EBV-associated cancers: Strategies for targeting the virus

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Latent EBV infection has been associated with the development of several cancers. Therefore, it may be considered as a unique opportunity to develop new drugs for preventing these cancers. To date there are no available drugs targeting latent EBV. Induction of viral lytic reactivation combined with treatment with antivirals has been suggested as a solution for the treatment of EBV-associated malignant diseases. Nevertheless, the increased probability of infecting other cells and therefore the risk of developing other EBV-associated diseases should not be discarded. Efforts have been made to find vaccines which could prevent primary infection and reduce the incidence of EBV-associated malignancies and some vaccines have already reached clinical trials. In addition, several other anti-EBV biological approaches have been described, such as the specific downregulation of viral oncoproteins using antisense, RNAi or DNAzymes strategies. Moreover, chemical approaches with natural and synthetic compounds, have shown antiviral potential against EBV. A combination of rational drug design with drug synthesis and biological assays is currently being used to search for new anti-EBV compounds. A review of different EBV-based strategies which may be valuable for the treatment and/or prevention of EBV-associated malignant diseases is presented.

Keywords EBV; cancer; treatment; prevention.

1. EBV infection

Epstein-Barr virus (EBV) is a human gamma-herpesvirus known to infect more than 95% of the human population [1-5]. EBV is mostly transmitted orally, through saliva, with primary infection being frequently acquired during childhood usually without causing symptoms. However, mainly in developed countries, EBV infection may occur later in life, in adolescence or even adulthood, causing the symptomatic disease named infectious mononucleosis (IM, also known as "Kissing Disease"). Following primary infection EBV persists in a latent state throughout life in B cells, usually without causing complications. However, latent long-term EBV infection may lead to more serious consequences and has been associated with the development of several human malignant diseases of lymphoid and epithelial origin [1-3].

Infection with EBV is biphasic (like the infection with other herpesvirus), with the virus being able to adopt two different forms of infection: a latent state (non-productive) in which no viral particles are formed, and a lytic replication (productive), in which new infectious viruses are produced [4, 5] and usually resulting in cell death and in virus dissemination to other cells [6, 7].

EBV infects mainly two different cell types: i) B lymphocytes (or B cells) preferentially, where the infection is mainly non-productive and ii) epithelial cells, in which lytic replication occurs, producing new viruses [8-10]. Following oral transmission with primary infection of epithelial cells from the oropharynx (which results in viral lytic infection and consequently in high rates of virus shedding into the throat), oropharyngeal mucosa B cells become infected and a latent growth-transforming infection begins [11]. This results in the proliferation of B-lymphoblastoid cell lines (LCLs) in the lymphoid tissue near the oropharynx and also in the appearance of a large number of EBV-infected B cells in the blood. Primary infection is controlled by a virus-specific adaptive immune response, which is however unable to completely eliminate EBV from the host [11, 12]. Therefore, viral DNA persists in B cells as an episome, maintaining a latent infection, with virus replication being usually minimal or undetectable [5, 8, 9]. In addition, some level of lytic infection is maintained in B cells without symptoms in immunocompetent EBV carriers, resulting in the shedding of virus in oral secretions and in possible transmission to other individuals [13, 14].

EBV episomal DNA is important for infection, as it is required for replication of the latent genome. During latency, EBV DNA acts like the cellular DNA, being associated with histones and replicating once during the S phase, depending only on the cellular machinery (although using a specific viral DNA origin sequence, *oriP*) and being equally transmitted to daughter cells [4, 15].

About 90 genes have been predicted to be encoded by the EBV genome [16]. During latency, only a restricted group of 12 may be expressed in different combinations (resulting in mainly three different programs of latency: type I, II, and III), while the rest remain silent until entering the replicative, lytic cycle [17]. Viral genes expressed during latency include: i) six EBV Nuclear Antigens (EBNA1, 2, 3A, 3B, 3C and –LP); ii) three Latent Membrane Proteins (LMP1, LMP2A and LMP2B); iii) two small nonpolyadenylated Epstein-Barr virus-encoded RNAs (EBER1 and 2) and iv)

highly spliced *BamHI A* rightward transcripts (BARTs) [6, 15, 17, 18]. Furthermore, these latency programs have also been shown to correlate with the type of EBV associated disease [19]. For example, while Burkitt lymphoma (BL) tumours usually present EBV in type I latency (in which EBNA1 is the only viral protein expressed), nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL) present type II latency. On the other hand, IM and lymphoproliferative disease present EBV in type III latency, which is the most complete latency program in which all the latency genes are expressed [20].

