The Status and Prospects of Epstein–Barr Virus Prophylactic Vaccine Development

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Epstein–Barr virus (EBV) is a human herpesvirus that is common among the global population, causing an enormous disease burden. EBV can directly cause infectious mononucleosis and is also associated with various malignancies and autoimmune diseases. In order to prevent primary infection and subsequent chronic disease, efforts have been made to develop a prophylactic vaccine against EBV in recent years, but there is still no vaccine in clinical use. The outbreak of the COVID-19 pandemic and the global cooperation in vaccine development against SARS-CoV-2 provide insights for next-generation antiviral vaccine design and opportunities for developing an effective prophylactic EBV vaccine. With improvements in antigen selection, vaccine platforms, formulation and evaluation systems, novel vaccines against EBV are expected to elicit dual protection against infection of both B lymphocytes and epithelial cells. This would provide sustainable immunity against EBV-associated malignancies, finally enabling the control of worldwide EBV infection and management of EBV-associated diseases.

Keywords: Epstein–Barr virus, vaccine, virus immunology, adjuvant, animal model

INTRODUCTION

Epstein–Barr virus (EBV) is a double-stranded DNA virus that belongs to the gamma herpesvirus family. It causes endemic infection in over 95% of the worldwide population (1), and is associated with diseases such as infectious mononucleosis (IM) and a broad range of lymphoid or epithelial malignancies (2, 3). It is estimated that approximately 2% of malignancies are caused by EBV infection, resulting in over 200,000 cases of EBV-associated cancer each year (4).

The transmission of EBV within the population is mainly mediated by saliva, and the infection involves both B lymphocytes and epithelial cells (5). Primary infection mostly occurs in early childhood with little or no overt symptoms (6). After the primary infection is established, EBV sustains a persistent infection in B lymphocytes, accompanied by the expression of specialized viral genes that maintain its latency, which is associated with B cell tumorigenesis (7). Therefore, a prophylactic EBV vaccine for establishing early protection against primary infection is critical for prevention both infectious diseases and EBV-associated malignancies. However, there is still no prophylactic vaccine against EBV in clinical use due to various reasons including antigen selection, vaccine platform used, and evaluation system for EBV vaccine assessment. Thus, in this review, we
summarize the challenges and opportunities encountered in the development of a prophylactic EBV vaccine.

ANTIGEN SELECTION FOR VACCINE DESIGN

Glycoproteins

Similar to other herpesviruses, EBV is an enveloped virus, comprising a membrane decorated with envelope glycoproteins (such as gp350, gp42, gH, gL, and gB), which are crucial for receptor recognition, attachment, and virus–host membrane fusion (8). As EBV can infect both B lymphocytes and epithelial cells, the glycoproteins involved in the infection process of each cell type differ. For B cell infection, EBV gp350 interacts with CD21 or CD35 on B cells to establish viral binding, followed by the binding of gp42 in complex with gHgL to HLA class II on B cells, after which gB eventually triggers the membrane fusion in endocytic vesicles (9–12). By contrast, EBV adopts a rather different and more versatile combination of ligand–receptor pairing during viral entry into epithelial cells. EBV can still use gp350 to establish attachment to CD21-expressing host cells (13), while BMRF2 (14) or the gH/gL complex binds to other host cell receptors to facilitate the infection of cells that lack CD21.

Currently, integrins (15), non-muscle myosin heavy chain IIA (NMHC-IIA) (16) and ephrin receptor A2 (17, 18) (EphA2) are recognized as receptors for EBV gH/gL, while neuropilin-1 (19) (NRP1) acts as the receptor for EBV gB during epithelial cell infection, indicating a more important role of gH/gL complex and gB during the recognition and attachment in comparison to B cell infection (Figure 1).

The complex molecular machine of surface glycoproteins brings challenges for not only understanding the complete fusion mechanism of EBV, but also choosing appropriate antigens for vaccine development. Therefore, an ideal prophylactic vaccine against EBV should be able to elicit potent neutralizing antibodies against EBV infection in both B lymphocytes and epithelial cells, which requires careful selection of antigens during vaccine design.

gp350

As the first-isolated and most abundant EBV glycoprotein, gp350 was the most studied antigen for vaccine candidates and is the core antigen for the majority of the currently-developed EBV vaccines (20–28). The first clinical trial of a recombinant viral vector encoding gp350 performed in China in 1997 proved that gp350-specific antibodies could be elicited in both seronegative and seropositive children (29). In later studies, recombinant
gp350 adjuvanted with AS04 was used as the vaccine in a phase II clinical trial among seronegative adults (30) and was shown to effectively reduce the incidence of IM compared to the placebo control group. However, this vaccine did not completely prevent EBV infection in the vaccinated population. In another phase I clinical trial gp350 was formulated with 0.2% Alhydrogel® as vaccine for reducing the risk of post-transplant lymphoproliferative disease (PTLD) (31). The vaccine failed to elicit neutralizing antibodies and control the viral titer in the majority of patients, possibly due to its low immunogenicity for immunosuppressed patients. Thus, despite the early and thorough study, gp350 exhibited only imperfect vaccination efficacy as single antigen in both preclinical and clinical trials (Figure 2A, Tables 1, 2).

