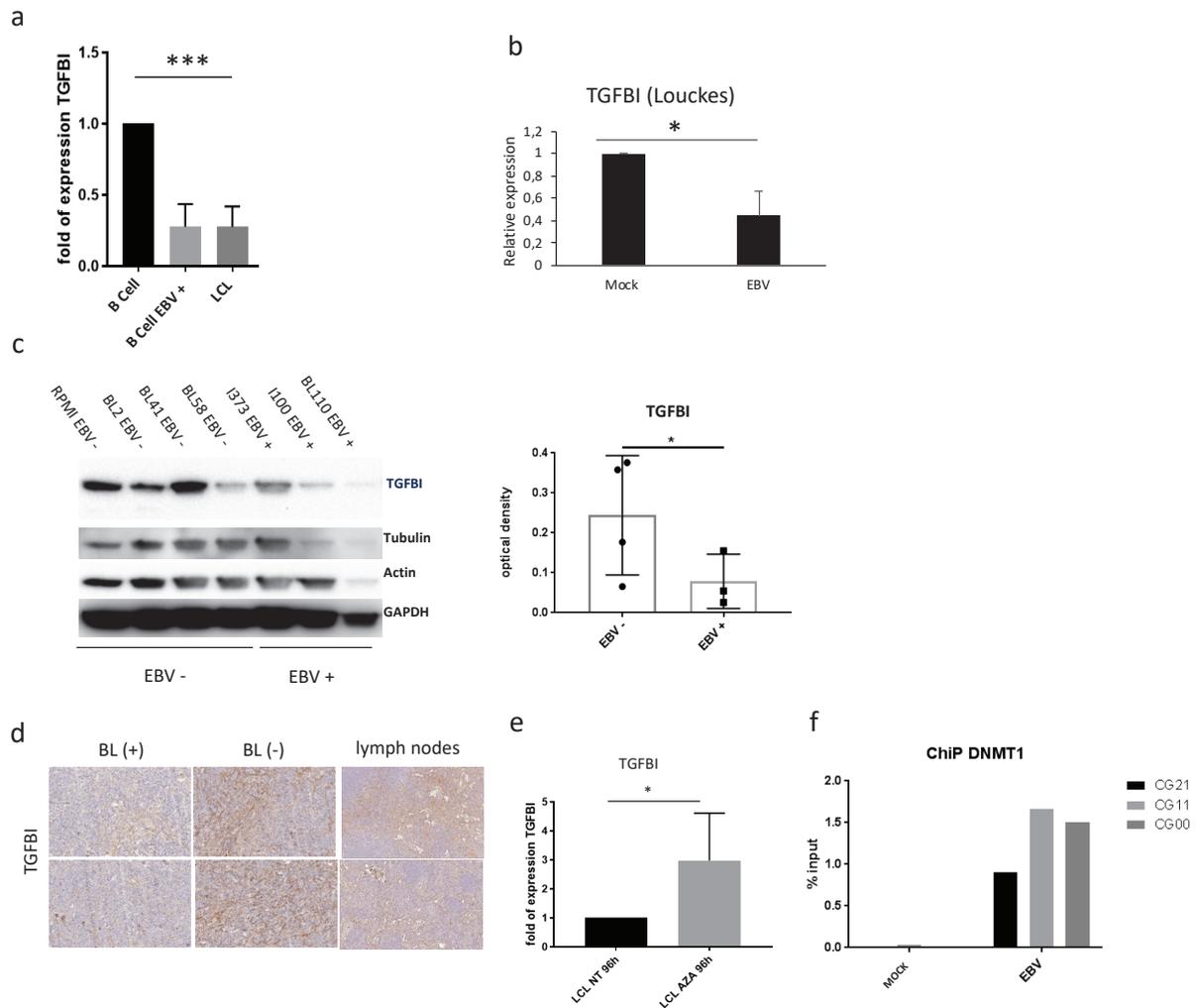


Figure 14. EBV infection leads to TGFBI silencing. (a) Primary B-cells from 3 independent donors were infected with EBV and collected to make dry pellets at t = 0h (B-cell), t = 48h B-cells EBV and t = 4 weeks (LCL). Cell pellets were processed and analysed for the expression level of TGFBI mRNA by RT-qPCR. (b) The EBV(-) cells (Louckes) were infected with EBV (EBV) or not (mock) and after 48h. The cell pellets were processed and analysed for the expression level of TGFBI mRNA by RT-qPCR. (c) Four EBV(-) and 3 EBV(+) BL cell lines were cultured and analysed by immunoblotting for TGFBI expression levels. The histogram shows the average TGFBI protein levels normalized to the β -actin, tubulin and GAPDH signals, measured by Image Lab software (Bio-Rad) in EBV(-) versus EBV(+) BL cells. (d) TGFBI levels in EBV(+), EBV(-) BL and lymph nodes samples were analysed by immunohistochemistry. The images shown were obtained in collaboration with Dr Lucia Mondo. (e) LCL were cultured for 96 h in the presence of DMSO (nontreated [LCL NT]) or 5-aza-2'-deoxycytidine (LCL Aza). Cells were then collected, and the TGFBI expression levels were analysed by RT-qPCR. The histogram shows the average TGFBI mRNA levels measured in 3 independent experiments. (f) Chromatin Immunoprecipitation (ChIP) was used to assess the recruitment of DNMT1 (2 μ g; Abnova MAB0079) on the three CpGs of interest in the presence or absence of the virus in Louckes cells.

- LMP1 is involved in TGFBI silencing

To gain insight into the mechanism by which EBV may regulate TGFBI, we focused on the viral protein Latent Membrane Protein 1 (LMP1). LMP1 is one of the main EBV transforming protein with known function in activating DNMT1 expression⁸² and inducing its recruitment on the promoter of KDM2B⁵⁰. Therefore, we stably expressed LMP1 in a BL EBV(-) derived cell line, the RPMI (Figure 15a). Analysing the TGFBI level we found that the expression of LMP1 in the RPMI cells induced a strong down-regulation of TGFBI compared to the cells expressing the empty pLXSN vector (Figure 15b). Importantly, the over-expression of LMP1 also had an effect on our CpGs of interest, as we detected an increased methylation level of CG11 and the CG21 in presence of LMP1 (Figure 15c).

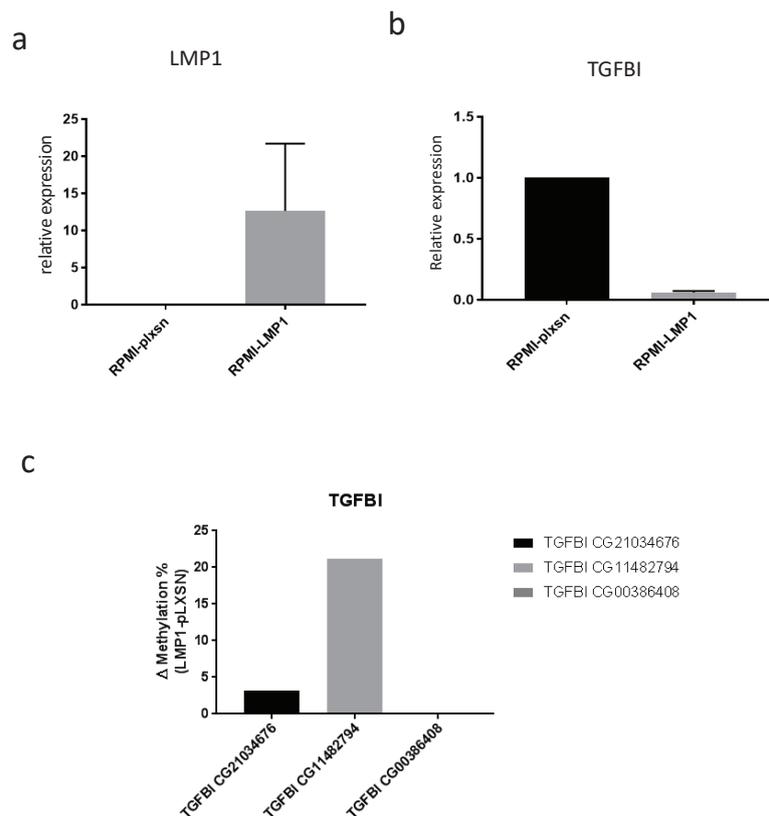


Figure 15. **LMP1 involved in TGFBI silencing.** (a-b) RPMI cell lines retro-transduced by the empty vector pLXSN (RPMI-pLXSN) or by a vector expressing LMP1 (RPMI-LMP1) in four independent transduction experiments. The expression levels of LMP1 (a) and TGFBI (b) were analysed by RT-qPCR. (c) The histogram represents the average difference in the percentage of methylation at the CpGs CG21034676, CG11482794 and CG00386408 between RPMI cells stably transfected by pLXSN-LMP1 and pLXSN respectively. The pyrosequencing results shown are representative of two independent experiments.