During its life cycle, EBV may also adopt a period of lytic reactivation leading to the production of new infectious virions. In contrast to what happens during latency, in which replication occurs from the *oriP* viral origin and has the main objective of duplicating EBV DNA for transmission to daughter cells, lytic replication initiates from different viral origins called *orilyt* [4, 21, 22]. In lytic phase, linear head to tail concatemers are produced from the template plasmid DNA, and will then be cleaved into linear DNA molecules of 150-200 kbp and packaged into virions [23, 24]. Also in contrast to latency, in which EBV depends only on the cellular machinery for its replication, lytic replication is highly dependent on proteins that are encoded by EBV itself [22, 25]. Reactivation of EBV is highly dependent on the expression of the two "immediate early" (IE) genes, BZLF1 and BRLF1, which code for the Zta and the Rta proteins, respectively. These proteins act as transactivators, which are responsible for the activation of several viral and cellular promoters, thereby resulting in the ordered expression of several other viral proteins.The viral proteins which are involved mainly in DNA replication are expressed first and the viral proteins involved in the structure of the virus are expressed at a later stage [4]. Although the mechanism underneath EBV lytic reactivation *in vivo* is still not fully understood [4], extensive host-driven methylation of the EBV genome, heterochromatin formation and/or cellular transcriptional repressors are probably involved in lytic gene repression and therefore in the maintenance of latency [26].

2. EBV-associated cancers

Unlike many other viruses whose "strategies" consist in using cells to produce viral proteins in order to make more viruses to infect more cells, EBV (like all the other herpesvirus) has also developed a "live and let live" strategy. This allows the virus to persist in latent form in the host (throughout his life) without leading to diseases, except by accident [27]. In fact, although looking harmless, in some few cases EBV infection may have more serious consequences, causing cellular malignant transformation and development of human cancers such as BL, HL, NCP and post-transplant lymphoproliferative disease (PTLD)[1, 2, 28]. In addition, its possible association with other malignant diseases such as breast and hepatic cancers has also been already suggested [20].

EBV infection adopts almost exclusively a latent pattern in these diseases, which does not come as a surprise knowing that some latent EBV proteins may transform cells while EBV lytic infection usually results in cell death [7]. Moreover, the fact that EBV-associated tumours only develop several years following the primary infection indicates that EBV may only be considered as one of the players in a multifactorial context that may lead to some cancers.

2.1. Burkitt lymphoma

The history of EBV description is tightly linked with BL. In fact, BL was firstly described by Denis Burkitt and was the first human cancer to be ever associated with a virus [29]. Contrary to what was initially expected, not all BL cases are EBV positive. Indeed, this cancer of the lymphatic system presents three clinical variants which differ in the presence, biology and association with EBV and are called endemic, sporadic or HIV-associated.

Endemic BL corresponds to the first description of BL by Denis Burkitt. One of its hallmarks is its extremely strong association with EBV, with more than 95% of the endemic BL being EBV-positive [30, 31]. Endemic BL occurs with an annual incidence of 6-7 cases per 100,000 children and with a peak incidence at the age of 6 or 7 years old [32]. It has an unusual pattern of presentation at extranodal sites, frequently starting as tumours in the jaw region [12, 31]. This variant of BL is highly prevalent in the region known as "Lymphoma Belt" (from West to East Africa between the 10° north to 10° south of the equator and continuing south down to the Eastern coast of Africa), being the most common cancer of children in this region (representing about 75% of the malignant diseases in children).

The sporadic BL variant occurs worldwide, being best characterized in Europe and in the United States of America (USA). The association between EBV and sporadic BL, although still significant, is not as strong as the one found for endemic BL, with only 15%-30% of the tumours being EBV positive [33, 34]. The pattern of presentation of these tumours also differs from the endemic BL, with tumours appearing mainly as abdominal masses and occasionally in leukemic form [33].