**gH/gL**

Since the gH/gL complex plays a critical role in the infection of B cells and especially epithelial cells, there is increasing focus on the gH/gL complex as the antigen for new vaccine candidates. The recently identified anti-gH/gL dual-tropic neutralizing antibody AMMO1 (63) further indicated that gH/gL may be an ideal antigen. In a study on rabbits (51), trimeric or monomeric gH/gL could elicit >100- and 18-fold higher EBV neutralizing antibody titers than monomeric gp350. Later nanoparticle vaccines displaying gH/gL or the gH/gL/gp42 complex were designed, and immunization assays in BALB/c mice demonstrated that the nanoparticle decorated with gH/gL or gH/gL/gp42 could elicit much higher neutralizing antibody titers than monomeric gH/gL or gH/gL/gp42 (55). Despite these promising results for gH/gL as a vaccine candidate, there are still no clinical trials examining whether gH/gL could provide broader protection than gp350 and possibly achieve complete protection from EBV infection.

**gp42**

gp42 is a subunit of the gH/gL/gp42 heterotrimer on the EBV virion membrane. It was identified as the ligand for HLA class II molecules mostly participating in B cell infection and was recently found to hinder the infection of epithelial cells (64, 65), indicating that it controls the tropism of EBV infection. The close structural connection and functional complexity suggested that a combination of gH/gL/gp42 as a complex antigen may be more potent than gp42 alone. Studies of the effect of immunization with the EBV viral fusion apparatus indicated that immunization using gH/gL in complex with gp42, either as monomers or nanoparticles, could elicit relatively higher neutralizing antibody titers against infection of both B lymphocytes and epithelial cells (55). Nevertheless, few studies investigated gp42 as the target for vaccine design. Moreover, its role in controlling the tropism of infection would complicate the protection efficacy of elicited antibodies against gp42, which may potentially influence the tropism of the original virus and enhance the efficiency of epithelial cell infection.

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**FIGURE 2** | (A) Hallmarks of prophylactic EBV vaccine development using EBV glycoproteins as antigens. Clinical trials are marked in yellow box and others are marked in gray box. (B) Current candidate platforms for EBV vaccines, including virus-based, protein-based and nucleic-acid-based vaccines.
### TABLE 1 | Summary of EBV vaccine animal trials.

| Year | Platform/ Adjuvant | Antigen | Animals | Results |
|------|--------------------|---------|---------|---------|
| 1984 | Subunit vaccine/ liposome, Freund’s adjuvant, lipid A | Full length membrane gp340 (gp350) purified from virus | Mice, rabbit | Antibody responses were induced similarly in mice and cotton-top tamarins, among which groups adjuvanted with liposome and lipid A elicited antibody responses earlier; Antibody responses in rabbits were rather weak (22). |
| 1985 | Recombinant vaccinia virus(WR strain) | gp340 | cottontop tamarins | Provided protection against malignant lymphoma (21) |
| 1986 | Subunit vaccine | gp340 produced by immunoadfinity chromatography from B95-8 | cottontop tamarins | No protection against malignant lymphoma (23) |
| 1988 | Recombinant subunit vaccine/ threonylmuramyl dipeptide adjuvant formulation. | gp350/gp220 produced by immunoadfinity chromatography from yeast and mammalian cells | cottontop tamarins | All of the mammalian cell-derived versions of the membrane antigen were found capable of inducing EBV-specific neutralizing antibodies as well as B95-8 (23). |
| 1988 | Subunit vaccine/ ISCOMS | gp340 incorporated into immune-stimulating complexes (ISCOMS) | cottontop tamarins | Provided protection against malignant lymphoma (34) |
| 1988 | Recombinant vaccinia virus (Wyeth or WR strains) | gp340 | cottontop tamarins | Only WR strain derived vaccine could offer protection against malignant lymphoma (24) |
| 1992 | Recombinant subunit vaccine/ threonylmuramyl dipeptide adjuvant formulation. | gp340, lack of membrane anchor region, produced using a bovine papillomavirus (BPV) expression vector | cottontop tamarins | 3/4 immunized cottontop tamarins showed protection against malignant lymphoma, 1/4 immunized cottontop tamarins developed idiopathic colitis due to low immune responses to gp340 (35). |
| 1993 | Replication-defective recombinant adenovirus vaccine | gp340/220 | cottontop tamarins | Provided protection against malignant lymphoma despite no detectable neutralizing antibodies in vitro (25). |
| 1994 | Subunit vaccine/alum | gp340 | Rabbits, cottontop tamarins | 3/5 immunized cottontop tamarins showed protection against malignant lymphoma (36). |
| 1996 | Recombinant vaccinia virus | gp340 | common marmosets challenged with M81 | Vaccinated group showed lower virus load compared to control group (26). |
| 1999 | Recombinant subunit vaccine/ alum VS Freund’s adjuvant | gp340 | rabbits | Elicited high neutralizing antibody titers; three immunizations with MSTOP gp350 elicited neutralizing titers of 3800±5400 in alum and 1,600 ± 3,400 in Freund’s adjuvant (27). |
| 2001 | Peptide epitopes | HLA A2-restricted epitopes from the latent, lytic and structural proteins | Humanized HLA A2/Kb mice | A maximal response to the epitopes within the structural proteins and low to moderate responses to the latent epitopes, indicating hierarchy of CTL responses between mice and humans (37). |
| 2003 | Recombinant poxvirus vaccine | Polyepitope protein comprising 6 HLA A2-restricted epitopes derived from LMP1 and LMP2A | Humanized HLA A2/Kb mice | Successfully reversed the out- growth of LMP1-expressing tumors in HLA A2/Kb mice (38). |
| 2009 | Epitope/HSP70 and incomplete Freund’s adjuvant | Mycobacterial HSP70 and LMP2A (S56-364) epitope fusion protein | Humanized HLA-A2.1 mice | Specific CTL responses; eliminated tumors in mice (40). |
| 2011 | Epitope/ HSP70 | Reconstituted complexes of MItHsp70 and LMP2A-epitopes | BALB/c mice (H-2d) | Specific CTL responses; protective activity and therapeutic efficacy against LMP2A-expressed tumor challenge (41). |
| 2011 | EBV-derived VLP | EBV-derived VLP, deleted or functionally inactivated six viral genes (EBNA2, LMP1, EBNA3A, -B, and -C, BZLF1) and LMP2 | BALB/c mice | Strong CD8+ and CD4+ T cell responses in a preclinical murine model (42). |
| 2011 | Combined immunization of DNA, AAV, and adenovirus vector vaccines | gp350 (1-470) | BALB/c mice | Combined immunization with DNA, AAV, and adenovirus vector vaccines induced specific cellular immunity better than any other combinations (43). |