- AFB1 exposure leads to TGFBI silencing

To assess a potential cooperation between EBV and AFB1 we next investigated the AFB1 effect on TGFBI. We exposed the RPMI and the Louckes cell lines to AFB1 for 24 hours and analysed the expression of TGFBI. We obtained a negative regulation of TGFBI in the cells treated with AFB1 compare to the untreated (DMSO) (Figure 16a). We performed the treatment on RPMI cells using two different concentrations of AFB1: 25 μM and 50 μM , and obtained a stronger inhibition with the concentration 50 μM , so for the next experiment we used this condition. From the same experiment, we analysed the protein levels and observed a decrease of TGFBI in the cells treated with AFB1 compared to the DMSO-treated cells (Figure 16b). These results are correlated with a dose-dependent down-regulation of TGFBI at the expression and protein level. The AFB1 also induced an augmentation of the methylation level in the CpGs of interest, as we observed by pyrosequencing (Figure 16c). Consistent with the increase of the methylation on the TGFBI promoter, we observed an augmentation of DNMT1 at the protein level and an increase in its recruitment on the three CpGs (Figure 16d). These data suggest that AFB1 exposure reduces TGFBI levels by increasing methylation levels of its promoter. This increase in the methylation level was associated with the recruitment of DNMT1 on TGFBI promoter, supporting the notion that AFB1 may be involved in silencing of TGFBI by inducing DNMT1-mediated hypermethylation of its promoter.

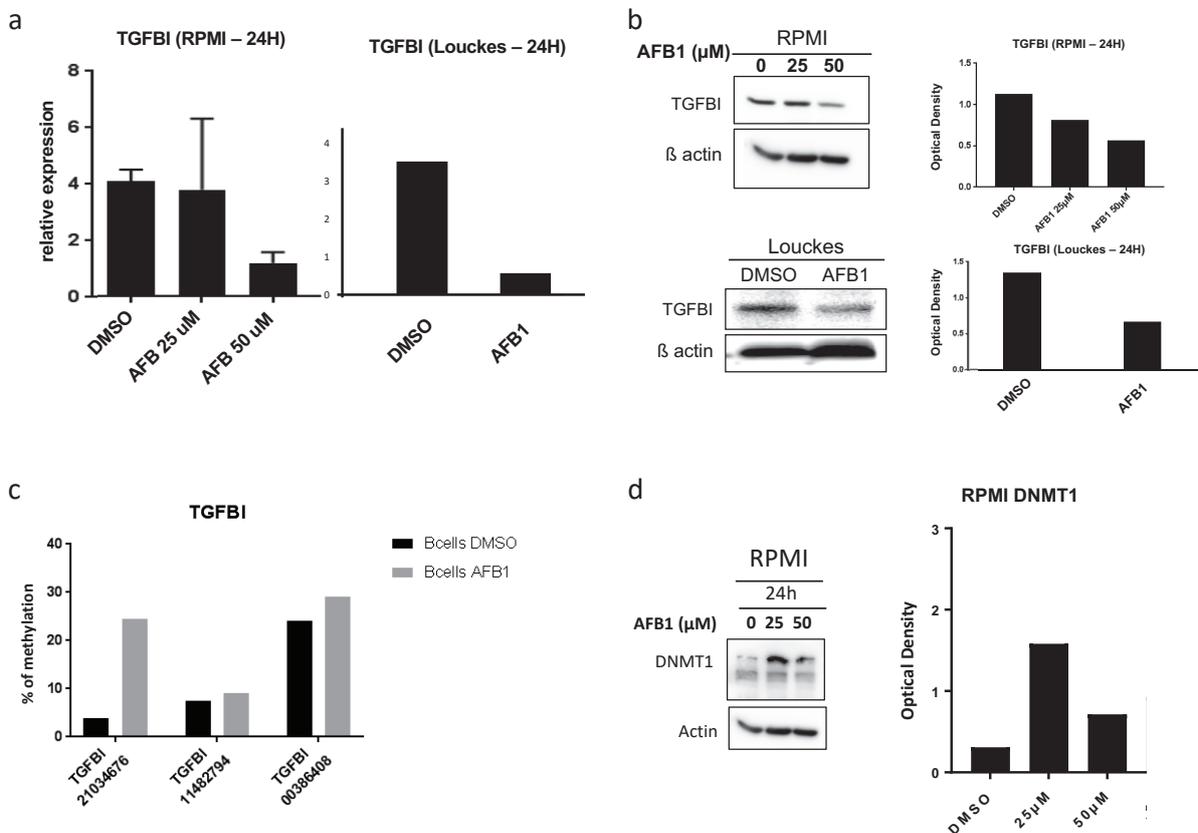


Figure 16. **AFB1 exposure leads to TGFBI silencing.** (a) BL EBV(-) derived cell lines (RPMI and Louckes) were treated with DMSO (the diluent of AFB1) or with AFB1 (25 μ M and 50 μ M for the RPMI and 50 μ M for the Louckes) and collected to make dry pellets at 24h. Cell pellets were processed and analysed for the expression level of TGFBI mRNA by RT-qPCR (b) RPMI and Louckes from the experiment (a) were analysed by immunoblotting for TGFBI levels. The histogram shows the TGFBI protein levels normalized to the β -actin, tubulin and GAPDH signals, measured by Image Lab software (Bio-Rad). (c) The methylation level of the CpGs CG21034676, CG11482794 and CG00386408 in B-cells treated by DMSO or AFB1 50 μ M as determined by pyrosequencing. (d) RPMI (a) were analysed by immunoblotting for DNMT1 levels. The histogram shows the DNMT1 proteins levels normalized to the β -actin, tubulin and GAPDH signals, measured by Image Lab software (Bio-Rad).

- EBV and AFB1 share a common pathway to regulate TGFBI

As we described above, both EBV and AFB1 may negatively regulate TGFBI. In addition, EBV and AFB1 are involved in the recruitment of DNMT1 and increase in the methylation on the TGFBI promoter. To substantiate these findings, we investigated the role of the EBV viral protein LMP1, known to activate the NF- κ B and JNK pathways⁵⁰, in this process. To this end, we inhibited the NF- κ B and JNK pathways, using the Bay11 and the JNKII inhibitor, respectively, in RPMI cells expressing LMP1, and examined the expression of TGFBI. Our results revealed a rescue of TGFBI expression in cells treated with the Bay11 but not in the cells treated with the JNKII inhibitor. This result suggests that LMP1 interferes with the NF- κ B pathway in order to down-regulate TGFBI (Figure 17a). The regulation is accompanied by a recruitment of DNMT1 on the CpG CG21 and CG11 (Figure 17b). In the cells expressing LMP1, there was a recruitment of DNMT1 on both CG21 and GC11, whereas the recruitment is lost when the cells were treated with Bay11 (Figure 17b). To verify if LMP1 also induced the recruitment of DNMT3a and DNMT3b, we performed a ChIP assay using DNMT3a and DNMT3b antibodies. As shown in Figure 17b, no increased recruitment of neither DNMT3a or DNMT3b on the TGFBI promoter (CG21 and CG11) was observed after the expression of LMP1 suggesting that LMP1 activates the NF- κ B pathway and it may reduce TGFBI expression by inducing specific recruitment of DNMT1 to its promoter (Figure 17c). Next Louckes cells were treated with AFB1 and an augmentation of PIKBa was observed, indicating that AFB1 also activates the NF- κ B pathway (Figure 17d). To test if the NF- κ B pathway has an effect on the expression of DNMT1 or its activity we performed two ELISA tests: one to evaluate the quantity of DNMT1 in the nucleus and another to measure the activity of the DNMTs (Figure 17e). We observed that in RPMI pLXSN (LMP1(-)) the Bay11 treatment failed to modify the activity, however when the cells were treated with AFB1 we observed an increase in the activity that is counteracted by the treatment with Bay11. In the presence of LMP1 (RPMI-LMP1), an

increase of DNMTs activity was observed in both DMSO and AFB1 treated cells whereas the DNMT activity decreased after the treatment with Bay11 (Figure 17e left). Similarly, the quantity of DNMT1 protein in the nucleus increased in presence of AFB1, LMP1 and LMP1+AFB1, while DNMT1 protein level decreased after the treatment with Bay11 in all the conditions except in the RPMI-LMP1 treated with DMSO (Figure 17e right).

