Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Rabbits immunized with Epstein-Barr virus gH/gL or gB recombinant proteins elicit higher serum virus neutralizing activity than gp350

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ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 26 April 2016
Accepted 3 June 2016
Available online xxxx

Keywords:
Epstein-Barr virus
Vaccine
Antibody
B cell
Recombinant protein
Rabbit
EBV gp350
EBV gH/gL
EBV gB

ABSTRACT

Epstein-Barr virus (EBV) is the primary cause of infectious mononucleosis and has been strongly implicated in the etiology of multiple epithelial and lymphoid cancers, such as nasopharyngeal carcinoma, gastric carcinoma, Hodgkin lymphoma, Burkitt lymphoma, non-Hodgkin lymphoma and post-transplant lymphoproliferative disorder. There is currently no licensed prophylactic vaccine for EBV. Most efforts to develop prophylactic vaccines have focused on EBV gp350, which binds to CD21/CD35 to gain entry into B cells, and is a major target of serum neutralizing antibody in EBV seropositive humans. However, a recombinant monomeric gp350 protein failed to prevent EBV infection in a phase II clinical trial. Thus, alternative or additional target antigens may be necessary for a successful prophylactic vaccine. EBV gH/gL and gB proteins coordinately mediate EBV fusion and entry into B cells and epithelial cells, strongly suggesting that vaccination with these proteins might elicit antibodies that will prevent EBV infection. We produced recombinant trimeric and monomeric EBV gH/gL heterodimeric proteins and a trimeric EBV gB protein, in addition to tetrameric and monomeric $gp350^{1-470}$ proteins, in Chinese hamster ovary cells. We demonstrated that vaccination of rabbits with trimeric and monomeric gH/gL, trimeric gB, and tetrameric $gp350^{1-470}$ induced serum EBV-neutralizing titers, using cultured human B cells, that were >100-fold, 20-fold, 18-fold, and 4-fold higher, respectively, than monomeric $gp350^{1-470}$. These data strongly suggest a role for testing EBV gH/gL and EBV gB in a future prophylactic vaccine to prevent EBV infection of B cells, as well as epithelial cells.

1. Introduction

Epstein-Bar virus (EBV) is a gammaherpesvirus that primarily infects B cells and epithelial cells. EBV has been strongly implicated as a co-factor in the development of Burkitt and other non-Hodgkin lymphomas, nasopharyngeal carcinoma, gastric adenocarcinoma, Hodgkin lymphoma, and NK/T cell lymphoma, overall accounting for over 200,000 cases of cancer world-wide each year [1–6]. EBV is the direct etiologic agent of most cases of infectious mononucleosis (IM) [7,8]. Patients developing IM exhibit a significantly increased risk for the subsequent development of Hodgkin disease [9,10] or multiple sclerosis [11,12]. EBV seropositive patients undergoing solid organ or stem cell transplantation are at risk of developing uncontrolled B cell proliferation due to EBV reactivation, termed post-transplantation lymphoproliferative disorder (PTLD) that can evolve into non-Hodgkin lymphoma [13]. A similar phenomenon also occurs in patients with AIDS.

Infection of B cells with EBV is initiated by binding of the EBV envelope protein gp350 to either the complement receptor (CR) 1/CD35 or CR2/CD21 [14,15]. Upon binding to B cell CR, EBV gp42 interacts with cell surface MHC-II, leading to its association with the heterodimeric protein gH/gL. GH/gL then undergoes a conformational change upon binding gp42, leading to activation of the EBV fusion protein gB, that directly mediates viral-host cell membrane fusion. Levels of expression of gB vary among different EBV strains and this positively correlates with infectivity [16].

Memory B cells are the major source of latent EBV [17], whereas EBV replication and transmission occurs predominantly within the nasopharyngeal epithelium [18,19]. The mechanism of EBV infection of epithelial cells remains an active area of investigation. EBV may infect epithelial cells directly by binding of EBV gH to integrins ($\alpha v\beta 5$, $\beta 6$, and $\beta 8$) expressed on the epithelial cell surface [20–22]. Alternatively, direct transfer of EBV from B cells

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http://dx.doi.org/10.1016/j.vaccine.2016.06.021
0264-410X/

to epithelial cells has been demonstrated [23,24]. EBV is also able to directly infect organotypic cultures of epithelial cells to establish a predominantly productive infection in the suprabasal layers of stratified epithelium [25]. Epithelial expression of CR is controversial [26,27] and a role for gp350 in EBV infection of epithelial cells has not been demonstrated. However, EBV gH/gL and gB are critical for infection of epithelial cells, as well as for B cells.

