Epstein-Barr Virus Down-Regulates Tumor Suppressor DOK1 Expression

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Abstract

The DOK1 tumor suppressor gene encodes an adapter protein that acts as a negative regulator of several signaling pathways. We have previously reported that DOK1 expression is up-regulated upon cellular stress, via the transcription factor E2F1, and down-regulated in a variety of human malignancies due to aberrant hypermethylation of its promoter. Here we show that Epstein Barr virus (EBV) infection of primary human B-cells leads to the down-regulation of DOK1 gene expression via the viral oncoprotein LMP1. LMP1 alone induces recruitment to the DOK1 promoter of at least two independent inhibitory complexes, one containing E2F1/pRB/DNMT1 and another containing at least EZH2. These events result in tri-methylation of histone H3 at lysine 27 (H3K27me3) of the DOK1 promoter and gene expression silencing. We also present evidence that the presence of additional EBV proteins leads to further repression of DOK1 expression with an additional mechanism. Indeed, EBV infection of B-cells induces DNA methylation at the DOK1 promoter region including the E2F1 responsive elements that, in turn, lose the ability to interact with E2F complexes. Treatment of EBV-infected B-cell-lines with the methyltransferase inhibitor 5-aza-2′-deoxycytidine rescues DOK1 expression. In summary, our data show the deregulation of DOK1 gene expression by EBV and provide novel insights into the regulation of the DOK1 tumor suppressor in viral-related carcinogenesis.

Introduction

Cellular transformation induced by oncogenic viruses often involves the activation of growth-promoting signaling pathways and the inactivation of tumor suppressor genes. The downstream of tyrosine kinase 1 gene (DOK1) has emerged as a newly identified tumor suppressor gene that encodes a multi-domain adapter protein and acts as a negative regulator of signaling pathways involved in several cellular functions. DOK1 inhibits cell proliferation, down regulates MAP kinase activity, and has an opposing role in leukemogenesis and promotes cell spreading, motility, and apoptosis [1,2]. Functional studies showed that mice lacking the DOK1 and/or DOK2 genes have a high susceptibility to the development of lung adenocarcinomas [3] and exhibit significant defects in their immune responses and immune cell development, often developing myelo-proliferative and autoimmune diseases, e.g. lupus-like renal disease [4,5]. The DOK1 gene locus is located in the human chromosome 2p13 region, which is frequently rearranged in a number of human tumors [6]. Oncogenic tyrosine kinases such as p210BCR-ABL, the causative mutation in chronic myelogenous leukemia (CML), and Src target DOK1 for ubiquitin-mediated proteasomal degradation [7], therefore promoting cell proliferation. We have reported a frameshift mutation of the DOK1 gene in chronic lymphoid leukemia (CLL) resulting in the expression of truncated DOK1 that is exclusively localized in the nucleus and loses its tumor suppressive activities, in contrast with the cytoplasmic wild type protein [8]. We also showed that DOK1 gene expression is repressed in a large proportion of head and neck cancer (HNC), lung cancer and Burkitt’s lymphoma [9], as a result of aberrant hypermethylation of its promoter region. The inactivation of DOK1 through promoter methylation also occurred in liver and gastric cancers [10,11]. Thus, DOK1 emerged as a tumor suppressor frequently altered in a variety of human cancers, making it a potential marker and therapeutic target in cancer control.

Epstein-Barr virus (EBV) is a γ-herpesvirus that is widespread in 90% of human populations. In the majority of individuals, EBV persists as a permanent, asymptomatic infection of the lymphocytes B-lymphocyte pool [12]. EBV occasionally causes infectious mononucleosis in adolescents [13] and is considered a human carcinogenic infectious agent. Indeed, EBV is associated with the development of different types of B-cell lymphoma such as Burkitt’s lymphoma (BL), Hodgkin disease, lympho-proliferative...
Many oncogenic viruses exhibit cellular transforming properties, often involving oncoproteins activation and tumor suppressor genes inactivation. The DOK1 gene is a newly identified tumor suppressor gene with altered expression via hypermethylation of its promoter in a variety of human cancers, including head and neck, lung, gastric and others. In addition, a correlation has been reported between DOK1 aberrant hypermethylation and the presence of oncogenic viruses such as hepatitis B virus (HBV) in hepatocellular carcinoma (HCC) and Epstein-Barr virus (EBV) in Burkitt’s lymphoma-derived cell lines. Here we report for the first time that EBV is directly involved in the inhibition of DOK1 expression in B-cells. We show that EBV leads to epigenetic repression of DOK1 through increased DNA methylation of its promoter and H3K27 tri-methylation. The LMP1 oncoprotein plays a key role in the repression of DOK1 expression. It promotes the formation and the recruitment to the DOK1 promoter of transcriptionally inhibitory complexes composed of E2F1/pRB/DNMT1 and of EZH2 which is part of the polycomb repressive complex 2. Interestingly, one or more additional EBV proteins cooperate with LMP1 in inducing massive DNA methylation at the DOK1 promoter, leading to the loss of E2F1 complexes recruitment and even stronger repression of DOK1 expression.