Based on these results, both EBV and AFB1 appear to affect the activity of the DNMTs, whereas only the AFB1 has an effect on the quantity of DNMT1. Further experiments are needed to confirm these preliminary data and an enzymatic assay specifically targeting DNMT1 should be particularly informative. Furthermore, an experiment inhibiting p65 and IKK should also provide important mechanistic insight. These additional data would allow a better understanding of the mechanism of interaction between the NF- κ B pathway, EBV and AFB1 and their impact on the DNMT1 activity and/or expression (Figure 17f).

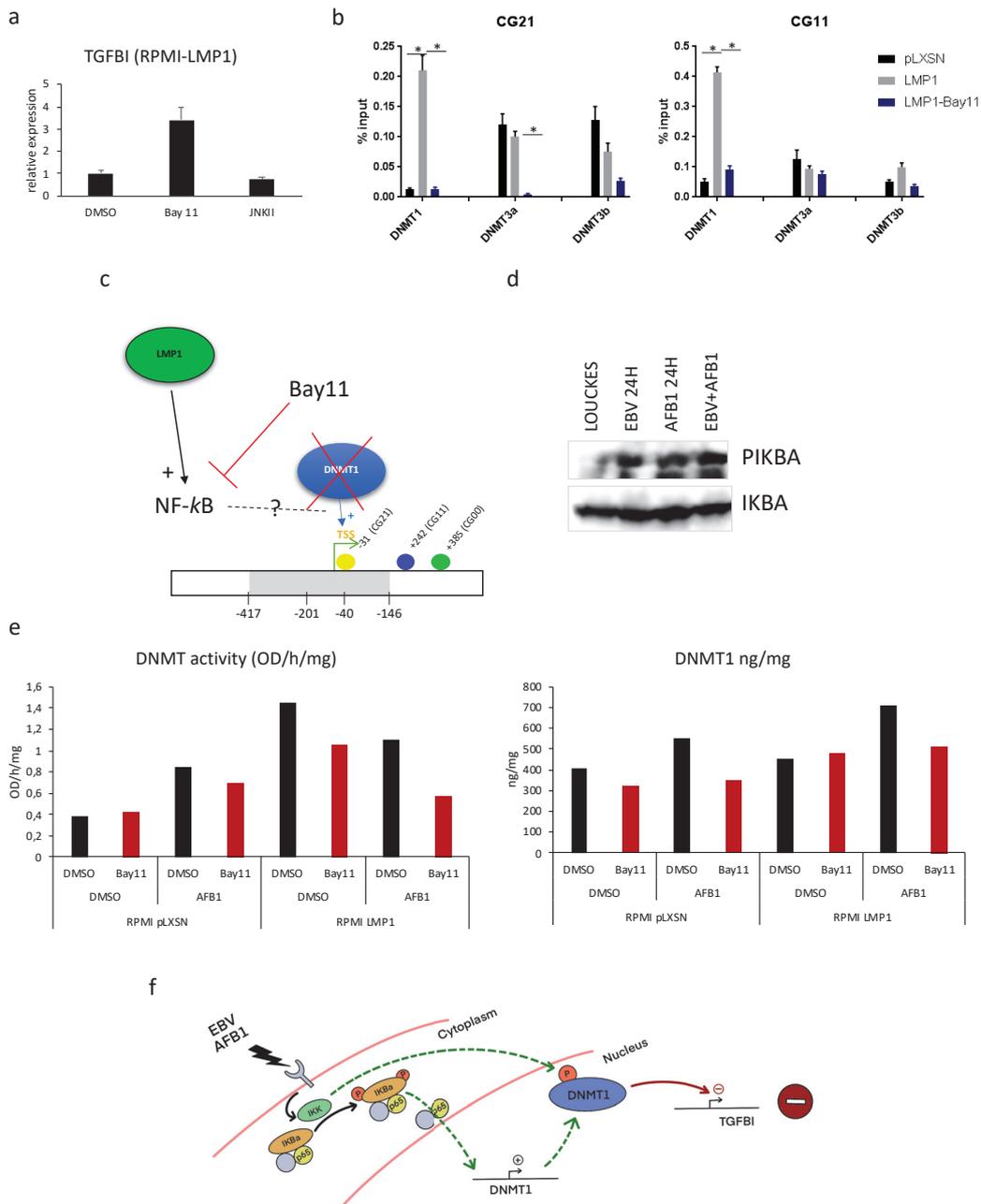


Figure 17. EBV and AFB1 share a common pathway to regulate TGFBI. (a) RPMI-LMP1 cells were treated for 2 h with BAY11-7082 (Bay11) (10 μ M) or with JNK inhibitor II (JNKII) (10 μ M) in three independent experiments. TGFBI mRNA levels were analysed by RT-qPCR. (b) RPMI pLXSN and RPMI-LMP1 cells, the latter of which were untreated or treated with BAY11-7082 (10 μ M) for 2 h, were fixed to perform ChIP with DNMT1, DNMT3a, DNMT3b or IgG antibodies. The eluted DNA was analysed by qPCR with primers designed to surround CG21034676 and CG11482794. The histogram shows the average percentage of recruitment in 3 independent experiments. (c) Representation of the hypothesis that the activation of the NF- κ B pathway by LMP1 causes the recruitment of DNMT1 on the TGFBI promoter. (d) Louckes infected or not by EBV and treated or not by AFB1 50 μ M were analysed by immunoblotting for Phospho-I κ B α (PIKBA) and I κ B α (IKBA) levels. (e) Measure of the DNMTs activity on the left (OD/h/mg) and the DNMT1 quantity in the nucleus on the right (ng/mg) on RPMI cell lines pLXSN or pLXSN-LMP1 treated by DMSO or AFB1 50 μ M and treated or not by Bay11 (μ M). The extraction and kit used are described in the Materials and Methods. (n=1) (f) Representation of our working model about the effect of EBV and the AFB1 on the NF- κ B pathway to modify the TGFBI expression either by a post-translational modification of DNMT1 or a modification of DNMT1 expression.

- EBV and AFB1 synergise in TGFBI down-regulation

The data presented so far indicate that EBV and AFB1 share a common pathway to down-regulate TGFBI, but do they have a synergic effect? To answer this question, we treated primary B-cells with AFB1 or DMSO for 24h (AFB1 pre-treatment), followed by infecting them with EBV for 24h. The cells were immortalised after 3 to 4 weeks in culture (LCL) (Figure 18a) and they were collected and analysed for the gene expression. We observed that TGFBI expression was significantly lower in the LCLs obtained from primary B-cells pre-treated with AFB1 (LCL from AFB1) compared to the LCLs obtained from the primary B-cells pre-treated with DMSO (LCL from DMSO). Infection with EBV was confirmed by the presence of LMP1 and BZLF1 (EBV viral genes) mRNA (Figure 18b). As expected, the methylome of the LCL from AFB1-treated primary cells was also impacted; EBV infection was found associated with a distinct pattern of methylation (EBV signature). Interestingly, we obtained a different methylome pattern in the LCL pre-treated with AFB1. The methylome analysis was performed on 3 different donors to confirm the results (Figure 18c). This difference was also present on the CpGs of interest, which showed a higher methylation level in LCLs from AFB1 compared to LCLs from DMSO (Figure 18d). Our data shows that EBV and AFB1 down-regulate TGFBI using a common pathway and synergistically induce an enhanced down-regulation of TGFBI through the methylation of the TGFBI promoter.