There is currently no prophylactic EBV vaccine in clinical use. Studies in non-human primates using gp350-based vaccination strategies have shown protection against EBV-induced lymphoma and EBV replication [28]. A phase II clinical trial conducted in EBV-seronegative adults using a recombinant monomeric gp350 protein versus placebo suggested a partial protective effect of gp350 vaccination on IM development [29,30]. However, the vaccine did not prevent asymptomatic EBV infection. A phase I trial of recombinant monomeric gp350 protein given to children with chronic kidney disease demonstrated only a minority of subjects developing detectable neutralizing serum anti-gp350 titers [31]. Collectively, these data suggest that a vaccine incorporating gH/gL and gB, in addition to gp350, may confer superior antibody-mediated protection against EBV infection through additive or synergistic blocking effects on B cells, as well as epithelial cells.

Monomeric proteins are relatively weak immunogens relative to proteins that are expressed in a multimeric manner or that are aggregated [32–37]. We previously constructed and expressed tetrameric and monomeric gp350 proteins in Chinese hamster ovary (CHO) cells [38], and demonstrated that immunization of mice with tetrameric gp350 elicited higher gp350-specific IgG and neutralizing serum antibody responses, relative to its monomeric counterpart [38]. In the current study, we produced recombinant trimeric and monomeric EBV gH/gL and trimeric EBV gB proteins in CHO cells. We immunized rabbits with these proteins in addition to tetrameric and monomeric gp350 to determine the relative EBV-neutralizing activity of the induced serum antibody in co-cultures of cell-free GFP-labeled EBV and B cells.

2. Methods

2.1. Design of DNA constructs and expression of monomeric and trimeric EBV gH/gL

The coding sequences for EBV gH and gL were downloaded from NCBI, reference sequence # NC_009334.1. The gL sequence coding 23-137 amino acids was used, and the signal peptide 1-22 was replaced with an IgGκ leader sequence. The gH sequence coding 19-678 amino acids was linked to the 3′ end of gL, separated by a 15 amino acid linker $(Gly_4Ser_1)_3$ sequence. The foldon trimerization domain coding sequence derived from T4 phage fibritin was linked to the 3′ end of gH, followed by a His_6 coding sequence. DNA coding for the trimeric gH/gL was synthesized by Blue Heron Biotechnology, Inc., cloned into pOptiVEV (Invitrogen), and verified by sequencing. The monomeric EBV gH/gL construct was made by PCR amplification of EBV gH/gL with the foldon coding sequence deleted, cloned into pOptiVEV, and verified by sequencing. Chinese hamster ovary (CHO) cells (strain DG44, Invitrogen) were transfected with pOptiVEC-gH/gL constructs and selected with gradually increased concentration of methotrexate (MTX) up to 4 μM. CHO cells were then loaded into "Fibercell" cartridges (FiberCell Systems, Frederick, MD) for protein production. Supernatants were concentrated and purified using cobalt affinity purification (Thermo Fisher Scientific).

2.2. Design of DNA construct and expression of EBV trimeric gB protein

The coding sequence for EBV gB was downloaded from NCBI, reference sequence # NC_009334.1. The sequence coding 23-732 amino acids was used. The signal peptide (1-22 amino acids) was replaced with an IgG κ leader sequence, and the coding sequence of the furin cleavage site (RRRRD) between amino acids 427 (L) and 434 (A) was replaced with a 15 amino acid $(Gly_4Ser)_3$ linker sequence. A His_6 sequence was linked to the 3′ end for protein purification. All the following steps were as described for EBV gH/gL.

2.3. Western blot analysis

Western blot analysis under reducing and non-reducing conditions was performed as previously described [38]. For Western blot analysis under modified non-reducing conditions, protein samples were mixed with LDS loading buffer without DTT, and resolved on 3–8% Polyacrylamide gels in Tris–Glycine native running buffer (Invitrogen). After PAGE, the gels were blotted on 0.45 μm nitrocellulose membrane, incubated with either HRP-labeled mouse IgG anti-His mAb (Thermo Fisher Scientific, Waltham, MA), or mouse IgG anti-EBV gH/gL mAb (clone E1D1, kind gift from Dr. L.M. Hutt-Fletcher, Louisiana State University Health Sciences Center, Shreveport, LA) or mouse IgG anti-EBV gB mAb (Virusys Corporation, Taneytown, MD) followed by polyclonal HRP-goat anti-mouse IgG (Thermo Fisher Scientific). Membranes were then incubated with SuperSignal West Pico chemiluminescent substrate, and signal captured on X-ray film.