EBV infection in B-cells leads to epigenetic repression and CpG methylation of the DOK1 gene and that LMP1 expression inhibits DOK1 promoter activity via the recruitment of inhibitory complexes including E2F1, pRB, DNMT1 and EZH2.

Results

EBV infection of primary human B-cells in vitro leads to down-regulation of DOK1 expression

Based on our previous results that showed the down-regulation of DOK1 expression in BL cell-lines [9], we evaluate whether this event was linked to infection with EBV, a key risk factor for this malignancy. Primary human B-cells, isolated from different healthy donors, were infected in independent experiments with recombinant EBV virus expressing the green fluorescent protein (GFP-EBV). The infection efficiency was evaluated by flow cytometry to monitor GFP expression (data not shown). The expression of EBV genes EBNA1 and LMP1, as well as DOK1 was determined by real-time PCR, and western blot at different time points post-infection ([Figure 1A and B]). EBV infection resulted in a strong reduction of DOK1 mRNA and protein levels, which was evident at 16 hours post-infection ([Figure 1A]). Similarly, DOK1 mRNA and protein levels were strongly down-regulated by EBV in three cancers B-cell lines (RPMM1, BJAB and Loukess) infected by EBV, as well as in EBV-immortalized lymphoblastoid cell lines (LCLs) ([Figure 1C and D]). Together, these findings highlight a role for EBV in down-regulating DOK1 gene expression.

LMP1 plays a key role in the inhibition of DOK1 expression

The EBV oncoprotein LMP1 is essential for EBV-induced B-cell immortalization by altering cellular gene expression via the activation of several signaling pathways [28]. To determine whether LMP1 can affect the expression of DOK1, we infected the RPMI cells with wild-type GFP-EBV or GFP-EBV lacking the LMP1 gene (EBVΔLMP1). The infection efficiency was monitored using flow cytometry for GFP expression ([Figure 2A]). In contrast to wild-type GFP-EBV, EBVΔLMP1 infection in primary B cells and in RPMI cells did not significantly decrease DOK1 mRNA or protein levels ([Figure 2B and C]). Re-expression of LMP1 in EBVΔLMP1 RPMI cells by retroviral transduction restored the ability of EBV to down-regulate DOK1 expression, while transduction of the same cells with empty retrovirus (pLXSN) did not affect DOK1 mRNA or protein levels ([Figure 2D and E]), highlighting the key role of LMP1 in this event. Accordingly, expression of LMP1 alone in RPMI cells was sufficient to reduce DOK1 mRNA and protein expression ([Figure 2D and E]), whereas expression of other viral proteins, such as EBNA1, 2, 3A, 3B, and 3C, did not lead to down-regulation of DOK1 protein levels [Supplementary Figure S1A-C]. In addition, transfection of RPMI with increasing concentrations of LMP1 expressing vector resulted in the decrease of DOK1 expression in a dose-dependent manner ([Figure 2F and G]). Together, these data underline the key role of LMP1 in EBV-mediated DOK1 down-regulation in infected B-cells.

LMP1 down-regulates DOK1 expression by altering the composition of the E2F transcription complex

We recently showed that the E2F1 transcription factor has a key role in activation of DOK1 transcription [29]. The 500 nucleotide upstream of the start site of the DOK1 promoter contains three E2F1 responsive elements (RE) which appear to have a role in
transcription activation; in particular the one at position $-490/-486$ (ERE1) [29]. Transient transfection experiments showed that LMP1 was able to efficiently inhibit the activity of $DOK1$ promoter cloned in front of the luciferase reporter gene (Figure 3A). The addition of upstream regions ($-1000/-500$ or $-2000/-500$) did not modify the pattern of LMP1 inhibition (Figure 3A). In addition, LMP1 was not able to further decrease the activity of $DOK1$ promoter harboring point mutations in ERE1 (Figure 3A). Together, these results suggest that LMP1 may exert its inhibitory activity targeting the regulatory complexes able to bind ERE1 within the $-500/+33$ region of the $DOK1$ promoter. Chromatin immuno-precipitation (ChIP) experiments using an anti-E2F1 antibody showed that infection with wild-type GFP-EBV significantly decreases the recruitment of E2F1 to ERE1 in
Figure 2. LMP1 plays a key role in EBV-mediated DOK1 silencing. RPMI cells were infected with GFP recombinant EBV wild type (GFP-EBV) or lacking LMP1 (EBVΔLMP1). (A) The infection was monitored using flow cytometry for GFP expression. (B and C) mRNA levels of EBNA1, LMP1, GAPDH and DOK1 in these cells were determined using real time PCR and the indicated proteins expression were analyzed using western blotting. Both RPMI
RPMI and two independent LCLs (Figure 3B), while EBVΔLMP1 did not have any impact on this event in RPMI (Figure 3B). Interestingly, LMP1 alone did not prevent the recruitment of E2F1 to the DOK1 promoter in RPMI cells (Figure 3B), although it is able to efficiently down-regulate DOK1 expression (Figures 2D, 2E, and 3A).