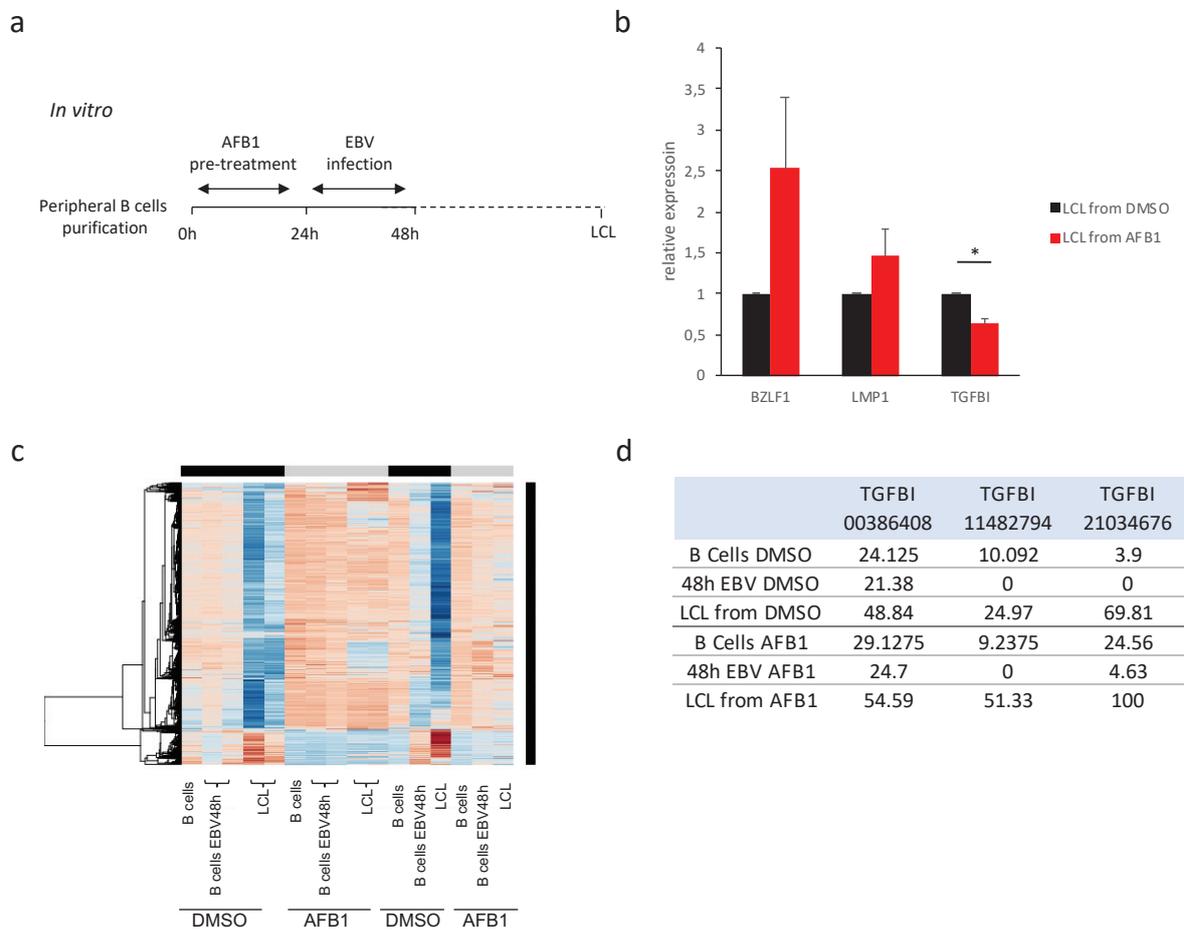


Figure 18. EBV and AFB1 synergise in TGFBI down-regulation. (a) Schema of the experiment: primary B-cells from different healthy donors were exposed to AFB1 ($50\mu\text{M}$) or to DMSO as negative control, for 24h. Cells were then infected by EBV and either collected 48h post-infection or let grow to generate Lymphoblastoid Cell Lines (LCL). (b) Primary B-cells from 3 different donors were treated as explained in (a) and RNA extracted at different time point from these cells retro-transcribed and analysed for the levels of TGFBI and two EBV viral genes by RTqPCR. (c) Methylation of primary B-cells from 3 different donors were analysed as described in (a). (d) DNA samples from cells prepared as in (a) were analysed for the methylation level of TGFBI CpGs.

- EBV proteins, other than LMP1, are also involved in TGFBI regulation

As described above, LMP1 plays a role in the down-regulation of TGFBI, however, LMP1 is not expressed during the latency I which is the one corresponding to the BL. Therefore, we checked if other viral proteins are involved in the regulation of TGFBI. To this end, we infected primary B-cells with an EBV wild type (EBV) and a recombinant EBV lacking the LMP1 gene (EBV Δ LMP1) (Figure 19a and b). The infection of primary B-cells with a recombinant EBV showed that, even without the LMP1 gene, EBV is still able to induce the down-regulation of TGFBI, at both the RNA and at protein level (Figure 19c and d). In addition, the results obtained in collaboration with Henri Gruffat from ENS Lyon (Figure 19e and f) suggest that, although LMP1 is involved in the regulation of TGFBI, it is not the only viral protein interfering with TGFBI expression. B-cells expressing LMP2A, EBNA1, EBNA2, EBNA3B, EBNA3C or EBNA1P have a lower level of TGFBI mRNA compared to B-cells expressing the empty vector (pBS) (Figure 19e). We also observed that in the presence of all the viral proteins transfected (ALL + LMP1) the TGFBI expression was comparable to that obtained after EBV infection and even lower than in the cells expressing all the viral genes except LMP1 (ALL) (Figure 19f). These data suggest that LMP1 and other viral proteins cooperate in the TGFBI down-regulation.

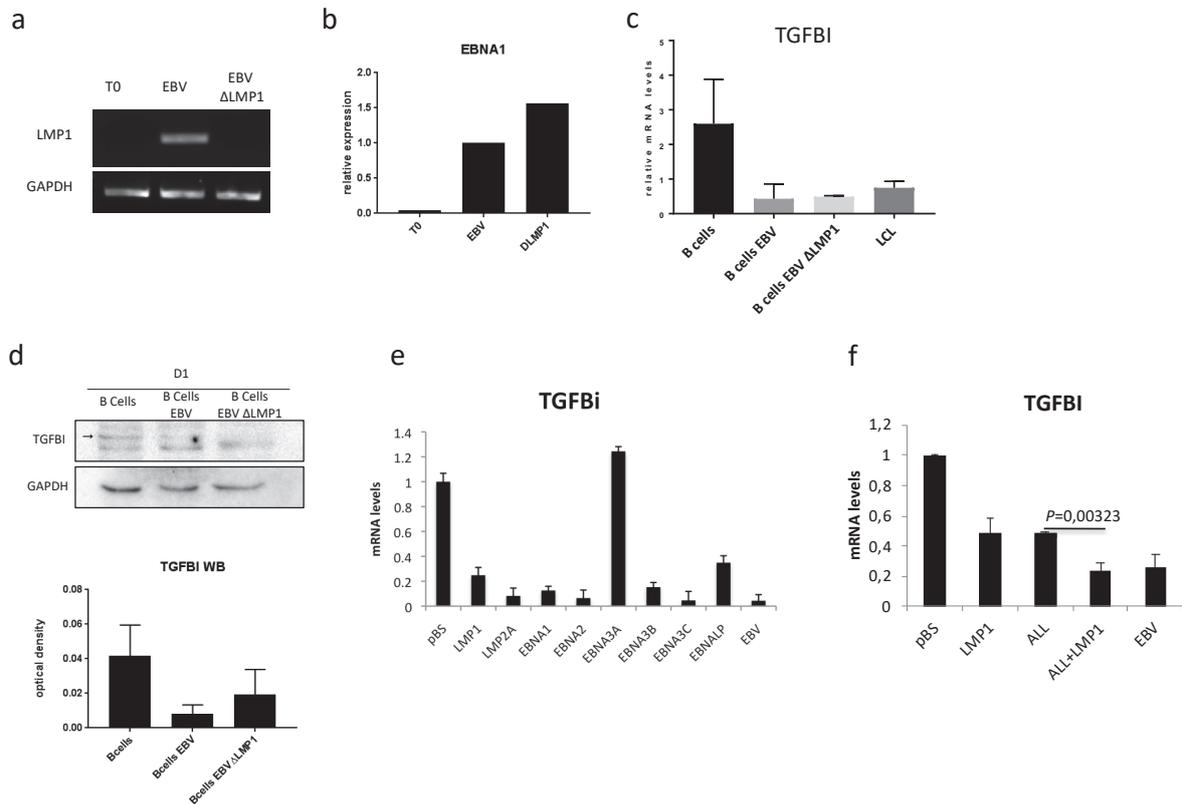


Figure 19. EBV proteins, other than LMP1, are also involved in TGFBI regulation. (a) B-cells from two donors were infected with EBV or EBV Δ LMP1 or not infected (T0) and collected at 48 h after infection. Reverse-transcribed RNA samples were analysed by RT-PCR for the LMP1 expression level. (b) Monitoring the infection of the B-cells by analysing the EBNA1 expression level by RT-qPCR. (c) RT-qPCR to analyse the TGFBI levels in cells from (a) and LCL. (d) B-cells (a) were analysed by immunoblotting for TGFBI levels. The histogram shows the TGFBI proteins levels normalized to the GAPDH signals, measured by Image Lab software (Bio-Rad). (e) Analysis by RT-qPCR of TGFBI level in B-cells expressing viral genes by transfection. (f) Analysis by RT-qPCR of TGFBI level in B-cells expressing the empty vector (pBS), LMP1 (LMP1), all the viral genes except LMP1 (ALL), all the viral genes including LMP1 (ALL + LMP1) or B-cells infected with EBV (EBV). Data obtain in collaboration with Henry Gruffat from ENS.