(Continued)
EBV gB is the fusion protein on the viral surface mediating viral–host membrane fusion and recognizing NRP1 on epithelial cells. Most prophylactic antiviral vaccines target the viral fusion protein, such as influenza HA (66–68), HIV env (69, 70), Ebola virus GP (71, 72), and coronavirus spike protein (73, 74), as the fusion proteins in these viruses not only drive membrane fusion but also recognize host membrane factors to initiate attachment and trigger the fusion process. Thus, in these viruses the functional domain of the fusion protein is considered an ideal vulnerable site for neutralization. Hence, the similarity between EBV gB and other comprehensively studied viral fusion proteins such as influenza HA and HIV env is likely to be a good general predictor of effectiveness of a new vaccine.

### TABLE 1 | Continued

| Year | Platform/ Adjuvant | Antigen | Animals | Results |
|------|-------------------|---------|---------|---------|
| 2013 | Replication-defective chimpanzee-derived adenovirus vectors | Rhesus Lymphohepatovirus EBNA-1 Homologue, rEBNA-1 pastoris | rhesus macaques | EBNA-1-specific T cells could be expanded by vaccination (45). |
| 2013 | Recombinant subunit vaccine | Truncated EBNA1 (E1ΔGA, codons 390–641), produced from methylotrophic yeast P. pastoris | BALB/c mice | Elicted CD4+ and CD8+ T cell responses (46). |
| 2015 | Newcastle disease virus (NDV)-virus-like particle | EBV gp350/220 ectodomain | BALB/c mice | Elicited neutralizing antibody responses, but not better than soluble gp350/220 (47). |
| 2015 | Dendritic cells pulsed with recombinant B2LF1 | B2LF1 | hu-PBL-SCID mice | Elicted specific cellular immunity; improved survival from fatal EBV-LPD (48). |
| 2015 | Self-assembling nanoparticles | gp350 D123-ferritin; gp350 D123-encapsulin | BALB/c mice; Cynomolgus Macaques | gp350-nanoparticle elicited 10- to 100-fold higher neutralization titer compared to soluble gp350 (49). |
| 2015 | Recombinant subunit vaccine/ TiterMax (CytRx) | native or denatured/alkylated gp350 produced from CHO | Rabbits | Denatured gp350 could induce binding antibodies but no neutralizing antibodies (28). |
| 2015 | Designed peptides, coupled with keyhole limpet hemocyanin (KLH), Sigma adjuvant system | Designed gp350 peptides to mimic gp350 amino terminus that interacts with 72A1 | BALB/c mice | The gp350 mimetic peptide bound to 72A1 antibody can block gp350 recognition (50). |
| 2016 | Multimeric subunit vaccine | trimeric gH/gL; trimeric gB; tetrameric gp350 | rabbits | Trimeric and monomeric gH/gL, trimeric gB, and tetrameric gp350 groups induced serum EBV-neutralizing titers >100-, 20-, 18-, and 4-fold higher, respectively, than monomeric gp350 (51). |
| 2016 | Multi-epitope vaccine | Chimeric multi-epitope protein referred to as EBV-LMP2m, which is composed of LMP2a195-232 and LMP2a195-232 | BALB/c mice | Elicited specific antibody and CTL responses (52). |
| 2018 | Subunit vaccine | Fc-fused gp350 dimer | BALB/c mice | Humanized NSG-A2 mice | Elicited higher specific antibody titers than gp350 monomer; elicited potent nAbs (53). Provide significant protection against wild-type EBV infection (54). |
| 2018 | EBV-derived VLP | Viral particle expressed both with lytic and latent proteins by insertion of latent protein epitopes into the major tegument protein BNRF1 | BALB/c mice | Elicited neutralizing antibodies (55). |
| 2019 | Self-assembling nanoparticles/ SAS adjuvant | gh/gl/ferritin; gh/gl/gp42- ferritin | BALB/c mice; Cynomolgus Macaques | Monkey immunized with gh/gl/gp42-ferritin nanoparticles elicited >40- and ~4-fold higher neutralization titers in B cells in comparison with soluble gh/gl and soluble gh/gl/gp42; in epithelial cells, gh/gl/ferritin and gh/l/gp42-ferritin nanoparticles showed >25- and ~4-fold higher neutralizing titers than the corresponding soluble glycoprotein vaccines (55). Elicited specific neutralizing antibodies more robust than soluble gp350 ectodomain (56). |
| 2020 | Newcastle disease virus (NDV)-virus-like particle/ aluminum hydroxide and monophosphoryl lipid A | gp350, gpB, gp42, gh, and gl pentavalent complex | rabbits | Elicited neutralizing antibodies (57). |
Table 2: Summary of EBV vaccine clinical trials.