We next analyzed the chromatin organization within the DOK1 promoter in the same cells by monitoring the tri-methylation of histone H3 at lysine 4 (H3K4me3) or at lysine 27 (H3K27me3) which are events associated with transcriptionally active or inactive chromatin, respectively. According to their ability to repress DOK1 expression, wild-type GFP-EBV or LMP1 alone induced an increase of H3K27me3 and a decrease of H3K4me3 within the DOK1 promoter compared with mock cells (Figure 3C). However, LMP1 was less efficient than the entire virus in promoting these epigenetic changes (Figure 3C). In summary, although LMP1 alone is not able to prevent the recruitment of E2F1 to the DOK1 promoter, it is capable of inducing epigenetic changes and inhibition of DOK1 transcription.

Based on these findings, we hypothesized that LMP1 mediates DOK1 down-regulation by altering the composition of the E2F1 complex. To explore this possibility, we performed oligo pull-down experiments using biotinylated DNA probes which contain a region of the DOK1 promoter encompassing the wild-type or mutatedERE1. Biotinylated DNA probes were incubated with protein extracts from RPMI cells transduced with empty retrovirus or with retrovirus expressing LMP1. In both extracts and as expected, E2F1 was found associated with the DNA, while only in the presence of LMP1 were three additional cellular proteins, which are usually part of negative regulatory complexes of transcription found associated with the DOK1 promoter fragment: (i) the E2F1 inhibitor retinoblastoma (pRB), (ii) the DNA methyltransferase DNMT1 and (iii) the polycomb-group (PcG) 2 member EZH2 (Figure 3D). Deletion of ERE1 prevented the association of E2F1 in both cellular extracts. In addition, in LMP1-containing extracts, mutation of ERE1 also significantly decreased the pRB and DNMT1 protein levels precipitated with DNA (Figure 3D), suggesting that both proteins are recruited in the same complex as E2F1. With regard to EZH2, its binding to the DOK1 promoter was less affected by the ERE1 mutation, indicating that it is recruited by a different complex. Although LMP1 is able to activate the NF-κB pathway, no binding of the p65 transcription factor was found in both cellular extracts (Figure 3D). ChIP-Rec-ChIP experiments in mock and LMP1-expressing cells confirmed the data obtained in the pull-down assay. Indeed, Rec-ChIP showed that a significant proportion of E2F1 complexes recruited to the DOK1 promoter contains pRB and DNMT1 proteins (80% and 40% respectively), but not EZH2 (Figure 3E), which appears to be associated with an independent complex.

Finally, the events occurring at DOK1 promoter were determined at early stages post-infection with EBV. We observed a significant enrichment of pRB, DNMT1 and EZH2 recruitment to DOK1 promoter in primary naive B cells infected with recombinant GFP-EBV virus for 48 hours. Consequently, an increase of H3K27 trimethylation (~5 folds) and CpG methylation (~10%) was detected (Supplementary Figure S3). Thus, early stage of EBV infection mimics the scenario observed in LMP1-expressing cells.

In summary, these data show that LMP1 initiates the repression of DOK1 expression by inducing the formation of transcriptional inhibitory complexes.

**LMP1-mediated NF-κB activation is required for DOK1 down-regulation**

LMP1 has the ability to activate different signaling pathways, such as NF-κB, MAPK p38, JNK, and MAPK/ERK [28]. To explore the potential role of these pathways in DOK1 down-regulation, RPMI cells infected with recombinant GFP-EBV were treated with different chemical inhibitors specific for these signaling pathways. No change was observed in mock or GFP-EBV cells treated with the chemical inhibitors of the MAPK p38, JNK, and MAPK/ERK pathways (SB203580, S600125 and PD98059, respectively) [data not shown]. However, DOK1 mRNA and protein levels were found to be considerably increased in GFP-EBV-infected cells treated with a specific inhibitor of NF-κB (Bay11), but not in mock cells (Figure 4A and B). Similarly, Bay11 treatment of LMP1-expressing cells increased the DOK1 mRNA and protein levels (Figure 4A and B). To further demonstrate the role of NF-κB signaling in EBV-mediated DOK1 down-regulation, we inhibited the NF-κB canonical pathway by expressing a non-degradable deletion mutant of IkBζ (Δ-IκBζ) that lacks the first 36 amino acids at the N-terminus containing the IKK-phosphorylated amino acid. Similarly to Bay 11, Δ-IκBζ expression in GFP-EBV RPMI cells led to an increase of transcript and protein levels of DOK1 (Figure 4C and D). Accordingly, transient transfection experiments using a plasmid containing the DOK1 promoter cloned upstream of the luciferase gene showed that Δ-IκBζ antagonized LMP1 in inhibiting the DOK1 promoter (Supplementary Figure S2).