- The biological role of TGFBI down-regulation by EBV

Because our results described above demonstrate that EBV down-regulates TGFBI, it is plausible to propose that TGFBI plays an important role in EBV infection. To examine the biological significance of TGFBI down-regulation by EBV, we over-expressed TGFBI in the EBV(+) cell line, Akata 2000 (Akata), and in the EBV(-) cell line, Louckes, together with three different concentration of the TGFBI expression construct (0,2 μ g; 0,5 μ g; 1 μ g) or the empty vector (pCDNA3) as a control (TGFBI 0) (Figure 20a). The cells were collected 48h after transfection and TGFBI levels were verified by RT-qPCR (Figure 20a). In the EBV negative cells, we observed a similar percentage of dead cells in all conditions, indicating that TGFBI over-expression does not have an effect on the viability of the Louckes. On the other hand, there is a marked effect of the TGFBI over-expression on the viability of the Akata cells, even if they are affected by the transfection without TGFBI expression (Figure 20b). We observed an enhanced effect of TGFBI over-expression on the Akata when the cell cycle of these cells was analysed by flow cytometry (Figure 20c). A higher proportion of cells in apoptosis (P3) in all three conditions of TGFBI over-expression was observed. In order to assess the number of viral DNA copies per cell in the Akata from Figure 20a, we performed a TaqMan PCR. As shown in Figure 20d, we observed a diminution of viral DNA copies per cell when TGFBI was over-expressed (Figure 20d). We further found that over-expression of TGFBI impacts the cell cycle of EBV(+) cell lines, favouring apoptosis. These results could be explained by the loss of viral DNA; therefore, in the absence of EBV, apoptosis could not be repressed.

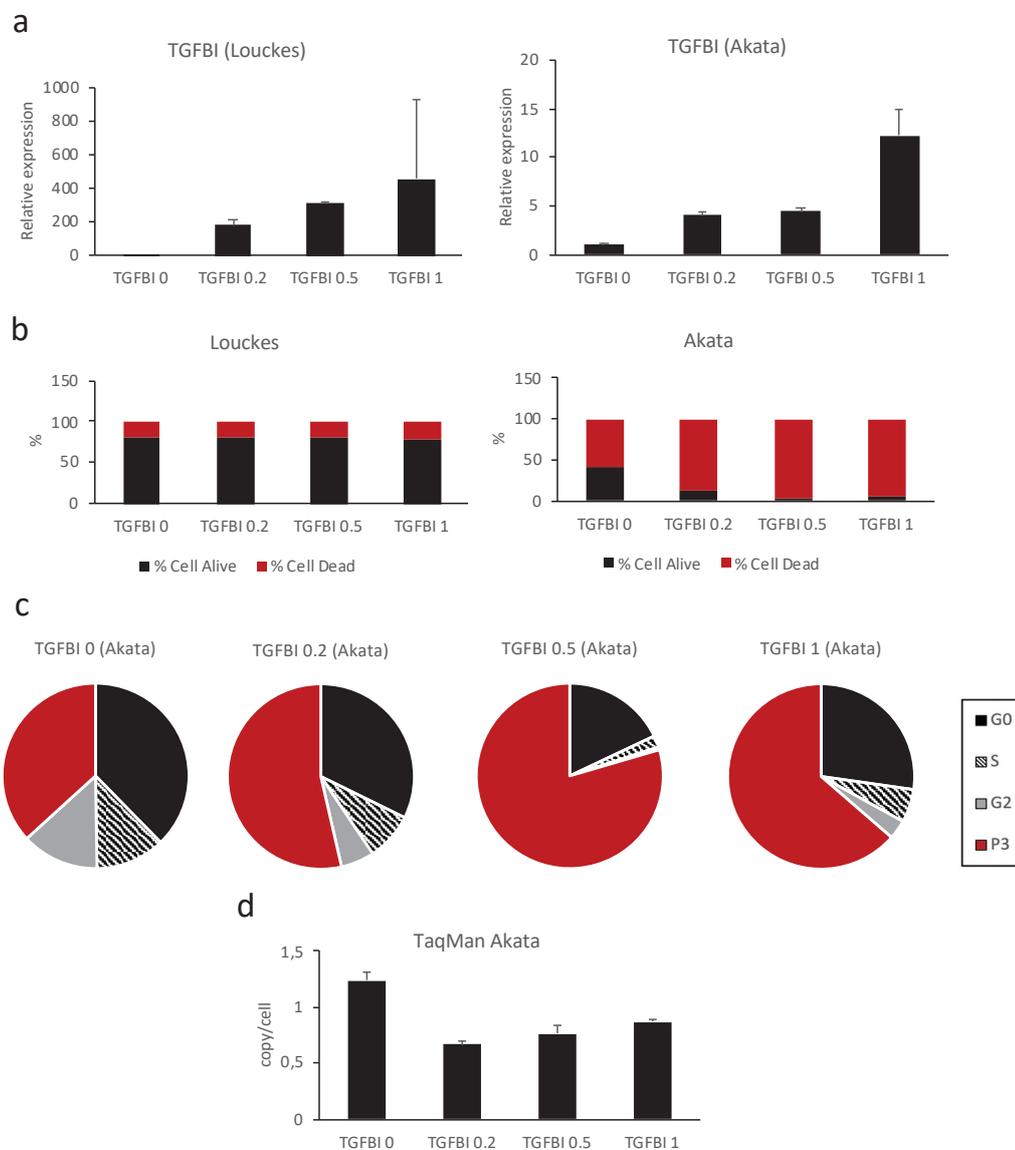


Figure 20. The biological role of TGFBI down-regulation by EBV. (a) Two different cell lines, the Louckes (EBV(-)) and the Akata 2000 (Akata) (EBV(+)) were transfected with a vector (pCDNA3) including the TGFBI coding region with the increasing concentration 0,2 μg ; 0,5 μg ; 1 μg (respectively TGFBI 0,2; 0,5 and 1) or with the empty vector (TGFBI 0) as a control. At 48 h after transfection, the cells were collected and the levels of TGFBI were evaluated by RTqPCR. (b) Percentage of the cells alive and dead from (a). (c) Transfected cells from (a) were ethanol fixed and processed for cell cycle analysis by flow cytometry. (d) DNA samples of cells from (a) were used to measure EBV genome copy number by TaqMan PCR.

- Exosomal epigenetic signature of BL

There is a growing interest in epigenetic signature as cancer biomarkers. Moreover, there is a need to develop markers for diagnosing BL at early stages, and to differentiate BL from other types of lymphomas. In our study, we aimed to investigate whether the methylation levels of 2 CpGs of interest (CG11 and CG21) in the exosomal DNA could be potential markers of BL. For this, we extracted DNA from the exosomes enriched from the supernatant of different cultured cells, followed by bisulfite conversion and methylation analysis of the CpGs by pyrosequencing (Figure 21). Consistent with our previous findings, low levels of methylation were found on exosomal DNA from the controls (lymph nodes and primary B-cells) and higher levels in the BL and LCL samples. Interestingly, the same high level of methylation is found in the exosome extracts (“lymphoblastoid cell line exosome”) from the same BL cell line (“lymphoblastoid cell line”), as shown in Figure 21. This result indicates that the methylation pattern of the two CpG CG11 and CG21 is preserved in the exosomes. These findings showing that the methylation pattern corresponding to the BL signature could be found in circulating DNA, which may prove attractive targets for the development of a potential future non-invasive biomarker.

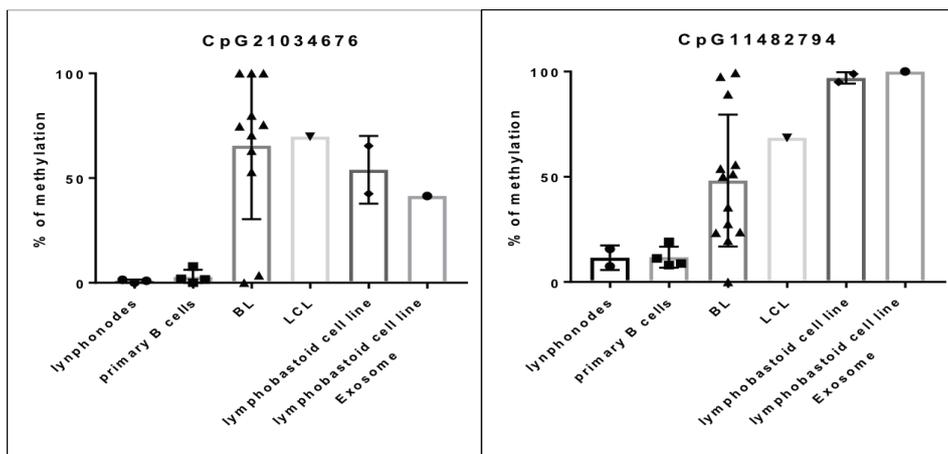


Figure 21 : **Exosomal epigenetic signature of BL.** Analysis by pyrosequencing of the methylation level of CpGs CG11 and CG21 in DNA from healthy lymph nodes, primary B-cells, BL cell line, LCL and BL-derived exosomes.