2.4. Size exclusion chromatography and multi-angle light scattering (SEC-MALS) analysis

For SEC-MALS analysis (performed by John Van Druff, FinaBioSolutions LLC, Rockville, Maryland), an Agilent 1100 series, Wyatt HELEOS 8, Wyatt Optilab T-rEX, a Waters 2497 series UV detector and a TSKGel G4000 PWxl column in series with a TSKGel G5000 PWxl column were used. Briefly, purified EBV monomeric gHgL, trimeric gHgL, and trimeric gB samples were spun through 0.45 micron centrifugal filters for 5 min in a tabletop centrifuge. 50 μL of each sample was injected. The column flow rate was 0.5 mL/min. Wyatt ASTRA software was used for data collection & analysis, and the Debye formalism was used to determine molecular weight.

2.5. Rabbit immunizations

Groups of 5 male New Zealand white rabbits, 12–15 weeks old were immunized subcutaneously with 25 μg of antigen adsorbed to aluminum hydroxide (alum; 0.25 μg alum/μg protein) and mixed with 50 μg of a 12-mer phosphorothioate-modified CpG-ODN (tcataacgttcc) optimized for rabbits (68). Rabbits were immunized on day 0, day 21, and day 42 and serum samples were taken before initial immunization, and 10 days following each immunization.

2.6. Determination of serum antigen-specific IgG titers

Serum titers of EBV gp350-, gH/gL-, and gB-specific IgG were determined by ELISA (69). Briefly, 96-well ELISA plates were coated with antigen in PBS (50 μL [containing 5 μg/ml] per well), blocked with bovine serum albumin, then serial dilutions of rabbit sera were added. Alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG antibodies (Southern Biotechnology Associates, Inc.) were then added followed by incubation with phosphatase substrate (Sigma). OD was measured at 405 nm.

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2.7. Preparation of peripheral blood naïve human B cells

Peripheral blood mononuclear cells (PBMC) were isolated from human healthy donor's buffy coats using Ficoll-paque plus (GE Healthcare, Sweden) density gradient centrifugation. Ack Lysis buffer (5 min at RT) was used to lyse erythrocytes in PBMC. Naïve B cells were subsequently isolated from PBMC by negative selection using a magnetic bead sorting kit (EasySep™ Human Naïve B cell enrichment kit). The purity of the naïve B cell preparation was between 90% and 95%.

2.8. Determination of serum EBV neutralization titers

Determination of serum in vitro EBV-neutralizing titers, using Raji cells (EBV-positive human Burkitt lymphoma cell line), were performed as described (66). Serial serum dilutions were mixed for 2 h with GFP-EBV (B95-8/F) in 96-well plates, followed by addition of Raji cells for 1 additional hour. Cells were then washed and recultured in medium alone for 3 days, fixed in paraformaldehyde and analyzed by flow cytometry for GFP+ Raji cells. The serum dilution that inhibited infectivity by 50% (EDI_{50}), based on reduction of the number of GFP+ cells, was calculated by non-linear regression analysis using Graph Pad Prizm 6. The EBV-neutralizing anti-gp350 mAb (72A1) was used as a positive control. Pre-immune sera and sera from rabbits immunized with alum + CpG-ODN alone served as negative controls. For determination of serum neutralizing titers using peripheral blood naïve human B cells, naïve human B cells were incubated with GFP-EBV and cultured in RPMI 1640 medium containing 100 ng/ml IL-4 (BioLegend, San Diego, CA) and 1 μg/ml CD40 antibody (R&D Systems, Minneapolis, MN).

3. Results

3.1. Production of EBV monomeric and trimeric gH/gL proteins

We previously designed DNA constructs to produce recombinant monomeric and tetrameric EBV gp350 proteins in CHO cells [38] (Fig. 1A). We now took a similar approach to design DNA constructs that encode for either a monomeric or trimeric EBV gH/gL heterodimeric protein (Fig. 1B). A 5′ Igκ leader sequence was introduced to promote protein secretion, followed by sequences encoding EBV gL and gH with a $(Gly_4Ser_1)_3$ linker in between to allow for proper folding of both proteins. To effect self-association of gH/gL into a stable trimeric form, a T4 bacteriophage fibritin trimerization domain (foldon) [39] was inserted 3′ to gH of the construct encoding trimeric gH/gL, followed by a His_6 tag to allow for efficient purification. A DNA construct encoding a monomeric gH/gL was also made, by excluding the foldon domain.