| Trial ID   | Published Year | Phase | Platform/Adjuvant | Antigen | Subjects | Observation index | Results |
|------------|----------------|-------|-------------------|---------|----------|-------------------|---------|
| NCT01147991 | 2007           | I     | Recombinant virus  | Major EBV membrane antigen BNLF-1 MA (gp 220–340) | EBV-positive and vaccinia-virus-exposed adults; EBV-positive, non-vaccinia-virus-exposed juveniles; and EBV and vaccinia virus-naive infants | EBV infection status for EBV negative infants | EBV-neutralizing titers increased in the vaccinated juveniles compared to adults; 9/9 vaccinated infants had specific neutralizing antibody response and only three of them vaccinated infants infected EBV while 10/10 unvaccinated infants got infected (29). |
| NCT00430534 | 2007           | II    | Subunit vaccine/AS04 | gp350 | Patients with advanced NPC | Clinical responses in 1-year follow-up: PR, partial response; PD, progressive disease | 9/16 patients had epitope-specific CTL responses; 2/16 patients had lesions shrunk (58). |
| NCT01256853 | 2008           | II    | Subunit vaccine/ AS04 | gp350 | Healthy adults (EBV + and EBV– both included) | Seroconversion rates were 100%; adjuvanted gp350 vaccine is better than non-adjuvanted in terms of GMTs for anti-gp350 ELISA responses (20). |
| NCT01147991 | 2014           | I     | Recombinant modified virus Ankara | EBNA1 and LMP2 | EBV-Positive NPC | Frequency of functional T-cell responses; levels of EBV genomes in plasma (to reflect tumor burden). | T-cell response rates: 7/14 for EBNA1; 6/14 for LMP2 (62). |

Proteins indicates that gB could be a promising target for vaccine development. In addition to the AMMO1 antibody targeting EBV gHgL, anti-EBV gB AMMO2/3/4/5 discovered by Snijder et al. also demonstrated a strong neutralization activity against epithelial cell infection (63), supporting the use of gB as a prophylactic vaccine candidate. In addition, the previously mentioned research studying the efficacy of immunization with trimeric gHgL in rabbits also explored gB,
which was also able to elicit higher neutralizing antibody titers than gp350 (51).

With the development of protein structure analysis, the fusion status of fusion proteins becomes increasingly important for elucidating the fusion mechanism and understanding the connection between conformational changes and the fusion process. Pre-fusion status is often regarded as the natural conformation on the viral membrane (75, 76) before interacting with the host cell. The discovery of pre-fusion status and artificial modification to freeze the fusion protein in the pre-fusion conformation (77–79) greatly promoted vaccine development in recent years. During the SARS-CoV-2 pandemic, the pre-fusion-stabilized spike protein variant S-2P (80, 81) provided an ideal antigen for the design of broad-use COVID-19 vaccines. Similarly, pre-fusion-stabilized HIV env BG505-SOSIP (69) and RSV F DS-CAV1 (77, 79) also provided an impulse for vaccine development, since they could elicit much higher neutralizing antibody titers than the post-fusion conformation. Therefore, there is increasing focus on the conformation of EBV gB. However, the currently available crystal structure of gB shows a post-fusion conformation at pH8.0 (82), and there is still no high-resolution structure of any pre-fusion gB from the herpesvirus family. Although recent cryo-electron imaging studies of gB displayed on vesicles (83), pseudo-virus membranes or virions (84) were highly suggestive of a potential pre-fusion conformation of gB from other herpesviruses; more evidence and structural studies are required to define the pre-fusion form of gB from EBV, which would greatly promote the use of this antigen as a vaccine candidate.

**Latent and Lytic Phase Proteins**

After primary infection, EBV undergoes a short period of replication in the oropharynx, after which further infection of B cells ensues, during which glycoproteins encoded by the EBV genome become eclipsed by certain lytic and latent genes, which drive the B cell transformation and latency as summarized by a review (85). Therefore, neutralizing antibodies against glycoproteins cannot induce the clearance of latently infected cells which do not express the target, while T cell-mediated immunity would be critical for controlling EBV infection during pre-latency and latency. With a deeper understanding of the role of T cell immunity in the control of EBV infection and extensive mapping of immuno-focused T cell epitopes of EBV antigens (86–97), the application of latent or lytic phase proteins as vaccine antigens has become a topic of continuing study. Elliott et al. used the EBNA3 HLA-B8 T cell epitope FLGRAYGL, adjuvanted with tetanus toxoid and Montanide ISA 720, as a vaccine in a phase I trial among EBV sero-negative adults (59). The results showed that despite good vaccine tolerance and reduced incidence of infectious mononucleosis, the vaccination did not protect the subjects from EBV infection. Other CTL epitopes based on LMP1 and LMP2A showed great potential in tumor treatment in preclinical studies (38–41, 52), but none displayed a clear viability as effective antigens to prevent primary infection. Thus, for prophylactic vaccine development, latent or lytic phase proteins could be used as auxiliary boosters for inducing adequate T cell responses, while the major glycoprotein antigens still play the key role in the prevention of primary infection.