- Conclusions

The data presented in this study show an effect of EBV and AFB1 on TGFBI expression. We based our work on previous studies⁷⁴, showing an AFB1 epigenetic signature from the blood DNA of Gambian children exposed to AFB1 and an EBV epigenetic signature in BL comparing BL EBV(+) to BL EBV(-). We found a third signature comparing the methylome of BL (EBV + and EBV -) to primary B-cells: the BL associated epigenetic signature. When we overlapped these three signatures, we obtained a list of cancer-related genes and among them, we selected TGFBI for detailed characterisation.

We focused on 3 CpGs found differentially methylated in the three arrays experiments, and we confirmed the results of the arrays by pyrosequencing with *in vitro* and *in vivo* samples. It is interesting to notice that the CpG CG11482794, that was found hypermethylated in the blood DNA of Gambian children exposed to AFB1, is also hypermethylated in BL samples *in vitro* and *in vivo*. We found that the EBV infection has an effect on the expression of TGFBI, which is down-regulated in the EBV(+) cells, and this regulation appears to have an underlying epigenetic mechanism. To gain a better understanding of the mechanism used by EBV to induce the TGFBI down-regulation, we first focused on studying LMP1, the main transforming protein of EBV. By expressing LMP1 in BL derived cell line EBV(-), we obtained a down-regulation of TGFBI coupled with methylation of the CpGs CG11 and CG21. Additional results obtained with our collaborator from ENS showed that other viral proteins can down-regulate TGFBI. This suggests that although LMP1 is involved in the down-regulation of TGFBI through an epigenetic mechanism, it is not the only viral protein involved in this regulation.

We then investigated the effects of the AFB1 on TGFBI. Similarly to EBV, AFB1 induces a down-regulation of TGFBI and an augmentation of the methylation on the CpGs of interest with an increase of the epigenetic enzyme DNMT1 at the protein level. These data led us to the same conclusion as with the viral infection: AFB1 has an impact on TGFBI regulation through

an epigenetic mechanism. Since EBV and AFB1 have the same effect on TGFBI, we also found that they share the same pathway. When we infected or treated EBV (-) cell with EBV or AFB1 we obtained an augmentation of the NF-kB pathway activation and an augmentation of the DNMTs activity. At this point, we built our working model (Figure 17f): EBV and AFB1 are regulating DNMT1 (to down-regulate TGFBI) either by modifying its expression or through a post-transcriptional modification. To test our working model, an enzymatic assay specific of DNMT1 would be informative. Moreover, to inhibit more specifically the NF-kB pathway, an experiment using siRNA against P65 and IKK (part of the NF-kB pathway) should be performed in order to better understand by which mechanism DNMT1 is regulated. Further experiments aimed at the analysis on the DNMT1 protein modifications may prove useful in gaining a better understanding of the underlying epigenetic mechanism.

We have shown that EBV and the AFB1 share the same pathway for the induction of TGFBI down-regulation, but do they synergise? By pre-treated primary B-cells with AFB1 before EBV infection we identified a synergic effect on the LCLs obtained from the B-cells in the down-regulation of TGFBI.

Lastly, we started to study the biological role of TGFBI regulation. We over-expressed TGFBI in the EBV(+) cells, the Akata 2000. The over-expression showed an increase in the apoptosis and a decrease in the number of viral DNA per cell. The diminution of the viral DNA load could be an explanation to the increased apoptosis, the virus cannot avoid the cell to enter in apoptosis. Further studies using the transfection in other EBV(+) cell line are needed to confirm that these data are not cell type-dependent.

Finally, it is known that epigenetic modifications are increasingly studied as potential biomarkers. In our case, we found a similarly high level of methylation in the circulating DNA of the CpGs CG11 and CG21. Therefore, methylation of the exosomal DNA represents an

attractive target for the development of a non-invasive biomarker, to help for early detection of BL or to help in the diagnostic of BL.

• Materials and Methods

• Cell culture and treatment.

Peripheral B-cells were purified from blood samples using the RosetteSep human enrichment kit (catalog number 15064; Stem Cell Technologies). LCL were generated in this study by infection of primary B-cells from different donors. The myeloma-derived RPMI-8226 cells (http://web.expasy.org/cellosaurus/CVCL_0014) and the BL-derived cell lines, including the BL EBV(-) Louckes cell line (http://web.expasy.org/cellosaurus/CVCL_8259), were obtained from the International Agency for Research on Cancer (IARC) Biobank. The Akata2000 cell lines (35 : Bryant H, Farrell PJ. 2002. Signal transduction and transcription factor modification during reactivation of Epstein-Barr virus from latency. *J Virol* 76:10290 –10298. <https://doi.org/10.1128/JVI.76.20.10290-10298> .2002.) were also used in the present study. Primary and immortalized B-cells were cultured in RPMI 1640 medium (Gibco, Invitrogen Life Technologies, Cergy-Pontoise, France) supplemented with 10% foetal bovine serum, 100 U/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (PAA; Pasching, Austria) or in advanced RPMI 1640 medium (catalog number 12633012; Life Technologies). EBV (the B95-8 strain) particles produced by culturing HEK293EBVgfp cells were used to infect B-cells. EBV infection of B-cells was performed either using a recombinant EBV-GFP genome or using the EBV mutant strain EBV Δ LMP1 lacking the entire LMP1. The percentage of GFP-positive cells was assessed by fluorescence-activated cell sorting (FACS; FACSCanto system; Becton, Dickinson) and spanned from 10% to 15% at 24 to 48 h postinfection in Louckes and RPMI cells and 60% to 80% when measured at 48 h post-infection in primary B-cells. Analysis of the cell cycle and apoptosis (sub-G1 phase) was performed by

ethanol fixing the cells and staining their DNA with propidium iodide at a final concentration of 5 $\mu\text{g/ml}$. Subsequently, cells were analysed by FACS. To block DNA methylation, cells were treated with 5-aza-2'-deoxycytidine ($\geq 97\%$; catalog number A3656; Sigma-Aldrich) at a final concentration of 10 μM for 48 or 96 h. To inhibit the different pathways, cells were treated with the I κ B α kinase inhibitor BAY11-7082 (Calbiochem) at a final concentration of 10 μM or with the JNK inhibitor II SP600125 (catalog number 420119; VWR International), used at a final concentration of 10 μM . Cells were preincubated with the different inhibitors for 1.5 h and 2 h.

- qPCR

Total RNA was extracted using an AllPrep DNA/RNA minikit (Qiagen). RNA reverse transcription to cDNA was carried out by the use of RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific), according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using a MesaGreen qPCR MasterMix Plus for SYBR assay (Eurogentec). For each primer set, the qPCR was performed in duplicate and the mRNA levels obtained were normalized to the average mRNA levels of three housekeeping genes (β -globin, β -actin, and GAPDH) measured in the same samples. For each PCR, a sample in which the DNA template was replaced with PCR-grade water was included as a negative control. To measure the EBV genome copy number per cell, total DNA was extracted using an AllPrep DNA/RNA minikit (Qiagen) and measured by use of a NanoDrop spectrophotometer (Thermo scientific). Similar amounts of DNA were used as a template for TaqMan PCR, performed according to the protocol described by Accardi et al. (36 : Accardi R, Gruffat H, Sirand C, Fusil F, Gheit T, Hernandez-Vargas H, Le Calvez-Kelm F, Traverse-Glehen A, Cosset FL, Manet E, Wild CP, Tommasino M. 2015. The mycotoxin aflatoxin B1 stimulates Epstein- Barr virus-induced B-cell transformation in *in vitro* and *in vivo*

experimental models. *Carcinogenesis* 36:1440–1451. <https://doi.org/10.1093/carcin/bgv142>).

The PCR primer sequences are indicated in Table 3. All the primers used for the first time in the present study were assessed for their efficiency (90% to 110%).