Western blot analysis of trimeric gH/gL protein using an anti-EBV gH/gL mAb under reducing conditions which disrupts native oligomers demonstrated a MW band of ~80 KDa, consistent with monomeric gH/gL (Fig. 2A). Under non-reducing conditions, a MW band of ~240 KDa, consistent with trimeric gH/gL was observed. To obtain a more accurate measurement of absolute molecular weight, we utilized SEC-MALS which does not rely on relative MW standards and yields absolute MW estimates from the angular dependence of scattered light intensity as a function of concentration [40]. SEC-MALS analysis of monomeric EBV gH/gL protein yielded a single band of MW 102 kDa consistent with the predicted MW of monomeric EBV gH/gL (Fig. 3A). Analysis of trimeric EBV gH/gL yielded two predominant bands of MW 321 and 1080 kDa consistent with the predicted MW of a trimeric EBV gH/gL (321 kDa), as well as indicating that a proportion of the total protein self-aggregated into a trimer of trimeric EBV gH/gL (Fig. 3B).

3.2. Production of EBV trimeric gB protein

The pre-fusion crystal structure of an EBV gB variant expressed in insect cells is a trimer [41]. The furin cleavage site within EBV gB

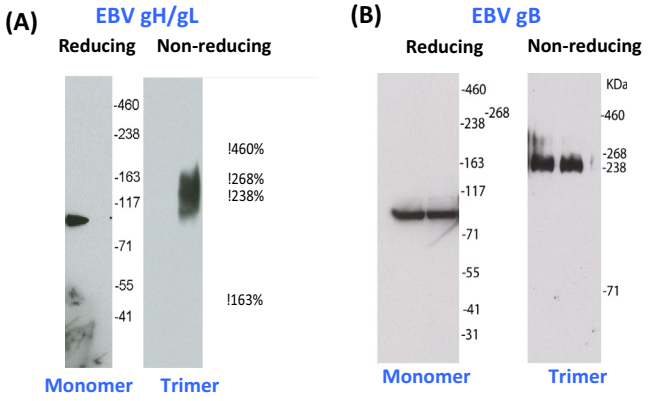

Fig. 2. Western blot analysis of monomeric and trimeric EBV gH/gL and trimeric EBV gB proteins under reducing and non-reducing conditions. Blots were developed with anti-EBV gH/gL and anti-EBV gB mAbs, respectively.

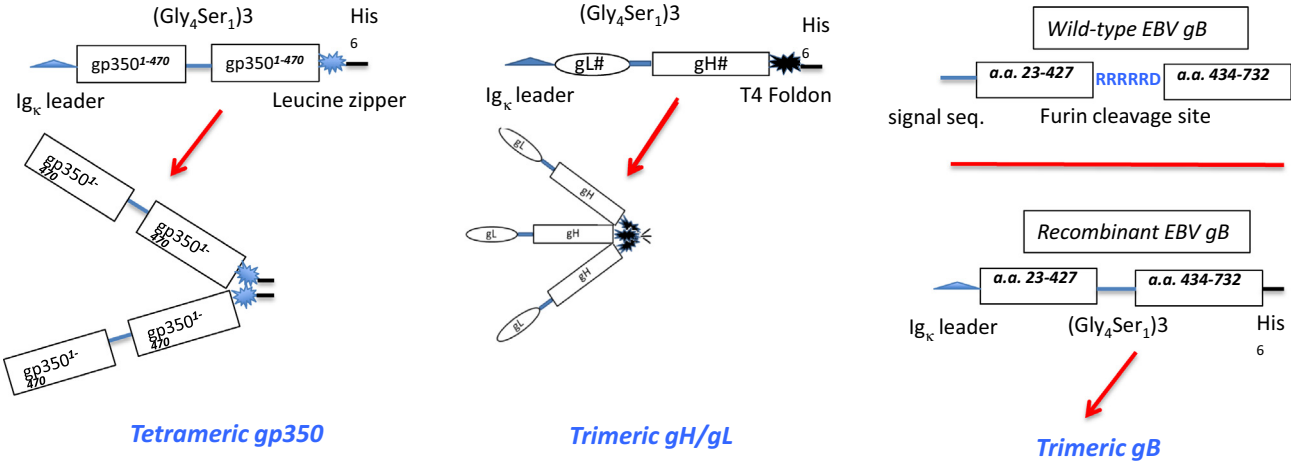

Fig. 1. DNA constructs encoding tetrameric EBV gp350, trimeric EBV gH/gL, and trimeric EBV gB. Constructs were stably transfected into CHO cells for protein expression.