The immunodeficiency-associated variant of BL is commonly associated with HIV infection. In fact, BL may even be the initial manifestation of AIDS. However, even though the incidence of BL in AIDS patients is high, only a minority of the AIDS-BL cases (30% - 40%) are EBV positive [1, 35]. This immunodeficiency-associated variant of BL has also been described in the setting of post-transplant patients who are taking immunosuppressive drugs [36-38]. In these cases the role of the immune response in the control of EBV latent infection seems to be clearly demonstrated, by the development of EBV-associated lymphomas in immunosuppressed individuals. Indeed, latent EBV infection is

associated with rapid tumour development in immunocompromised individuals, such as the post-transplanted ones or even some individuals with AIDS [15, 39, 40].

2.2. Hodgkin lymphoma

HL is an usual tumour of B-cell origin, characterized by the presence of mononuclear Hodgkin (H) cells and their multinucleated variant Reed-Sternberg (RS) cells within a background of non-neoplastic cell populations comprising T- and B-lymphocytes and other cell types [41]. This lymphoma may be divided into two major types, classical and nodular lymphocyte predominant HL, with EBV infection being found associated only with the first type (the classical HL) [41, 42]. About 50% of classical HL cases are EBV positive [43]. Although the exact mechanism of EBV influence in the development of HL is not fully understood, the fact that EBV latent proteins are widely expressed in these tumours suggests a role for EBV in several cellular pathways, in particular facilitating cellular proliferation [42].

2.3. Nasopharyngeal carcinoma

NPC is a tumour arising from the epithelial cells that cover the surface and line of the nasopharynx [44]. Together with BL and HL, NPC is one of the diseases that is strongly associated with EBV latent infection. NPC is classified in three different subtypes: the keratinizing squamous cell carcinoma, the nonkeratinizing squamous cell carcinoma and the undifferentiated or poorly differentiated carcinoma [44]. Although the association between EBV and the keratinizing carcinomas is still under debate, the latter two sub-types, besides being more frequent, are strongly associated with EBV (about 100% association) which seems to indicate that EBV infection occurs at some point before the expansion of the malignant initial clone of epithelial cells [15].

NPC is a relative rare disease in most populations worldwide occurring in less than 1 per 100,000 persons, per year [45]. However, when analysing southern China populations, the disease is more commonly found. Indeed, in this region the incidence has been described as more than 20 cases per 100,000 persons [1, 46, 47]. Other world regions such as the isolated northern populations of Eskimos and Greenlanders also show a high incidence, together with a higher prevalence in male rather than female individuals. It has also been observed that, in the USA, the majority of NPC patients are Chinese-Americans together with workers exposed to fumes, smoke or chemicals [32]. Since its incidence is so different between ethnic groups and regions, several factors have been proposed to be involved in the pathogenesis of NPC. On the one hand, a genetic link must not be discarded, mainly due to the fact that certain HLA (human leukocyte antigens, central component of MHC) profiles are associated with increased risk of NPC, but some other hereditary factors have also been proposed [30]. On the other hand, environmental and dietary factors appear to have an important role, with chronic exposure of the nasopharyngeal mucosa to chemical carcinogens or to other physical substances possibly influencing the risk of developing NPC [48]. In fact, an association between NPC and eating highly salted foods, such as the salted fish commonly used in southern China, has been described [49, 50]. Moreover, other factors such as exposure to smoke [51], use of herbal medicines (rich in phorbol esters which are known to reactivate EBV infection) [52], or vitamin C deficiency at a young age [53, 54] have also been described as contributing factors to the development of NPC.

2.4. Post-transplant lymphoproliferative disease

EBV also seems to be an important factor that may be involved in the development of PTLD, which consists of a group of disorders resulting from complications from both solid organ or bone marrow transplantations. Although the risk of development of PTLD varies according to several factors such as the type of transplant, age of the individual or even the immunosuppressant regimen [55], primary infection with EBV after transplantation is considered the strongest factor involved [56]. EBV is detected in more than 90% of B-cells in early-occurring PTLD (within the first year after transplant) and in 60%-80% in late onset PTLD [55, 57]. EBV-associated PTLD may be due to viral reactivation following the transplant or due to primary EBV infection, acquired from the donor following the transplant [58]. In most cases, PTLD arises from patients who were EBV negative prior to the transplant [30]. The fact that EBV latent proteins are widely expressed in this disease supports a probable role for EBV in the PTLD pathogenesis [59]. It seems that, as is also observed for HL, 50% of PTLD B cells lack functional B-cell receptor (BCR) which is essential for B-cell survival. Therefore, one of EBV functions is probably to protect these cells from death by apoptosis in the absence of antigen stimulation [35] .