Table 5 : Primer used for qPCR and ChIP-qPCR

Gene	Sequence	
	Forward	Reverse
qPCR		
TGFBI	GTCCACAGCCATTGACCTTT	GAGTTTCCAGGGTCTGTCCA
LMP1	CCAGTCCAGTCACTCATAACG	CCTACATAAGCCTCTCACACT
EBNA1	GGTCGTGGACGTGGAGAAAA	GGTGGAGACCCGGATGATG
DNMT1	GAGGAAGCTGCTAAGGACTAGTTC	ACTCCACAATTTGATCACTAAATC
β-globin	GCATCTGACTCCTGAGGAGA	AGCACACACACCAGCACATT
Actin	CTGGGAGTGGGTGGAGGC	TCAACTGGTCTCAAGTCAGTG
GAPDH	GCCAAAAGGGTCATCATC	TGCCAGTGAGCTTCCCGTTC
BZLF1	AATGCCGGGCCAAGTTTAAGCA	AATGCCGGGCCAAGTTTAAGCA
ChIP-qPCR		
CG11482794	CTCCATGGCGCTCTTCGT	CCCCGACTACCTGACCTTC
CG21034676	AAGGGCTGGGAAAAGTCTGAG	GGCTCCAGGGAAAGTGAGAG
CG00386408	CTGCGGAAGGTCAGGTAGTC	ACCTCCCTCCCTCTCTCCTT

- TGFBI overexpression

The TGFBI coding region was cloned into a pCDNA3 vector. Akata 2000 and Louckes cells were transfected with increasing concentrations of TGFBI pCDNA3 (0.2; 0.5; and 1 μg) or with the pCDNA3 vector as a control by electroporation using a Neon transfection system (10-μl tips; pulse voltage, 1,350 V; pulse width, 30 ms; pulse number, 1). At 48 h after transfection, the cells were collected and processed for RNA/DNA extraction. The levels of TGFBI were evaluated by RTqPCR using TGFBI-specific primers, indicated in Table.

- Immunoblotting and antibodies

Whole-cell lysate extracts were obtained using lysis buffer, as previously described (37 : Giarre M, Caldeira S, Malanchi I, Ciccolini F, Leao MJ, Tommasino M. 2001. Induction of Prb degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome P16ink4a-imposed G1 cell cycle arrest. *J Virol* 75:4705–4712. <https://doi.org/10.1128/JVI.75.10.4705-4712.2001>). The cell extracts were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for immunoblotting using standard techniques. The following antibodies were used for immunoblotting : TGFBI (Beta Ig/TGFBI Antibody; BIO-TECHNE SAS; NBP1-88606), Tubulin (SIGMA ALDRICH FLUKA SUPELCO; T4026-.2ML) DNMT1 (DNMT1 antibody (60B1220.1) 0.1mg; NB100-56519 BIO-TECHNE SAS) DNMT3a (Anti DNMT3a antibody [64B1446], 100 ug; AB13888; abcam) DNMT3b (Anti-Dnmt3b [52A1018] antibody; AB13604; abcam) PIKBa (Phospho I κ B α (Ser32/36) (5A5) Mouse mAb; 9246S; Cell Signaling Technology) IKBa (I κ B α Antibody; 9242S ; Cell Signaling Technology) LMP1 (S12 monoclonal antibody), β -actin (clone C4; MP Biomedicals), and GAPDH (Mouse Monoclonal Anti-GAPDH; SC-32233; CLINISCIENCES). Images were produced using a ChemiDoc XRS imaging system (Bio-Rad).

- Immunohistochemistry

Immunohistochemistry analysis for TGFBI (ref) was performed by an automated staining system (Ventana BenchMark Ultra; Roche Diagnostics, Monza, Italy) on formalin-fixed, paraffin-embedded 4- μ m-thick sections. An UltraView universal detection kit (Ventana) using a horseradish peroxidase multimer and DAB (as the chromogen) was used.

- Chromatin immunoprecipitation

ChIP was performed with Diagenode Shearing ChIP and OneDay ChIP kits according to the manufacturer's protocols. The following antibodies were used: TGFBI (ref), DNMT1 (catalog

number MAB0079; Abnova), DNMT3a (ref) DNMT3b (ref) and IgG (Diagenode). The eluted DNA was used as a template for qPCR with primers designed on the TGFBI gene. The primers used for quantitative ChIP are listed in Table. The value of binding obtained for each antibody was calibrated on the input sample and normalized to the values for IgG.

- Bisulfite modification and pyrosequencing

The cells were collected and resuspended in a lysis buffer (1% SDS, 0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris pH8 + Proteinase K 500ug/ml) for 2 hours at 55 °C. The DNA saturation and precipitation have been made with NaCl (6 M) and isopropanol, then clean with 70% ethanol and resuspended in water. The DNA was measured by use of a NanoDrop spectrophotometer (Thermo scientific). Next we performed a bisulfite conversion of the DNA using the EZ DNA methylation Kit (Zymo Research) and modified DNA was eluted in 16 ul of water. We performed the pyrosequencing, as described Hernandez-Vargas, H. et al. Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. PLoS One 5, e9749, doi: 10.1371/journal.pone.0009749 (2010). The primers are indicated in Table 6.

Table 6 : Primers used for pyrosequencing

Pyrosequencing primers	Sequence		
	Forward	Reverse ¹	Sequencing primer
CG11482794	GTTTTGGTTTTGTTTTGGG	TCCCTCCCTCTCCTTCC	GGGTTYGTTAAG
CG21034676	TGGGTGTTTAGGGTAGTTAGGG	CCCAAACCAAACCAAAC	AGTTTATTTAATTTG
CG00386408	GTTTTGGTTTTGTTTTGGG	TCCCTCCCTCTCCTTCC	TGAATTGGGTTGGG

¹ : The reverse primer was labelled with biotin at the 5' end.

- DNMTs assays

Before to proceed to the tests, we did a protein nuclear extraction with the kit ab1134474 from abcam. Then using 10 ug of nuclear extract we did the DNMT1 Assay (DNMT1 Assay Kit ab113469 – abcam) and the DNMT activity assay (DNMT Activity Quantification Kit

(Colorimetric) ab113467 - abcam). We used the ELISA reader Multiskan GO (Thermo scientific) with the software: SkanIt Software Version 5.0.

- Exosome study

For the exosome study, we extracted the exosomes from the supernatant when we was pelleting the cells. For the exosome purification we used the exoEasy Maxi Kit from Qiagen, then for the DNA extraction the QIAamp DNA mini from Qiagen. For the Bisulfite conversion we had to use the Zymo Low DNA Methylation Kit and proceed to the pyrosequencing as describe before.

DISCUSSION

EBV is one of the 9 known human herpesviruses, infecting 90% of the world adult population. In most of the cases the infection has no effect on the health of the individual, nevertheless, in rare cases EBV contributes to the development of neoplasia. For this reason, EBV is one of the seven viruses classified as oncogenic¹². The studies constituting this thesis are focused on EBV and its effects on KDM2B and TGFBI expression/pathways. For both studies, the starting points are the epigenetic signatures obtained *via* whole-genome methylation analysis.

The methylation analysis showed that a regulatory region on the promoter of KDM2B is hypermethylated in the BL EBV(+) compared to the BL EBV(-) samples, confirming our previous data⁸³. This data is in line with other studies, showing that this region is methylated in some cancer-derived cell lines and that KDM2B levels appear to be deregulated in cancers of haematological origin⁸⁴.

To better understand the relation between the methylation of KDM2B and EBV, we treated eBL cell lines with 5-aza-2'-deoxycytidine, a chemical that blocks DNA methylation. The treatment led to a rescue of the KDM2B mRNA level, indicating that DNA methylation contributes to the silencing of KDM2B expression in these cells. The same treatment does not change the level of the KDM2B transcript in sBL EBV(-) derived cell lines, suggesting that EBV infection plays a direct role in eBL pathogenesis by altering the cellular epigenome and deregulating the expression of genes with a key role in lymphomagenesis.

Supporting this hypothesis, the *in vitro* infection of primary B-cells with EBV led to a rapid down-regulation of KDM2B expression. Our data also show that down-regulation of KDM2B by EBV is not due to a specific B-cell activation status, but it needs the viral gene expression. We also show that the KDM2B down-regulation is mediated by DNMT1 recruitment on the promoter and its DNA methylation in EBV-infected cell.

It is known that LMP1 can alter DNMT1⁸²; similarly, here it has been shown that cells stably expressing LMP1 display lower levels of KDM2B, and this ability depends mostly on an NF-

kB pathway activation. The loss of the KDM2B down-regulation is found by blocking the NF-kB pathway *via* a specific chemical inhibitor and by using an LMP1 molecule mutated on its NF-kB-activating domain.