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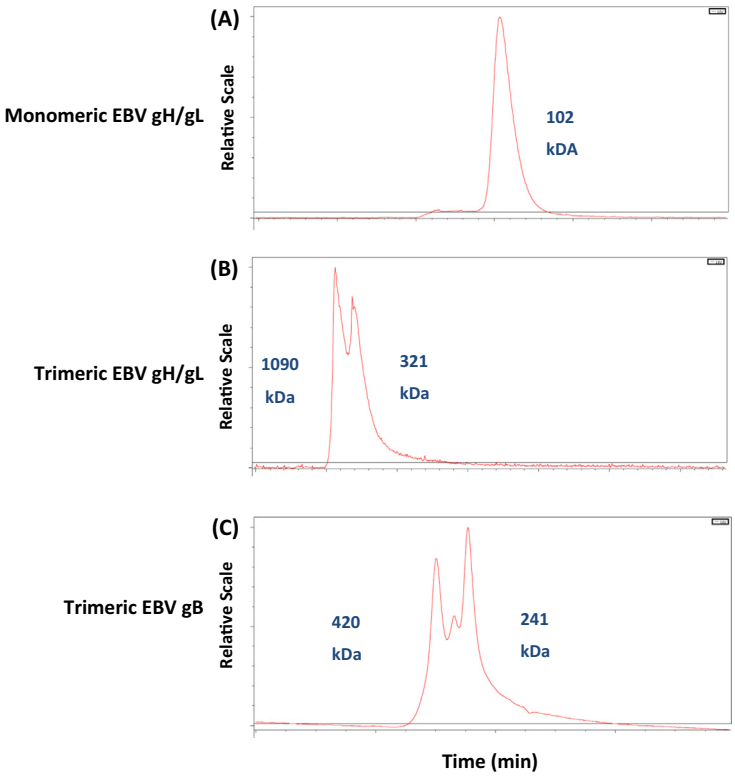

Fig. 3. SEC-MALS analysis of monomeric and trimeric EBV gH/gL and trimeric EBV gB proteins.

may be critical for the natural folding of EBV gB protein into its terminal trimeric form (63), but inclusion of this site leads to low yields of recombinant non-trimeric human cytomegalovirus (HCMV) gB [19]. We reasoned that a recombinant trimeric EBV gB could be produced by insertion of a flexible $(Gly_4Ser)_3$ linker in place of the furin cleavage site to allow for terminal protein folding and efficient expression (Fig. 1C).

Western blot analysis under fully reducing conditions using an anti-gB mAb demonstrated the EBV gB protein to be the predicted size of the monomeric form (~80 KDa) (Fig. 2B). Under modified native conditions, that allows for detection of the native form of EBV gB protein, we observed a uniform band with the predicted size of a trimeric EBV gB (~240 KDa). SEC-MALS analysis of trimeric gB yielded two predominant bands of MW 241 kDa and 420 kDa, consistent with the predicted MW of trimeric EBV gB, as well as indicating that a proportion of the total protein self-aggregated to form a dimer of trimeric gB (Fig. 3C).

3.3. All EBV proteins induced high total serum titers of antigen-specific IgG

We directly compared monomeric and trimeric EBV gH/gL, trimeric EBV gB, and monomeric and tetrameric EBV gp350 for elicitation of total serum titers of antigen-specific IgG. Groups of 5 adult rabbits each were immunized s.c. with 25 μg of protein in alum + CpG-ODN, then boosted in a similar fashion on days 21 and 42 post-immunization. The CpG-ODN sequence was optimized for use in rabbits (68). As illustrated in Fig. 4, each of the 5 EBV proteins induced augmented serum IgG responses following the first booster immunization, and with monomeric $gp350^{1-470}$ and monomeric gH/gL, further significant augmentation in serum IgG titers following the second booster immunization. Tetrameric EBV $gp350^{1-470}$ induced >20-fold serum gp350-specific IgG titers relative to monomeric EBV $gp350^{1-470}$ following the first and second booster immunizations (Fig. 4). Trimeric EBV gH/gL induced