The LMP1 protein has two important C-terminal cytosolic domains named C-terminal activation region 1 (CTAR-1) (residues 194–232) and 2 (CTAR-2) (residues 351–386). Both the CTAR1 and CTAR2 domains have the ability to activate the NF-κB pathway through their interactions with tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) [30], and TNFR-associated death domain protein (TRADD) [31], respectively. In particular, the CTAR2 domain is required for the activation of the canonical NF-κB pathway, while the CTAR1 domain is critical for the stimulation of the non-canonical NF-κB pathway [32]. The LMP1 mutants AxAxAxA (mutated CTAR1), 378 stop (deleted in CTAR2) and AxAxA/378 stop (mutated CTAR1 and deleted CTAR2) were expressed in RPMI cells. Both LMP1 378 stop and AxAxA/378 stop mutants failed to down-regulate the DOK1 gene, but not the LMP1 AxAxA mutant, which still retained its ability to suppress DOK1 expression at similar levels of wild-type LMP1 (Figure 4E and F). Therefore, LMP1 down-regulates DOK1 expression through its CTAR2 domain. In addition, we investigated whether the LMP1-mediated NF-κB activation plays a role in the formation of inhibitory complexes and their recruitment to the DOK1 promoter. LMP1-expressing RPMI cells were cultured in the presence of NF-κB inhibitor Bay11. No significant change in
Figure 3. LMP1 represses DOK1 promoter activity through the recruitment of E2F1/pRB/DNMT1 inhibitory complex. (A) RPMI cells were transfected with the indicated firefly luciferase reporter pGL3-DOK1 promoter constructs along with increasing amounts of pcDNA3 LMP1. Renilla luciferase was used as an internal control for the reporter assay. After 48 hours, cells were collected and processed for luciferase activity measurement. The data are average of three independent experiments. (B) RPMI cells, RPMI cells infected with GFP-EBV recombinant virus, or GFP-EBV-DLMP1, RPMI cells transduced with empty pLXSN (V) or expression vector pLXSN-LMP1, and LCLs and their original primary B-cells were subjected to quantitative ChIP assay using anti-E2F1 (KH 95) antibody or IgG. The DOK1 promoter was amplified by real-time PCR using specific primers flanking the E2F-response element located at (–498/–486). Data were calculated as percentages of enrichment of input. Error bars indicate the standard deviation from three independent experiments performed in triplicate. (C) The same cells from (B) were subjected to ChIP assay using the anti-H3K27 trimethylation antibody, anti-H3K4 trimethylation antibody or IgG. The DOK1 promoter and GAPDH promoter were amplified by real-time PCR. (D) In

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H3K27me3 and the increase of H3K4me3 levels (infected cells (RPMI or LCLs) as well as in LMP1-expressing RPMI protein levels was observed upon exposure to 5-Aza in GFP-EBV-infected with EBV (Figure 5A). In addition, high levels of DOK1 led to a significant increase of subG0 population and AnnexinV-positive cells (Figure 5B and C). Together, these data demonstrate the role of DOK1 in inhibiting cell proliferation induced by EBV and promoting both cell growth arrest and apoptosis.