Furthermore, the decreased T-cell immunosurveillance observed in PTLD patients due to the immunosuppression regimen also helps the EBV infection to persist in these cells and to transform them [30, 35].

3. Approaches for the treatment and/or prevention of EBV-associated cancers

In general, therapeutic strategies for EBV-associated cancers do not take into account the presence of the virus and therefore do not differ from the corresponding EBV-negative ones. Conventional therapies are usually based in radio- and chemotherapy aiming at destroying the rapidly proliferating cells. Beside the fact that most EBV-associated

tumours respond poorly to these conventional approaches, severe short- and long-term side effects including secondary malignancies are also frequent [60-63]. Therefore, the presence of EBV inside cells may be considered as a unique opportunity to develop more efficient strategies for EBV-associated cancers [64, 65]. The development of strategies aiming at targeting the virus may have impact in reducing the burden of EBV-associated diseases. Strategies may include the induction of the lytic EBV cycle, the suppression lytic EBV, targeting latent EBV, or monoclonal antibodies and vaccines against EBV.

3.1. Induction of lytic EBV infection

An EBV-based approach which has been proposed for treatment of EBV-associated cancers consists in the induction of the EBV lytic cycle [7, 66, 67]. This therapeutic approach is not only based on the fact that an increase in the number of lytically-infected cells will result in the direct killing of the tumour cells but also in the fact that the existing antivirals are only active against lytic EBV.

In summary, EBV present in latent state in several EBV-associated malignancies could be induced to enter lytic cycle through different strategies, some of them so far only tested *in vitro*.

One of such strategies uses gene delivery techniques to express the IE proteins (Zta or Rta) in tumour cells, under the control of a strong promoter [68], and was proven to be sufficient for causing the expression of the viral lytic EBV program. Indeed, expression of EBV lytic proteins was detected in different types of EBV-positive cell lines following infection with adenoviral vectors containing BZLF1 or BRLF1 expression cassettes [68, 69]. In addition, in a xenograft model of NPC in nude mice which were injected with such vectors, the expression of lytic proteins was accompanied of a reduction of the xenograft tumor size [65, 68].

Another approach to induce lytic EBV infection in cancer cells is to use several EBV lytic inducers. Several agents have been shown to induce lytic reactivation namely demethylating agents, such as 5-azacytidine [70] and agents that induce histone acetylation, such as sodium butyrate [7]. Interestingly, the induction of lytic EBV by butyrate and other histone deacetylase (HDAC) inhibitors may also be associated with their ability to induce demethylation and reactivation of methylated, silenced genes through repression of DNA methyltransferase 1 (DNMT1) [65, 71].

Moreover, the use of radiation or chemotherapeutic drugs, commonly used in the treatment of several cancers, has also been shown to induce lytic EBV replication both *in vitro* and *in vivo*. For example, etoposide, doxorubicin and cisplatin were shown to induce reactivation of EBV in BL cells [72, 73]. Gemcitabine and doxorubicin induced lytic EBV infection in EBV transformed B cells as well as in SCID mice inoculated with these cells [66]. Other chemotherapeutic drugs such as cisplatin, 5-fluorouracil and taxol have also shown EBV inducing abilities in other EBV-associated models [74]. The ability of these chemotherapeutic drugs to promote lytic EBV induction is mediated through the activation of several different transduction pathways namely MAPK, p38 and PI3K but not of apoptosis *per se* [64, 74, 75]. Interestingly, aspirin was also shown to be able to reactivate EBV into lytic replication and therefore induce cytotoxicity in some EBV-positive cells such as cells from the Raji BL cell line [76]. Moreover, DNA damage response and unfolded protein response to endoplasmic reticulum stress may also be involved in EBV lytic reactivation [60]. Indeed, the proteasome inhibitor bortezomib has been shown also to induce lytic reactivation [77-79].