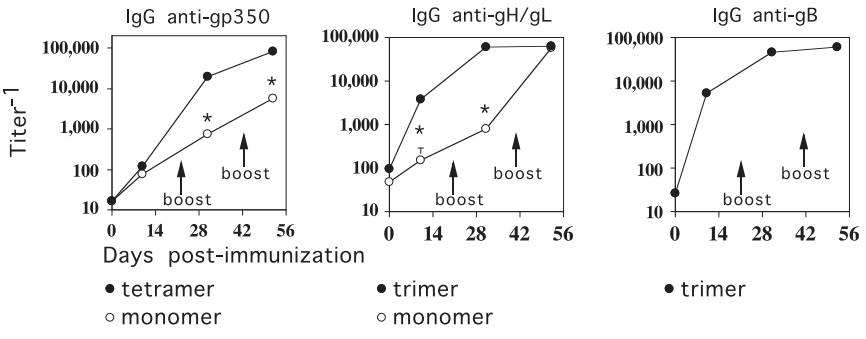

Fig. 4. Serum titers of antigen-specific IgG following immunization with monomeric and multimeric EBV proteins. Groups of rabbits, 12–15 weeks old (n = 5) were immunized s.c. with 25 mg of either monomeric or tetrameric EBV gp350, monomeric or trimeric EBV gH/gL, or trimeric EBV gB in alum + CpG-ODN adjuvant, and boosted on day 21 and day 42. Sera were obtained 10 days following each immunization for measurement of serum titers of antigen-specific IgG by ELISA. Significance *p.

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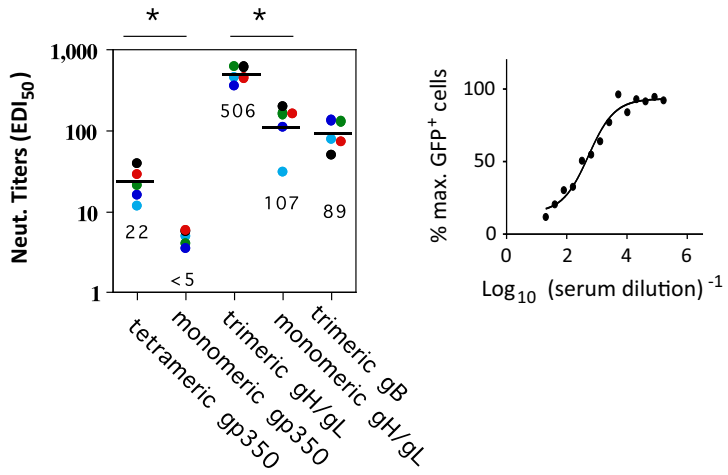

Fig. 5. Measurement of serum neutralization titers in response to monomeric and multimeric EBV proteins using Raji Burkitt lymphoma cells. Sera obtained 52 days post-immunization (10 days following the second boost) as described in Fig. 4 were used to determine EDI_{50} EBV neutralization titers of individual serum samples by flow cytometric analysis of GPF + Raji cells in the presence of GFP-EBV and varying concentrations of sera. Significance *p.

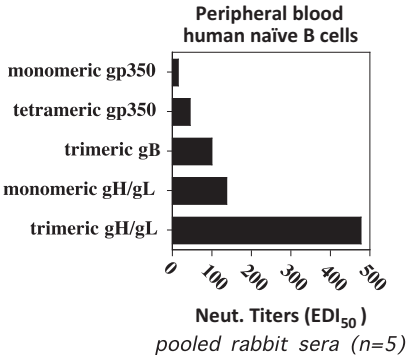

Fig. 6. Measurement of serum neutralization titers in response to monomeric and multimeric EBV proteins using peripheral blood human naïve B cells. Groups of sera obtained as described in Fig. 4 were pooled and used to determine EDI_{50} EBV neutralization titers as described in Fig. 5 using peripheral blood human naïve B cells.

>30-fold and >90-fold enhancements in serum gH/gL-specific IgG titers following the primary immunization and the first booster immunization, respectively with the titers equalizing by the second booster immunization (Fig. 4). These data confirm our previous study in mice using tetrameric and monomeric $gp350^{1-470}$ [38], that multimerization of proteins induce marked increases in immunogenicity.