Discussion

Several studies have demonstrated that the loss of DOK1 function is a key event in human carcinogenesis [1,3,4,9,33]. Indeed several mechanisms of DOK1 inactivation have been characterized so far DOK1 expression was found to be silenced by hypermethylation of its promoter in a variety of human cancers, including, head and neck, lung, gastric and liver cancer as well as in Burkitt’s lymphoma-derived cell lines [9,10,11]. In addition, DOK1 was found to be mutated in chronic lymphocytic leukemia (CLL) [8]. At the protein level, DOK1 is targeted for proteasome degradation triggered by oncoprotein kinases (OTKs) such as p210bcr-abl and oncogenic forms of Src [7]. A recent study has provided evidence that DOK1 inactivation also occurs in virus-induced cancers [10]. Indeed, a correlation between DOK1 aberrant hypermethylation and the presence of hepatitis B virus (HBV) has been reported in hepatocellular carcinoma (HCC) [10]. Similarly, the expression of DOK1 mRNA was found to be down-regulated in cell lines derived from Burkitt’s lymphoma [34], a pathological condition associated with EBV infection. However, these initial findings do not provide evidence about whether the down-regulation of DOK1 expression is directly induced by the viral proteins or is a consequence of the chromosomal alterations occurring during the carcinogenic processes. In this study, we demonstrate for the first time that EBV is directly involved in the inhibition of DOK1 expression. Our data show that the EBV LMP1 oncoprotein plays a key role in this event. Indeed, an EBV mutant lacking the entire LMP1 gene was unable to inhibit DOK1 transcription, while re-expression of LMP1 in cells infected with the EBVΔLMP1 mutant fully restored the ability of EBV to decrease DOK1 mRNA and protein levels. Expression of LMP1 alone in human cancer B-cells was sufficient to efficiently inhibit DOK1 transcription by promoting the formation of a transcriptional repressor complex containing E2F1, pRB, and the DNA methyl-transferase DNMT1. In addition, deletion of the E2F1-binding element (ERE1) strongly affected the binding of three cellular proteins to the DOK1 promoter, and a Re-ChIP assay confirmed that E2F1 is the carrier of pRB and DNMT1. We also observed that LMP1 promotes the recruitment of the histone-lysine N-methyl-transferase EZH2 independently of E2F1, leading to an increase in the level of H3K27me3. In agreement with the recruitment of the two epigenetic enzymes, an increase in H3K27me3 and DNA methylation levels was detected at the DOK1 promoter.
Figure 4. LMP1-mediated NF-κB activation is required for EBV-related DOK1 down-regulation. RPMI cells transduced with empty retroviral pLXSN (Vector), expression vector pLXSN-LMP1, or infected with GFP-EBV recombinant virus were treated with Bay11 or the equivalent volume of DMSO (Mock). (A) mRNA levels of LMP1 and DOK1 were measured by real time PCR, and normalized to GAPDH expression. (B) The indicated proteins were detected using western blotting. RPMI cells were transfected with pcDNA3 empty plasmid (Vector), expression vector pcDNA3-LMP1 and/or expressing the super-repressor IκBα (DIκBα), while RPMI cells infected with GFP-EBV recombinant virus were transfected only with pcDNA3 empty (Vector) or expression vector of the super-repressor IκBα (ΔIκBα). After 48 hours, cells were collected for analysis. (C) mRNA levels of LMP1 and DOK1 were measured by real time PCR, and normalized to GAPDH expression. (D) The indicated proteins were detected using western blotting. RPMI cells were transfected with empty pLXSN (Vector), or expression vector pLXSN-LMP1 wild type (WT), LMP1 mutant for the CTAR1 domain (Δx3A), and CTAR2 domain (378 stop), or both CRAT1 and 2 domains (Δx3A/378 stop). After 48 hours, cells were harvested for expression analysis. (E) mRNA levels of LMP1, GAPDH and DOK1 were measured using real time PCR. (F) The indicated proteins were detected using western blotting. DOK1 protein levels were quantified from two independent immunoblots and normalized to the corresponding β-actin level (bottom of B, D and F). Stable RPMI cells with empty pLXSN (Vector), or expression vector pLXSN-LMP1, were treated with Bay11 or the equivalent volume of DMSO (Mock). (G) Cells were subjected to quantitative ChIP assay using the indicated antibody or IgG. The DOK1 promoter was amplified by real-time PCR using specific primers flanking the E2F-response element located at -498/~486. Data were calculated as percentages of enrichment of total input. Error bars indicate the standard deviation from two independent experiments performed in triplicate. (H) In vitro DNA pull-down assay.
It has previously been shown that LMP1 is able to increase the expression and activity of DNA methyl-transferases (DNMT1, 3a, and 3b), which could explain the increase of the DOK1 promoter methylation. Interestingly, DNA methylation was strongly enhanced in B-cells infected by the entire virus compared with cells expressing only LMP1. Thus, it is likely that additional viral products may cooperate with LMP1 in promoting DOK1 silencing via DNA methylation. No down-regulation of DOK1 was observed when EBNA1, 2, 3A, 3B, and 3C are expressed in RPMI cells. In addition, none of these viral proteins further stimulate DNA methylation at DOK1 promoter when co-expressed with LMP1 (data not shown). Thus, a more complex pattern of viral gene expression may be involved in the hyper-methylation of DOK1 promoter. Most importantly, we show that in EBV-infected B-cells the DNA methylation extends over a large region of the DOK1 promoter including ERE1 that loses the ability to recruit the active form of E2F1. Inhibition of DNA methylation significantly increases DOK1 transcription in LMP1-expressing cells as well as EBV-infected cells.

In summary, based on our findings, a two-step model can be proposed for EBV in the inhibition of DOK1 expression (Figure 7). In the first step, LMP1 favors the formation and recruitment of transcriptional repressor complexes containing E2F1/pRB/DNMT1 and EZH2. These complexes induce epigenetic changes in the DOK1 promoter region, leading to its inhibition. In the second step, LMP1 in collaboration with other EBV proteins leads to further increase of DNA methylation which in turn results in a loss of all transcriptional regulatory complexes and a strong repression of the DOK1 promoter. These data corroborate our previous studies that highlighted the key role of E2F1 and DNA methylation in the regulation of DOK1 expression [29]. Our data also show that the LMP1-induced DOK1 down-regulation is linked to activation of the NF-kB canonical pathway. Indeed, NF-kB activation by LMP1 plays a role in the formation and recruitment of inhibitory complex E2F1/pRB/DNMT1 to the DOK1 promoter. Although we did not observe any recruitment of p65 to the DOK1 promoter, neither by DNA-pull-down assay nor by chromatin immuno-precipitation (data not shown), we cannot exclude the involvement of other NF-kB transcription factors.