One of the advantages of inducing EBV lytic cycle is that the effects obtained following EBV induction may be enhanced by the concomitant use of antiviral drugs. In fact, the majority of the antiviral agents used to date against herpesvirus (anti-herpetic), such as acyclovir or ganciclovir, are only effective during the lytic phase of the virus life cycle. This is due to the fact that the majority of these drugs are acyclic nucleoside analogues which action requires phosphorylation by the virus-encoded kinases (EBV thymidine kinase and the BGLF4 gene product, PK) which are only expressed during the lytic phase [75, 80]. Once phosphorylated, these antivirals prevent the synthesis of viral DNA but also inhibit the host cell DNA polymerase and kill the tumour cell.

Thus, studies using both cell lines and mice models clearly indicate that the use of EBV lytic inducers sensitize cells to the effects of ganciclovir [7, 64, 66, 74, 81]. In fact, the combination of both these agents (lytic inducer and ganciclovir) has shown to be more effective in treating EBV-positive tumours than using each of the therapeutic approaches alone [75]. In a recent study, the use of a cytolytic virus activating therapy (CLVA) which combined gemcitabine and valproic acid (for virus activation and tumour clearance) with the antiviral ganciclovir (to block virus replication and kill proliferating virus-infected cells) resulted in increased cytotoxicity towards NPC cells than chemotherapy alone [82]. Interestingly, when this therapeutic strategy was tested in 3 patients with end-stage NPC, refractory to conventional treatment, disease stabilization and improved quality of life were observed, although the levels of viral DNA in the circulation (probably originating from apoptotic tumour cells) were also increased.

Nonetheless, although lytic EBV induction is considered a good approach to target EBV-associated cancers, it may present serious risks to the host. Indeed, by increasing the probability of viral dissemination and transmission from cell to cell, lytic infection may increase the number of latently infected cells thereby promoting the development of EBV-associated diseases [75]. Accordingly, studies show an increased probability of developing EBV-associated lymphomas following treatment of patients (such as those with rheumatoid arthritis or polymyositis) with drugs that induce EBV lytic cycle (such as methotrexate) [70, 83].

Thus, the use of some agents in the treatment of EBV-associated cancers, as well as in the treatment of other pathologies in EBV-infected patients, must be considered carefully.

3.2. Suppressing lytic EBV

The use of antivirals following primary infection (in a case of IM) has still not proven to significantly shorten or ameliorate the disease and therefore is not frequently chosen as the first treatment option [75]. However, the fact that they reduce EBV lytic infection therefore reducing the EBV genome load may be relevant for the prevention of EBV-associated diseases.

Several compounds have been described as having antiviral activity against EBV. As previously mentioned, the majority of the antiviral agents used against herpesvirus to date, such as the acyclic nucleoside analogs acyclovir or ganciclovir, are only effective during the lytic phase of the virus life cycle. Maribavir is an alternative antiviral, described to be active against EBV [84]. Although its mechanism of action is not fully understood, it seems to not only inhibit the essential replication gene named early antigen-diffuse (EA-D [BMRF1], the EBV DNA polymerase processivity factor) but also to inhibit EBV transcription in EBV-infected cells [67, 84, 85].

Furthermore, other natural and synthetic compounds, including moronic acid [86], derivatives of betulinic [87] and glycyrrhizic acids [88, 89] and flavonoids such as (-)-epigallocatechin gallate (abundant in green tea) [90] seem to inhibit EBV lytic cycle. Although their mechanism of action is not fully understood, some of them seem to interfere with the viral reactivation by affecting the IE proteins. In a study in the BL cell line Raji, the chemopreventive agent curcumin was also found to be an effective agent for inhibition of EBV reactivation through inhibition of BZLF1 gene transcription [91]. Interestingly, the use of antisense and RNAi technologies to reduce Zta expression has proven to effectively inhibit EBV lytic cycle *in vitro* and could also be of potential use to develop anti-EBV treatments aiming at suppressing lytic infection [92-94].

3.3. Targeting latent EBV

To date there are no effective drugs that can be used for latent EBV infection [95]. One of the explanations for this is the fact that, while in latency, EBV episomes use the cellular machinery for replication and therefore it is difficult to target EBV without also affecting the host cell. Therefore, new strategies aiming at latent EBV are being developed, some of them trying to target the EBV episome while others trying to aim at EBV latent proteins or at cellular products that are EBV-associated.

3.3.1. Interfering with the EBV episome

EBV latency is assured by the maintenance of EBV genome in its episomal state. During this phase, the EBNA1 protein assumes control of both initiation and maintenance of EBV replication by binding to *oriP* [96, 97]. Therefore, a reduction in the total number of episomes in the cell may be possible by interfering with EBNA1 or with the binding of EBNA1 to EBV's origin of plasmid replication *oriP*.