Hence, during EBV vaccine development, rational and careful antigen selection is necessary to ensure both robust and comprehensive immunity against EBV infection. There is still a lot of space for extensive study on immunization efficacy of single glycoproteins, especially gH/gL or gB. Additionally, combinatorial use of multiple antigens as vaccine candidates, including glycoprotein sets or glycoprotein-latency protein combinations, deserves further study for eliciting both sufficient neutralizing antibody titers and T cell responses.

**Vaccine Delivery Platforms and Formulations**

**Delivery Platform for Vaccine Design**

The outbreak of the COVID-19 pandemic has brought significant challenges for global vaccine development, prompting a continuous stream of innovative designs of candidate vaccines against SARS-CoV-2, and thus giving great impetus to next-generation vaccine development. The rapid application of the first clinically used mRNA vaccine developed by Moderna and BioNTech (98, 99) achieved great success in combating SARS-CoV-2 and demonstrated that innovation of new vaccine designs could accelerate the procedures of vaccine development, provide more flexible platforms for antigen delivery, and improve immunization efficacy. Nevertheless, traditional platforms for vaccine development, such as weakened or inactivated virus (100–102), still account for the majority of currently available vaccines and have demonstrated their value during the COVID-19 pandemic due to their outstanding stability, immunogenicity, and convenience in distribution. Therefore, a wider array of adequate platforms for vaccine design is also critical for EBV vaccine development (Figure 2B).

**Virus-Based Vaccines**

Because EBV tends to establish a latent infection of host cells, a general approach to induce EBV replication and cell lysis requires complicated procedures and results in a low yield of live virus. Consequently, the development of attenuated virus or inactivated virus vaccines based on authentic EBV is challenging due to limited viral material. Thus, there are few reports on the use of inactivated or attenuated EBV as vaccine candidates. Alternatively, modification of EBV the genome for direct production of defective virions without genomic DNA could be a viable approach for EBV-derived vaccine development. EBV-derived virus-like particles (VLPs) are based on different EBV mutants with various deletions of sets of oncogenic genes or DNA packaging genes (103), produced by inducing cell lines to enter the lytic phase, followed by purification from cell supernatants by centrifugation. Multiple studies developed
several EBV VLPs (delta BFLF1/BFRF1, delta BBRF1, delta BFLF2, delta TR terminal repeats) (42, 104–107) by deletion of certain critical genes to obstruct virus replication and DNA packaging. However, the possibility of repacking of EBV DNA would bring safety concerns to such designs. In addition to the construction of VLPs based on EBV itself, a Newcastle disease virus-like particle (ND VLP) platform was also used for the presentation of EBV antigens such as gp350/gp220, combinations of gHgL-EBNA1 or gB/LMP2, and even pentavalent gp350/gH/gL/gp42/gB (47, 56). It may be easier to produce VLPs by additional co-transfection of NDV-F for particle assembly, benefiting the rapid development of safe VLP vaccines.

Another approach for the development of virus-based vaccines is using viral vectors as carriers to deliver targeted antigens by generating recombinant vaccinia virus. After inserting specific sequences encoding EBV antigens into the genome of vaccinia virus, the recombinant virus can infect host cells and drive the expression of exogenous antigen in the cells, leading to antigen processing and presentation via the classic MHC-I pathway and activation of antigen-specific cytotoxic CD8+ T cells (108, 109). In addition, through maintaining the certain degree of replication function, attenuated self-replicated vaccinia virus could stimulate an even higher immune response than replication-defective virus. The vaccinia virus also acted as a self-adjuvant by expressing a broad range of pathogen-associated molecular patterns (PAMPs), increasing the whole immunogenicity of vaccine. Currently, modified vaccinia virus Ankara (MVA) (61, 62, 110, 111), adenovirus (ADV) (60, 112, 113) and Varicella-zoster virus (VZV) (114) have been used as a vector to generate an EBV antigen-carrying recombinant live virus vaccine. However, this technology was more commonly used for developing therapeutic vaccines for the treatment of EBV-associated tumors due to the favorable stimulation of cellular immunity, while few trials investigated its use in prophylactic vaccines since the first human test using gp350 as antigen and smallpox-based vaccinia virus as viral vector (29) due to the uncertain safety and reported adverse events of this platform (115).