Until now, several studies reported that DNA methylation patterns were higher in EBV positive tumors compared to the EBV-negative ones and that EBV infection was clearly demonstrated to induce specific methylation epigenotypes that lead to silencing of multiple tumor suppressor genes such as BIM, p16[INK4A], p14 [ARF], E-cadherin and pTEN in EBV–associated nasopharyngeal and gastric cancers [17,35,36,37,38]. While these events are believed to be caused by elevated levels of DNMTs induced by LMP1 and 2, the mechanisms establishing the methylation patterns themselves are unknown. As DNA methyltransferases have little specificity in vitro, we propose the notion that LMP1 triggers DOK1 gene repression through the recruitment of DNMT1 to its promoter in a specific manner via E2F1-binding to its response element, and this event might be an early step for EBV-induced DNA methylation. As some of the genes listed above are targets of E2F1 [39,40], it will be interesting to see whether their methylation patterns are also specific to the recruitment of the inhibitory complex E2F1/pRB/DNMT1. Moreover, EBV appears to have an initiator role of epigenetic alterations and therefore inducing oncogenesis, however, the latency expression patterns of EBV genes differ in different cancers, which make unclear the contribution of the virus to some types. One explanation would be that EBV-induced epigenetic changes, such as EBV-mediated DNA methylation of DOK1 promoter, are stable events and could also persist even after the changes in EBV latent gene expression. As DOK1 gene silencing was found to be related to its promoter hypermethylation in gastric cancer [11], it will be important to investigate whether these events are associated with the presence of EBV in these cancers and others.

In conclusion, the present study sheds light on the association between EBV infection and DOK1 down-regulation in B-cells. It provides novel insights into the regulation of DOK1 in viral-related carcinogenesis, and could define it as a potential cancer biomarker and an attractive target for epigenetic-based therapy.

Materials and Methods

Expression vectors

Cellular and viral genes were expressed using the retrovector pLXSN (Clontech, Palo Alto, CA) or the expression vector pcDNA-3 (Invitrogen). The pLXSN-LMP-1 and the mutants LMP-1AxAxA, LMP-1 378 stop, and LMP-1AxAxA/378 stop constructs have been previously described [41]. The pGL3 basic luciferase reporter (Promega) and pGL3 containing the DOK1 promoter constructs have been described previously [29], The NF-kB super-repressor Δ-IκBz, which lacks the coding sequence of the first 36 N-terminal amino-acids, was kindly provided by Dr Elliott Kieff (Harvard Medical School, Boston, Massachusetts, USA). The expression plasmids pDEST-myc-EBNA1, pSG3-EBNA2, pDEST-myc-EBNA3A, pDEST-myc-EBNA3B, pDEST-myc-EBNA3C were kindly provided by Dr Evelyne Manet (ENS, Lyon, France).

Cells, transfection, and chemicals

RPMI 8226 cells were kindly provided by Dr Christophe Caux (Centre Léon Bérard, Lyon, France). The EBV-negative immortalized B-cells, BJAB were previously described [42], and the Louckes cells were kindly provided by Dr Evelyne Manet (ENS, Lyon, France). The primary B-cells were isolated from total blood of healthy donors using negative selection EasySep or RosetteSep (StemCell Technologies). Primary naïve B cells and RPMI cells were infected with recombinant GFP-EBV, and GFP-EBV/LMP-1 as described in [43,44,45], RPMI pLXSN-empty or pLXSN-LMP1 cell lines were generated as described previously [41]. Expression of LMP-1 wild-type, LMP-1 AxAxA, LMP-1 378 stop, and LMP1 AxAxA/378 stop mutants in RPMI was achieved by transduction with recombinant retroviruses [41]. The EBV-immortalized lymphoblastoid cell lines (LCLs) were generated in this study by infecting primary B-cells isolated from different donors with recombinant EBV expressing GFP, as described previously [41]. Primary and immortalized B-cells were cultured in RPMI 1640 medium (GIBCO, Invitrogen life Technologies, Cergy-Pontoise, France) supplemented with 10% FBS, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (PAA, Pasching, Austria). Expression plasmids were transiently transfected in cells using Xtreme gene 9 reagents (Roche) according to the manufacturer’s protocol.

For treatment, cells were incubated in media containing different reagents: with a final concentration of 1 μM of the NF-kB pathway inhibitor Bay11 in dimethyl sulfoxide (DMSO) for 6 hours. Inhibition of DNA methylation was performed by incubation for...
Figure 5. S-Aza treatment rescue DOK1 expression in EBV infected cells. Cells were treated with 1 μM methyl-transferase inhibitor S-Aza-deoxycytidine (S-Aza) for 4 days or equivalent volume of DMSO (Mock), then collected for analysis. (A) DNA methylation levels of the DOK1 promoter were measured using pyrosequencing. Each bar represents the percentage of methylation for individual CpG sites. (B) Quantitative ChIP assay using anti-E2F1 (KH 95) antibody or IgG. The DOK1 promoter was amplified by real-time PCR using specific primers flanking the E2F-response element located at (−498/−486). Data were calculated as percentages of enrichment of input. Error bars indicate the standard deviation (SD) from two independent experiments performed in triplicate. (C) The mRNA expression levels of LMP1, GAPDH and DOK1 were determined using real time PCR. (D) The indicated proteins were analyzed using western blotting. DOK1 protein levels were quantified from two independent immunoblots and normalized to the corresponding β-actin level (bottom). (E) ChIP assays were carried out using anti-H3K27 trimethylation antibody, anti-H3K4 trimethylation antibody or IgG. The DOK1 promoter and GAPDH promoter were amplified by real-time PCR. Data were calculated as percentages of enrichment of input.
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4 days with 5-aza-2’-deoxycytidine (5-aza) at 1 μM (Sigma) dissolved in DMSO. Cells were then harvested for analysis.