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, has been used to eradicate EBV episomes from latently infected BL cell lines [98]. The mechanism beneath this event, although not yet fully understood, seems to involve an alteration in the replication timing and in chromatin organization of *oriP* and the destabilization of episomal maintenance [99]. Although in some cases the loss of EBV episome promoted by HU is not total, this event is observed in different cell types [98, 100, 101]. Furthermore, HU has been efficiently used *in vivo* for the treatment of EBV-associated primary central nervous system lymphoma in the setting of advanced AIDS [102]. However, the use of HU as treatment option may increase the risk of acquired cellular DNA mutations [103]. Moreover, acquired resistance to HU was observed following treatment of Raji BL cells with this drug [104].

Several strategies have been attempted *in vitro* to downregulate EBNA1 expression. Indeed, through the use of antisense oligonucleotides for EBNA1, a reduction in this protein expression was observed with the concomitant reduction in the EBV episomal copy number [105]. In addition, the use of a specific EBNA1 ribozyme suppressed EBNA1 mRNA and protein expression in LCLs and significantly reduced the number of EBV genomes [106]. More recently, using RNA interference (RNAi), downregulation of EBNA1 has been achieved in several EBV-infected cell lines. Of note is the fact that this downregulation of EBNA1 in EBV-positive tumour cell lines resulted in the inhibition of cell growth/survival [107-109]. Interestingly, a previous study in which EBNA1 expression was downregulated (with siRNAs in the BL Akata cell line) showed that EBV influenced the expression of cellular anti-apoptotic protein Bcl-xL and reverted cellular sensitivity to etoposide [110].

Another approach to target EBV episome is by interfering with its replication at *oriP*, which requires host cellular replication proteins as well as the viral EBNA1. Some of these cellular proteins are the same proteins required for the replication of cellular DNA at the origin of replication complexes (ORCs). In fact, geminin, an inhibitor of the mammalian replication initiation complex, also inhibits replication from *oriP*. Thus, drugs that affect the processes involving ORCs may also prove of additional value to interfere with EBV episome [64, 111].

3.3.2. Interfering with EBV latent proteins

Since the role of EBV in tumour development is associated with the function of several of its latent proteins, it is possible that by interfering with their expression the role of EBV in oncogenesis could be reverted. Thus, several

strategies aiming at decreasing the expression of latent proteins, such as EBNA1, LMP1 and LMP2, have been developed with the use of antisense, rybozimes, DNAzymes and RNAi. In fact, by downregulating LMP1 in LCLs, a decrease in Bcl-2 anti-apoptotic protein was observed together with a decrease in proliferation, an increase in apoptosis and an increase in sensitivity to apoptotic stimulus [112, 113]. Furthermore, in NPC cells, combination of the LMP1-specific DNAzymes and radiation treatment significantly induced apoptosis [114] and LMP-1 suppression by short hairpin RNA (shRNA) significantly altered cell motility, substratum adhesion, and transmembrane invasion ability [115]. In addition, LMP2B silencing in BL Akata cells reduced the activation of EBV lytic replication [116].

Cidofovir, an antiviral which is an acyclic nucleoside phosphonate analogue, has been shown to have anti-proliferative activity against EBV-infected cells. In contrast to the other antiviral drugs, it seems to bypass the first phosphorylation steps by viral-encoded kinases. Interestingly, treatment of BL cells with cidofovir resulted in downregulation of LMP1 and EBNA2 proteins, together with a decrease in Bcl-2 and increase in Bax, which was associated with inhibition of proliferation and stimulation of apoptosis in these cells [117], indicating that this approach may also help killing the tumour cells.

3.4. Monoclonal antibodies and vaccines against EBV

Monoclonal antibodies against a specific protein have been used to treat several human pathologies, some of which known to be associated with EBV. For example, rituximab, a monoclonal antibody against CD20 (a B-cell specific protein), has been used for the treatment of early lymphoproliferative disease in post-transplant diseases [118]. However, this antibody targets all B cells expressing CD20 and does not identify the EBV expressing cells. Studies using monoclonal antibodies targeting CD70 [75], which is expressed at the surface of cells infected with latent type II or III EBV (although not type I) resulted in growth inhibition of CD70 expressing cells. This means that, at least for some EBV-associated pathologies, the use of these antibodies may be advantageous. However, there is still need to further develop this strategy in order to efficiently produce better antibodies that specifically recognize EBV proteins.