**Protein-Based Vaccines**

With the rapid development and great progress in structure-guided protein modification and design (116–122), recombinant proteins have gradually become an effective approach for accurate antigen immunization. As gp350 was firstly applied as antigen for EBV vaccine design, gp350 modification to promote immunization efficacy was also a focus during the early exploration of protein-based vaccines against EBV. In the late 20th century, soluble gp350 protein was successfully expressed as a vaccine antigen (33). Subsequent attempts to enhance the immunogenicity and improve the immunization efficacy aimed to increase the valency or target the protein to antigen presenting cells (APCs) using a variety of methods such as multimerization, nanoparticle assembly and fusion-protein design. For multivalency, tetrameric gp350 was designed by fusing two separate gp350 (1–470) to a C-terminal leucine-zipper with or without T cell epitopes, and the results showed that tetrameric gp350 could elicit higher neutralizing antibody titers than monomeric gp350 (51). Additionally, by fusing gp350 to ferritin or encapsulin, multivalent gp350 nanoparticles (49) were generated and immunization of mice or monkeys showed that nanoparticles elicited much higher neutralizing antibody titers than soluble monomeric gp350. Further, virus challenge experiments also demonstrated that gp350 nanoparticles provide better protection against EBV infection and improve the survival of challenged monkeys. In an effort to both increase the valency and enable APC-targeting, gp350 was fused with the Fc domain of mouse IgG2a (53, 123), rendering a dimeric antibody-like antigen which could target FcγR on antigen-presenting cells to prolong the retention time for recognition. In addition, the fused protein simplified the purification and detection.

Comparatively few studies investigated using other glycoproteins or latent phase proteins as antigens. Trimeric gHgL constructed by fusing gHgL to a C-terminal trimeric T4 bacteriophage fibritin and native trimeric gB were also tested as immunogens (51), and the results showed that trimeric gHgL could elicit higher neutralizing antibody titers than monomeric gHgL. Recently, analogous methodology was adopted to design gHgL or gHgL/gp42 nanoparticles by fusing the antigen to the 24-mer ferritin, whereby the neutralizing antibody titers of the nanoparticle-immunized groups were significantly higher than in the monomer groups as previously mentioned.

Instead of using the full length or a major segment of the protein, some studies attempted to use specific epitopes as antigens to induce site-specific immune responses and thereby achieve accurate immunization. Jerome et al. designed two 72A1-gp350 blocking peptides that mimic the interacting region of gp350 (50), which demonstrated that the neutralization epitope of the glycoprotein could be an ideal vaccine antigen. Afterwards, Zhang et al. inserted different tandem gp350 epitopes into HBC149 to construct a gp350 epitope-displaying VLP (57), and the neutralizing antibody titers of some gp350 epitope-VLP groups were even higher than that of gp350ECD123, a shortened version of the gp350 ectodomain, which compares favorably to the anti-gp350 nAb 72A1.

**Nucleic-Acid Vaccines**

The rapid and successful application of nucleic-acid SARS-CoV-2 vaccines demonstrated their great potential in viral vaccine development. This method, based on synthetic nucleic acids, enables large-scale manufacturing with almost perfect uniformity. Despite such advantages, the use of synthetic nucleic acids for EBV vaccine development is still in the early exploration phase. Krzyztof et al. developed DNA vaccines based on three EBV latency genes (EBNA1, LMP1 and LMP2A) (124) and found that the vaccine based on EBNA1 and LMP2A could elicit robust T cell immunity. Although mRNA vaccines are highly potent and can be rapidly manufactured, the development of an mRNA vaccine for EBV still awaits the first step. As expected, Moderna has announced its great ambitions in EBV mRNA vaccine development, with the candidate mRNA-1189 encoding all the major glycoproteins (gp350, gB, gH/gL, gp42).
Adjuvants for Vaccine Formulation

Adjuvants incorporated in components of the antigen for vaccine formulation can modulate the immune response. In addition to the original immunogenic profile of the selected antigen, a carefully selected adjuvant can broaden the use or enhance the efficacy for immunization. For vaccine platforms such as inactivated virus or protein-based subunit vaccines/VLPs, the loss of bioactivity greatly diminishes the immunogenicity of the antigen itself, which further requires powerful adjuvants for pre-stimulation of immune recognition, prolongation of antigen retention, as well as both humoral and cellular immunity enhancement (125–129).

The development of gp350-based vaccines inspired the exploration of adequate adjuvants for EBV vaccines. In the late 20th century, adjuvants such as Freud’s adjuvant, lipid A, immune-stimulating complexes (ISCOMS), and aluminum hydroxide (32, 34–36, 130) were used in the formulation of gp350 vaccines. Some may show superior immunization efficacy compared to unadjuvanted gp350 as immunogen, since an immunization trial of unadjuvanted gp350 subunit vaccine on cotton-top tamarins gave unsatisfactory results, with no protection against incidence of malignant lymphoma in spite of eliciting antibodies against gp350. With the use of more complicated adjuvant systems in recent years, a higher immunization efficacy achieved in preclinical studies supports the case for further clinical trials. However, due to the paucity of studies on other protein-based immunogens as vaccines against EBV, only a limited number of adjuvants were tested. For example, the VZV gE-based vaccine (Shingrix) was the first clinically approved herpesvirus vaccine providing protection against herpes zoster in older adults and immunosuppressed patients, while containing only VZV glycoprotein gE adjuvanted with AS01b (131). Although VZV gE was not used as a prophylactic vaccine antigen to prevent VZV infection, an appropriate combination with the adjuvant made gE an ideal antigen (132), with benefits for controlling latent VZV infection. This result was based on a systematic screening of appropriate adjuvant systems (133). This study also offers insights for EBV vaccine development, confirming that smart selection of adjuvants can also contribute to the development of a powerful vaccine against EBV by enhancing both initial protection from primary infection and secondary protection from reactivation or expansion of latent infection.