Quantitative RT-PCR
Total RNA was extracted using TRIzol reagent (Life Technologies). Reverse transcription was performed using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) according to the manufacturer’s protocol. Real-time PCR was performed using the following gene-specific primers:

**DOK1**:
Fw ATGGACGGAGCAGTGATGGA, Rev CCCAGGTCTTCTCCACCTC

**LMP1**:
Fw CCCCGCTTCTCTTCCACCTAG, Rev GCCAAAGATGAGGCCACAA

**EBNA1**:
Fw GGACCCGCCCAACAACCTG, Rev CTCCTGCGCCCTTTCCACCTG

**GAPDH**:
Fw GAAGGTGAAGGTCGGAGTC, Rev AAGATGGTGATGGGATTT

Data were analyzed using the ΔΔCT method.

Antibodies and immunoblotting
The following antibodies were used: anti-DOK1 (ab8112, Abcam), anti-E2F1 (KH-95; Santa Cruz Biotechnology), anti-β-Actin C4 (MP Biomedicals), anti-LMP1 (S12), anti-phosphor IκBα (#9246, Cell Signaling Technology), anti-total IκBα (#9242, Cell Signaling Technology), mouse IgG, rabbit IgG (Santa Cruz Biotechnology), anti-p65 (#3034, Cell Signaling Technology), anti-H3K4me3, and anti-H3K27me3 (Epigentek), anti-EZH2 (AC22; Cell Signaling Technology), anti-pRB (4H1, Cell Signaling Technology), anti-DNMT1 (60B1220, Abnova), anti-EBNA1 (1EB12, Santa Cruz Biotechnology), anti-EBNA2 (Novocastra), anti-EBNA3A (Exalpha), anti-EBNA3C (ab16128, Abcam). Immunoblotting was performed as described previously [29].

Reporter assays
Cells were transfected with 0.250 μg of pGL3 or DOK1 promoter constructs along with other experimental plasmids using X-tremeGENE 9 (Roche Diagnostics). The Renilla construct was included for normalization of transfection efficiency. At 48 hours after transfection, cells were harvested and the enzyme activities of firefly and Renilla luciferases were measured using the Dual-Luciferase reporter assay system (Promega). The luminescence signal was quantified using an Optocomp I luminometer (MGM Instruments). Each condition was used in triplicate and replicated in different independent experiments.
Chromatin immuno-precipitation (ChIP)

For each reaction, 10^6 cells were cross-linked with 1% formaldehyde, harvested and subjected to sonication to shear the chromatin into fragments of 0.2 kb, immuno-precipitated with 2 μg of appropriate antibody, and then processed according to the standard protocol for ChIP analysis from Cell Signaling Technology. Low cell ChIP kit (Diagenode) was used for primary B cells and infected with EBV for 48 hours. 50,000 cells per reaction were processed according to the manufacturer’s protocol.

The input and immuno-precipitated DNA from both methods (standard and low cell) were then analyzed by real-time PCR using primers flanking the E2F-response element (−498/−486) of the DOK1 promoter: Fw GCCAAAACCGAGGACTTTCG, Rev CATCACTGCTCCGTCCCATGG, or primers for GAPDH promoter: Fw GACGGCCGCATCTTCTTGT, Rev CCTGGTGACCCAGGGCC. Data were calculated as a percentage of enrichment of input.

Re-ChIP assay

Following the initial anti-E2F1 ChIP (performed as above using 10^6 cells and 10 μg of anti E2F1 KH-95 antibody), up to the final wash step with TE buffer, E2F1–chromatin complexes were eluted by the addition of 10 mM dithiothreitol (DTT) and incubated for 30 minutes at 37°C. Supernatants were diluted 1:20 with re-ChIP buffer (1% Triton X-100; 20 mM Tris-HCl, pH 8.1; 2 mM EDTA; 150 mM NaCl; supplemented with protease inhibitors), and immuno-precipitated a second time (IP 2) using 4 μg of antibody against pRB, DNMT1, and EZH2. IgG was used as negative control. The Re-ChIP mixtures were incubated overnight at 4°C with rotation. Isolation and purification of associated DNA were carried out as described for the standard ChIP experiment. The binding of each factor was determined by real-time PCR as previously described. Data were calculated as a percentage of enrichment of total input.