Over the past decades, efforts have been made in order to find a vaccine that could prevent EBV infection, with the main goal of preventing IM but also to reduce the incidence of EBV-associated malignancies. However, several factors presented problems to the development of such a vaccine. Apart from the information obtained from experiments with vaccines for other herpesvirus, indicating that it is unlikely that a primary EBV infection could be entirely prevented through the use of a vaccine, limited experimental information may be obtained regarding the efficacy of a possible vaccine since EBV only infects humans (and few related primates). Nevertheless, some useful information has been obtained from a very restricted number of phase I/II clinical trials in humans [119].

In the quest for an EBV vaccine, attention has been given to the EBV gp350 envelope glycoprotein which is a major target for neutralizing antibodies [120, 121]. A group of EBV-negative individuals were voluntarily administered the gp350 vaccine and were followed over a 3 year period for EBV infection and also for IM [119, 122]. Results showed that the vaccine, although having little effect on the frequency of silent seroconversion, greatly reduced the frequency of IM. A gp350 based vaccine is under development [123]. Nevertheless, the fact that this type of vaccine does not seem to prevent infection, nor can be active against latent EBV, further indicates that there is the need to develop a vaccine that targets other EBV proteins. In fact, the use of a peptide vaccine designed from EBNA3 proteins has already been tested in a phase I trial [124] and studies aiming at developing a novel cocktail vaccine expressing both lytic (EBV gp350 and gp110) and latent EBV proteins (EBNA2 and EBNA3) have also been described [125]. In addition, a dendritic cell vaccine targeting LMP1 and LMP2 (expressed in NPC) has reached a phase II trial of immunotherapy in EBV-positive metastatic NPC patients but had limited efficacy [126]. Very recently, a phase I trial of a recombinant vaccinia virus designed to boost T-cell immunity to EBNA1/LMP2 has been conducted, to determine safety and the immunogenic dose [127]. Moreover, the use of non-transforming virion like particles (VLPs) has been shown to facilitate the specific and rapid expansion of EBV-specific $CD4^+$ T cells and thus VLPs are being considered for the development of a potential EBV vaccine [128, 129].

3.5. Search for new anti-EBV agents: a role for computational analysis

Since there is a need to find an agent that efficiently targets EBV, mostly during its latent phase, new strategies need to be developed. Major information is arising from the association of laboratory studies with computational analysis. In fact, through the use of computational analysis new targets may be found. Furthermore, analysis of the structural features of the targets and their possible interaction with several molecules is also possible though chemoinformatics. Indeed, through docking models, it is possible to choose the best molecules that associate with a given target and to score this association according to their binding affinities. This allows sorting the molecules and chemical groups that best fit the model and therefore further optimize those molecules to improve their binding to the target.

The knowledge obtained through this strategy may lead to new information regarding the requirements needed for a molecule to bind EBV target proteins, and therefore to the development of compounds that may efficiently target EBV. Interestingly, by virtual screening 90,000 compounds using computational docking programs with the solved crystal structure of EBNA1, it was possible to identify a new class of compounds that inhibit EBNA1-DNA binding in biochemical assays [130]. Furthermore, some of these compounds inhibited EBNA1 transcription activation in cell-

based assays and reduced EBV genome copy number of a BL cell line. More recently, using computational molecular docking complemented with *in vitro* studies in BL Raji cells, three novel sulfated small molecules capable of decreasing EBV viral load as well as LMP1 expression have been identified [131].

These studies support the idea that *in silico* screening presents a good approach for the development of new anti-EBV agents. Ultimately, the goal would be the combination of rational drug design with drug synthesis and biological assays, further supported by animal models and clinical studies, which would provide the means to efficiently develop a targeted anti-EBV therapy.

4. Concluding remarks

EBV infection plays a causal role in the development of some cancers such as BL, HL, NPC and PTLD. The unique presence of EBV in EBV-associated diseases allows the development of viral targeted therapies. The possibility of developing strategies which target viral activity, in particular the virus in its latent state, may have great impact in the treatment and prevention of EBV-associated diseases.

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