Therefore, an appropriate platform and adjuvant systems also determine the immunization efficacy of the vaccine, and not just the antigen. The COVID-19 pandemic exemplifies the effective and rapid development of vaccines against broadly distributed infectious pathogens. Both mature, extensively tested technologies like inactivated virus (100) and emerging technologies like mRNA vaccines (98, 99) gave satisfactory results, demonstrating the unlimited opportunities of the available vaccine design platforms and encouraging further comparative studies on the use of a variety of platforms for EBV vaccine development. For virus-based vaccines, breakthroughs in the mass production of live EBV could be a solution for inactivated vaccine development, since the latency-preference and complicated induction procedures seriously hinder its manufacture. For the emerging protein- or nucleic acid-based vaccines, convenient modification of antigens to strengthen their immunogenicity and viable co-valency of multiple antigens to broaden the immune response spectrum are promising future approaches for vaccine development. Since the licensed VZV vaccine took the first step in clinical herpes virus immunization, it has brought home the lesson that appropriate adjuvants used in vaccine formulation can greatly enhance the immunization efficacy. Additionally, the rising application of specific toll-like receptor (TLR) agonists (134–136) provides additional alternatives in the selection of adjuvants to achieve specific immunization responses.

EVALUATION SYSTEMS FOR VACCINES

Animal Models

Animal models are necessary and critical for the evaluation of infection or protection status against infectious disease pathogens and developing therapeutic drugs or vaccines. During the evaluation of vaccines against most pathogens, challenge experiments in animal models are considered the gold standard for the final assessment of vaccine efficacy (137–141). However, due to the restricted host tropism of EBV, a human herpesvirus, there is a limited range of susceptible candidate animal models (142–144) (Figure 3). Non-Human Primates

The great similarity between humans and non-human primates (NHPs) encouraged the use of NHPs as challenge models for EBV vaccine evaluation (145). The fact that New- and Old-World NHPs are naturally infected by EBV-related herpesviruses or lymphocryptoviruses (LCVs) further demonstrated the potential value of NHP in EBV vaccine evaluation.

In the late 20th century, the discoverer of EBV, Epstein et al. as well as Emini et al. used cotton-top tamarins and common marmosets (Callithrix jacchus) for gp350-based vaccine evaluation of both neutralizing antibody titers and challenge protection (146, 147). However, cotton-top tamarins are no longer a viable NHP model because of their critically endangered status, and the common marmoset is also listed on the IUCN Red List, which basically rules out these two NHPs from general use in EBV vaccine evaluation (148).

By contrast, rhesus macaque, as one of the Old World NHPs, has enjoyed broad use as an animal model for a variety of human viral infections, mostly due to its relatively larger population and successful artificial breeding. Although it is susceptible to its species-specific LCV (rhLCV), which shares a high level of genomic sequence similarity with EBV (149), EBV cannot stably infect and immortalize the B cells of rhesus macaques (150), which restricts the use of this animal model in challenge experiments. Therefore, the majority of EBV immunization studies used rhesus macaques as the animal model for evaluation of specific T cell responses (45, 151–153). And thus, instead of using EBV as challenge virus, rhLCV could be used as
an equivalent virus for determining the immune protection from EBV infection. Singh et al. evaluated the protection efficacy of the anti-EBV gHgL neutralizing antibody AMMO1 via rhLCV challenge in rhesus macaques, and the protected animals showed higher plasma EBV neutralizing activity.

Other Mammalian Models
As the most widely used animal model, mice play an important role in EBV vaccine evaluation. Most prophylactic EBV vaccine studies used mouse immunization to primarily assess the serum antibody titer and neutralizing antibody titer (43, 44, 46). However, because mice cannot be naturally infected with EBV, humanized mice are used as an alternative animal model for EBV challenge experiments (37, 48, 54). These chimeric animals are constructed by transferring human CD34-positive hemopoietic stem cells into immunocompromised mice (154, 155). This model is appropriate for evaluating the efficacy of therapeutic treatment for immediate EBV challenge, rather than the eliciting of an adaptive immune response by a prophylactic EBV vaccine, since the mice have an incomplete immune system even after reconstitution and lack human epithelial cells. A humanized mouse model was also used to evaluate the protective efficacy of AMMO1 (63), and the results showed that the AMMO1 antibody could inhibit EBV infection.

Some studies also used rabbits as animal models for EBV vaccine evaluation (22, 27, 28, 51, 56, 155, 156). The anti-EBV VCA titer and EBV DNA level could be detected in the blood of most rabbits after intravenous, intranasal, or peroral inoculation. However, only a portion of rabbits showed positive EBERs, LMP1, or EBNA in splenectomized samples, and even fewer rabbits displayed sustained EBV positivity, accompanied by a heterogeneous host reaction (157, 158). The uncertainty of the infection status hindered the use of rabbits as a challenge model, and most research studies only used rabbits as an immunization model for serum response evaluation (159).