DNA pull-down assay

Cells were lysed by sonication in HKMG buffer (10 mM HEPES, pH 7.9; 100 mM KCl; 5 mM MgCl2; 10% glycerol; 1 mM dithiothreitol (DTT); and 0.5% NP-40) containing protease and phosphatase inhibitors. Cellular debris was removed by centrifugation. Then, 1 mg of total lysate was pre-cleared with 40 μl of streptavidin-agarose beads (Thermo Scientific) for 1 hour at 4°C, with rotation, and incubated with 2 μg of biotinylated PCR product oligonucleotides and 20 μg of poly (dI-dC) for 16 hours at 4°C, with rotation. Biotin-oligonucleotide-protein complexes were collected with 60 μl of streptavidin-agarose beads for 1 hour at 4°C, with rotation, washed twice with HKMG buffer, separated on SDS-PAGE, and detected by western blotting. The biotinylated double-stranded oligonucleotides were amplified using the same primers as for ChIP with 5’ biotin.

DNA extraction and pyrosequencing

Genomic DNA was extracted using the QIAamp DNA minikit (Qiagen) and bisulfite converted using the EZ DNA Methylation-Gold kit (Zymo Research). Converted DNA was then subjected to Pyrosequencing (Qiagen) as previously described [46]. The primers used to measure the methylation of DOK1 promoter were: Fw GAGGTTGGAGAGATTGG, Rev BIOTIN-CCA-CACICACACACTCAA, and sequencing primer AGTTTTGGGGGTTGT. The percentage of methylation was evaluated as the mean of each CpG analyzed.

Figure 7. Schematic model of DOK1 gene regulation in EBV-infected cells. (A) In uninfected cells, DOK1 expression is activated via the recruitment of the active form of the E2F1 transcription factor to its response element located at (−498/−486) on the DOK1 promoter. (B) In cells expressing the oncoprotein LMP1, DOK1 is down-regulated through the recruitment of the inhibitory complexes E2F1/pRB/DNMT1 and EZH2 to its promoter region. These complexes lead to the induction of partial DNA methylation and the increase of H3K27 trimethylation levels, respectively. (C) In EBV-infected cells, DOK1 is repressed through heavy DNA methylation of its promoter region and the increase in H3K27 trimethylation level. These events likely induce conformational changes in the chromatin, which become less permissive to E2F1 transcription factor recruitment.

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Flow cytometry analysis
To determine cell cycle profile, cells were collected 48 hours post-transfection with empty pcDNA3 (Vector) or expression vector pcDNA3–Flag-DOK1, washed twice with PBS 1×, and then cell pellets were re-suspended in 70% ethanol while vortexing, in order to prevent cell clumps. After ethanol fixation (30 minutes at 4°C) the cells were re-washed in PBS 1× and finally re-suspended in PBS 1× + 100 μg/mL RNase (Roche) + 25 μg/mL of Propidium iodide (Sigma).

Apoptotic cells were detected using the PE Annexin V apoptosis detection kit I (BD Pharmingen) according to the manufacturer’s instructions.

Stained cells for cell cycle and for apoptosis were detected using the BD FACScanto II flow cytometer (BD Biosciences) and analyzed using FACSDiva software.

Ethics statement
Blood samples from healthy donors were provided by the Etablissement Français du Sang (EFS, Lyon, France) after being anonymized. All participants signed a written informed consent.

Supporting Information

Figure S1 Expression of latent EBV proteins EBNA1, 2, 3A, 3B, and 3C failed to down-regulate DOK1 gene expression. RPMI were transfected with 0.5 μg of empty vector or expression vector of myc-EBNA1 (A), EBNA2 (B), myc-EBNA3A, 3B, or 3C (C). After 48 hours post-transfection, the expression of the indicated proteins was determined using western blotting. (TIF)

Figure S2 Inhibition of LMP1 mediated NF-κB activation leads to the rescue of DOK1 promoter activity and protein expression. (A) RPMI cells were transfected with pG5L3 basic vector, or containing the DOK1 promoter construct (−500/+33) along with pcDNA3 empty (Vector), expressing LMP1 or different amounts of the super-repressor 1κBz (Δ1κBz). The Renilla luciferase was used as an internal control for the reporter assay. After 48 hours, the cells were harvested and the luciferase activities were measured. (B) The expression of the indicated proteins was determined using western blotting. (TIF)

Figure S3 Early stage infection with EBV leads to epigenetic repression of DOK1 expression in primary B cells. (A) Primary B cells were isolated from healthy donor blood using negative selection, and then infected with GFP-EBV recombinant virus. Genomic DNA was extracted at different time points 12, 16, 24, 36, and 48 hours post infection, and DNA methylation of DOK1 promoter was measured using pyrosequencing. (B) Primary B cells were infected with GFP-EBV recombinant virus for 48 hours. Quantitative low cell ChIP assay was performed to measure the individual recruitment of E2F1, pRB, DNMT1, and EZH2 to the DOK1 promoter, and the levels of histone 3 modifications (H3K27 trimethylation or H3K4 methylation). Non infected primary B cells were used as control. Data was calculated as percentage of enrichment of total input. Statistical significance was measured using Student’s t test (*, p value<0.05). (TIF)

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Author Contributions
Conceived and designed the experiments: MS BSS MT. Performed the experiments: MS CF CC. Analyzed the data: MS RA JY BSS MT. Contributed reagents/materials/analysis tools: MS EM HG ZH. Wrote the paper: MS BSS MT.

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