Recently published data showed that HPV16 E6/E7 transforming proteins inhibit the expression of miRNA-146-5p, known to target the KDM2B transcript, which results in an increase of the KDM2B expression level in HPV16-infected cells. In contrast, LMP1 induces miRNA-146-5p *via* its ability to activate NF-kB⁸⁵. Our data show that blocking miRNA-146-5p in primary B-cells before infection leads to a partial rescue of KDM2B levels compared with those in untreated cells. It is therefore possible that the virus uses alternative mechanisms to target KDM2B. In line with these results, our data also indicate that proteins other than LMP1 appear to be able to mediate down-regulation of KDM2B. EBV may need to tightly control KDM2B levels and activity to regulate expression of its own genes as well as that of cellular genes. Our results show that an over-expression of KDM2B induces a down-regulation of viral genes. Altering KDM2B expression in B-cells before EBV infection or in latently EBV-infected B-cells led to the deregulated expression of all analysed viral genes. This was consistent with the recruitment of KDM2B to their respective promoters, as observed by ChIP experiments. Moreover, KDM2B depletion in EBV-infected B-cells led to increased viral gene expression. Taken together, our data show a novel interplay between EBV infection and the host epigenome. EBV alters KDM2B expression *via* an epigenetic mechanism involving LMP1 and other viral proteins expressed during viral latency. The KDM2B, through its histone modifying activity, plays a role in regulating the expression of viral and host cell genes. These data, in addition to the observed deregulated KDM2B levels in BL-derived cell lines, indicate that altered expression of this epigenetic enzyme could contribute to B-cell transformation. Future studies aimed at investigating the functional importance of KDM2B gene methylation and down-regulated expression during the EBV-mediated lymphogenic process are warranted.

The second part of our work focused on the TGFBI gene. TGFBI is found differentially methylated in three different epigenetic signatures: AFB1, BL and EBV signatures^{50,83}. When overlapping the 3 signatures, three CpGs have been identified as differentially methylated (CG00386408 (CG00), CG11482794 (CG11) and CG21034676 (CG21)). Interestingly, TGFBI promoter has been often found hypermethylated in different types of cancer^{77,78,79}; however, no studies were available on Burkitt's Lymphomas and TGFBI.

Our methylome-wide results have been validated by pyrosequencing with *in vitro* samples and *in vivo* specimens, showing a higher level of methylation in the BL compared to the primary B-cells and the lymph nodes. When analysing expression levels, it was noted that TGFBI levels are also impacted. The eBL specimens and derived cell lines showed a low level of TGFBI at the mRNA and the protein level, suggesting that TGFBI down-regulation is an important process in the development of BL. Moreover, the blocking of the DNA methylation on B-cells immortalised by the virus leads to a rescue of the TGFBI mRNA level, indicating that the DNA methylation could be involved in the down-regulation of TGFBI. Because LMP1 was known to up-regulate DNMT1⁸⁶ and to be one of the main EBV transforming proteins, we expressed LMP1 in EBV(-) sBL-derived cell lines. In these cells, LMP1 induced a negative regulation of TGFBI and augmentation of CG11 and CG21 methylation. Our data also show that other EBV viral proteins are involved in the regulation of TGFBI.

The dietary contaminant aflatoxin B1 is carcinogenic for human and it has been already shown to be involved, in collaboration with viruses, in the development of cancers. A previous study by Accardi et al. 2015⁷³ showed the cooperation between AFB1 and EBV in the BL development, and our study was the first showing the impact of EBV on TGFBI expression *via* epigenetic mechanisms.

The EBV(+) BL-derived cell lines showed a lower level of TGFBI at the expression and the protein level. This down-regulation seems to be associated with an epigenetic event, as an

augmentation of the methylation on the 3 CpGs of interest and an increase of the DNMT1 protein level can be observed and in both cases for EBV and AFB1 down-regulate TGFBI and increase the methylation of its promoter. Our data show that EBV and AFB1 share a common pathway to deregulate TGFBI. It has already been reported that EBV activates DNMT1 activity *via* LMP1⁸² and that LMP1 activates the NF- κ B pathway⁵⁰. This study shows that, by blocking the NF- κ B pathway *via* a specific chemical inhibitor, it was possible to rescue the TGFBI expression, leading to the conclusion that LMP1 might need an active NF- κ B pathway to regulate TGFBI. Furthermore, the data of this thesis indicate that LMP1 induces the recruitment of DNMT1 on the CpGs CG11 and CG21 *via* an active NF- κ B pathway; the inhibition of the NF- κ B pathway associates with the loss of DNMT1 recruitment. This recruitment *via* LMP1 appears to be specific of DNMT1, as no effect of LMP1 has been observed on the recruitment of DNMT3a and DNMT3b. Further investigations are needed to better understand the interaction of AFB1 with the NF- κ B pathway. For instance, to compare of the TGFBI expression levels in BL EBV(-) derived cell line treated with AFB1 and with an inhibitor of the NF- κ B pathway or with AFB1 and the JNK pathway inhibitor may prove informative. A ChIP assay assessing the recruitment of DNMT1 on CG11 and CG21 in the presence of AFB1 and of an NF- κ B pathway inhibitor could give an insight on the possibility that EBV and AFB1 share the same mechanism of down-regulation of TGFBI. This study shows that AFB1 and EBV have a similar effect on the activity of the DNMTs, activity that is lost after a Bay11 treatment.

The results of this thesis helped to build a working model in which EBV and AFB1 are regulating DNMT1, that in turn down-regulates TGFBI either by modifying its expression or through a post-transcriptional modification. It would be of interest to target IKK and p65 to gain a better mechanistic understanding of the NF- κ B pathway role in DNMT1 activity. As mentioned before, the AFB1 is a carcinogen known to cooperate with viruses, and it shares a

common pathway of down-regulation of TGFBI with EBV. In order to understand if EBV and AFB1 synergise in the regulation of TGFBI, primary B-cells were treated with AFB1, then infected with EBV and immortalised (LCL). The expression level of TGFBI was found to be lower in the LCL originating from the primary B-cells treated by AFB1 compared to the untreated LCL cells. This was associated with differential DNA methylation pattern in AFB1 treated cells and an increase in the methylation of the three CpGs was observed.

To understand the biological effect of TGFBI down-regulation, the gene was over-expressed in EBV(+) and EBV(-) BL-derived cell lines. The over-expression of TGFBI did not impact the cells without the virus, on the other hand, the EBV(+) cells exhibited a higher level of apoptosis after over-expressing TGFBI. To further confirm these findings, the same experiment needs to be performed using different cell lines in order to prove that this effect is not cell line dependent. Over the past few years, epigenetics has become a hot topic and epigenetic signatures are considered as good cancer biomarkers candidates. The epigenetic signatures would allow reduced sampling invasiveness and it could improve the diagnostic and prognostic^{87,88,89}. With this in mind, the exosomal DNA from lymphoblastic cell lines EBV(+) was extracted and its methylation level was analysed by pyrosequencing. Similar levels of methylation on the exosomal DNA was found when compared to the genomic DNA. This data could be interesting for the development of a non-invasive epigenetic biomarker.

Taken together, our data show that the promoter of TGFBI is methylated in BL at the same positions than in the DNA of children exposed to aflatoxins, suggesting that early-life exposure to the toxin could alter the methylation levels of genes associated to BL and play a causative role in its development. TGFBI, considered as a tumour suppressor gene, has its expression altered *via* an epigenetic mechanism involving the viral protein LMP1 which regulates epigenetic enzyme (DNMT1) using cellular pathway (NF- κ B). In addition, epigenetic

signatures on TGFBI promoter, detectable in exosomal DNA of transformed B-cells, could be potentially used as a non-invasive biomarker of lymphomagenesis.

Together, the work of this thesis identified epigenetic biomarkers of the oncogenic viruses and aflatoxin (each alone or in viral–aflatoxin combinations) and provided insights into the mechanism (altered epigenetic pathways) underlying the observed increased predisposition to infections and specific human malignancies. Ongoing and future work is aimed at investigating the role of epigenetic regulator genes with “driver” potential (“epidrivers”) in Epstein–Barr virus (EBV)-mediated immortalization/transformation of B-cells, and the role of epidrivers in the maintenance of EBV latency and the transformed phenotype of BL cell lines. Moreover, these studies will be complemented by our ongoing collaboration in which we explore the roles of EBV and DNA methylation in the causation and the acquisition of immune resistance in nasopharyngeal cancer cells.

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