Recently, it was found that the Chinese tree shrew (Tupaia belangeri subsp. chinensis) could also be a viable animal model for EBV vaccine evaluation. Following intravenous injection of virus, 8/10 tree shrews displayed symptoms of EBV infection including detectable expression of EBV-related genes and increase of anti-EBV antibodies. Despite positive results in early challenge, only a small portion of tree shrews showed EBER, LMP, and EBNA2-positive cells in spleen or mesenteric lymph node samples. The negative staining for EBV markers in the lungs and nasopharynx also indicated that epithelial cell infection might also be absent in the tree shrew animal model (160, 161).
Assessment of Immune Protection Efficacy

After confirming the design of a vaccine and immunization methods, assessment of immune protection efficacy would be critical for vaccine evaluation (162). For prophylactic vaccines against infectious pathogens, the key index revealing the efficacy of immunization protection is the neutralizing antibody (nAb) titer (163, 164), since neutralizing antibodies can efficiently block the virus from interacting with the host receptor, preventing viral attachment and membrane fusion. Therefore, a higher anti-EBV neutralizing antibody titer indicates better protection against EBV infection and could theoretically also reduce the incidence of EBV-associated malignancies. Although the presence of neutralizing antibodies is theoretically sufficient evidence for protection against viral infection, the value of this index in predicting the incidence of malignancies remained unclear (165, 166). A large cohort study conducted in Taiwan (167) indicated that EBV B cell neutralization capability of the serum or the anti-gp350 antibody titer was associated with lower risk of nasopharyngeal carcinoma. However, Zhu et al. recently performed a prospective cohort study on EBV glycoprotein-targeting neutralizing antibody titers in plasma samples from nasopharyngeal carcinoma (NPC) patients and healthy controls, which revealed that there was no significant difference in neutralizing antibody titers against EBV glycoproteins, including gp350, gH/gL, gp42, and gB (168).

During the evaluation of immune reaction against EBV, the T cell response is also considered critical part, especially for eliminating latent infection and adaptive immune responses against EBV-associated tumors (58, 60–62, 169, 170). A review concluded that T cell responses participate in the control of EBV in all phases of infection (171, 172). However, the majority of vaccine studies evaluating the T cell response were based on latent-phase proteins such as LMP and EBNA, while studies on T cell responses induced by EBV glycoproteins or T cell epitope mapping for glycoproteins were relatively rare. Thus, further studies on the T cell response elicited by EBV glycoproteins for controlling both primary infection and regulating immunological surveillance against EBV-associated malignant diseases could provide guidance for improving the evaluation systems for the assessment of prophylactic vaccine efficacy.

CONCLUSION AND PROSPECTS

In recent years, prophylactic vaccines against EBV received significant attention, since the latest achievements in fundamental virology, vaccine technology and synthetic biology have brought new opportunities for vaccine development. Early research studies on gp350 as a vaccine candidate revealed intrinsic shortage of gp350 in eliciting sufficient humoral immunity against primary infection. But still these studies become the forerunner for exploration of EBV glycoproteins as vaccine candidates. Recent progress in the discovery of epithelial cell receptors and elucidation of the infection mechanism of EBV highlights the critical function of gH/gL and gB during virus–host interaction and membrane fusion, indicating that they could be ideal major vaccine target for eliciting robust neutralizing antibody. Besides glycoproteins, although immunization with lytic and latent phase proteins is not able to provide protection against primary infection, the strong T cell immune response elicited by these proteins benefits the establishment of lasting immune surveillance of EBV latent infection and reinforcement of anti-EBV immunity after primary humoral defense. Additionally, an appropriate vaccine platform can improve the immunogenicity of certain antigens and enhance immune recognition. The adoption of protein modification via multimerization or fusion with immune cell-targeting domains may provide more possibilities for protein-based vaccines, while the application of synthetic nucleic acids as delivery systems could be the next milestone in the evolution of general vaccine design for not only EBV but all pathogens. Beyond vaccine design, a finer system for the evaluation of vaccine efficacy is also crucial for the development of a successful vaccine. A suitable animal model for EBV challenge is required. Further studies on the EBV-susceptibility of non-NHP models or viable NHP models would be as important as the innovation in EBV vaccine design. And it remains unclear whether T cell responses should be listed in the assessment system for determining the protection efficacy of EBV prophylactic vaccines, urging more intensive research on the connection between elicited cellular immunity and protection from both primary infection and malignancies.

Prospectively, with the advancement in understanding of immunity against EBV infection, more vaccine targets would be discovered, and using combinatorial antigens as vaccine candidate may display even promising immunization efficacy. The emerging vaccine platforms such as nanoparticle or mRNA nucleic acids as delivery systems could be the next milestone. Besides, immune cell-targeting domains may provide more possibilities for vaccine development. Additionally, an appropriate vaccine platform can improve the immunogenicity of certain antigens and enhance immune recognition. The adoption of protein modification via multimerization or fusion with immune cell-targeting domains may provide more possibilities for protein-based vaccines, while the application of synthetic nucleic acids as delivery systems could be the next milestone in the evolution of general vaccine design for not only EBV but all pathogens. Beyond vaccine design, a finer system for the evaluation of vaccine efficacy is also crucial for the development of a successful vaccine. A suitable animal model for EBV challenge is required. Further studies on the EBV-susceptibility of non-NHP models or viable NHP models would be as important as the innovation in EBV vaccine design. And it remains unclear whether T cell responses should be listed in the assessment system for determining the protection efficacy of EBV prophylactic vaccines, urging more intensive research on the connection between elicited cellular immunity and protection from both primary infection and malignancies.

AUTHOR CONTRIBUTIONS

CS wrote the original manuscript and generated the figures. XC generated the summary table of animal trials and clinical trials. YK and MZ provided guidance and reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

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