3.4. EBV monomeric gHgL, trimeric gHgL and trimeric gB induced significantly higher serum neutralization titers compared to monomeric and tetrameric $gp350^{1-470}$

Flow cytometric analysis of GFP-labeled EBV entry into Raji Burkitt lymphoma B cells has been demonstrated to be an accurate and efficient way to measure serum EBV neutralizing titers [42]. We utilized this method to determine the effective dilution of serum antibody, from rabbits immunized 3× with monomeric versus tetrameric EBV $gp350^{1-470}$, monomeric versus trimeric EBV gH/gL, and trimeric EBV gB, that inhibits infectivity of 50% of Raji B cells (EDI_{50}). As illustrated in Fig. 5, tetrameric EBV $gp350^{1-470}$ induced significantly higher EDI_{50} titers than monomeric EBV $gp350^{1-470}$ (EDI50 22 versus <5, respectively). Of note, trimeric gH/gL induced significantly higher EDI_{50} titers than monomeric gH/gL (EDI_{50} 506 versus 107, respectively), that was markedly and significantly higher than that induced by tetrameric $gp350^{1-470}$. Similarly, EBV gB induced significantly higher EDI_{50} titers (EDI_{50} 89) than tetrameric $gp350^{1-470}$, that was comparable to that elicited by monomeric gH/gL. Compared to monomeric $gp350^{1-470}$, trimeric gHgL, monomeric gHgL, trimeric gB, and tetrameric gp350 elicited >100-, 20-, 18-, and 4-fold higher EDI_{50} titers respectively. Similar EBV neutralization titers were obtained from sera that were pooled from each of the groups in Fig. 5, utilizing GFP-EBV and naïve peripheral blood human B cells from healthy donors (Fig. 6), except that monomeric and tetrameric $gp350^{1-470}$ showed slightly higher EDI_{50} titers compared to those calculated using Raji cells (Fig. 5).

4. Discussion

We make the novel observation that EBV gH/gL and EBV gB proteins elicit antibodies in rabbits that block EBV entry into Raji Burkitt lymphoma and naïve peripheral human B cells, and appear to be significantly more potent on a per weight basis than EBV gp350. Specifically, trimeric and monomeric EBV gH/gL and trimeric EBV gB induced serum neutralization titers >100-fold, 20-fold, and 18-fold higher, respectively than monomeric $gp350^{1-470}$. Further, tetrameric, relative to monomeric $gp350^{1-470}$ induced >4-fold higher serum neutralization titers than its monomeric counterpart. The truncated $gp350^{1-470}$ contains all of the reported neutralizing epitopes of the full-length molecule [43–45].

To our knowledge, we are the first to report production of a fully trimeric recombinant EBV gB protein. EBV, HCMV, and HSV-1 gB proteins have all been reported to be naturally expressed as a trimer, and thus this form may represent the ideal gB vaccine protein [41]. A recombinant non-trimeric HCMV gB protein, that was modified to remove the furin cleavage site, has been tested in a phase II clinical trial for prevention of HCMV infection in HCMV-seronegative women. Women were immunized i.m. with HCMV gB + MF59 adjuvant (squaline in water emulsion) at 0, 1, and 6 months (n = 234) or placebo [sodium chloride solution] (n = 230) [46]. Following a 42-month observation period a total of 18 infections were documented in the vaccine group and 31 in the placebo group (P = 0.02) for a vaccine efficacy of 50%. In another phase II study, patients awaiting solid organ transplantation who were HCMV seronegative, and thus at high risk for HCMV infection ($donor^{+}recipient^{-}$) were vaccinated with gB/MF59 [47]. The vaccine was effective in preventing viremia in 5 out of 11 subjects, compared to 0 out of 5 subjects in the placebo group.

Our data thus provide a strong rationale for further testing of EBV gB, as well as EBV gH/gL proteins in humans for inclusion in

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a prophylactic EBV vaccine. Such a vaccine could potentially reduce the incidence of not only infectious mononucleosis [7,8], but the development of EBV-related B cell and epithelial malignancies [1–6], and possibly a number of autoimmune diseases, such as multiple sclerosis [11,12]. In contrast to EBV gp350, EBV gH/gL and EBV gB also have the potential to induce antibodies that will block EBV entry into epithelial cells [20–22], although it remains to be determined whether such antibodies would block B cell to epithelial cell transfer [23,24]. Since epithelial cells are the major cellular site of EBV replication they may play an important role in both establishing and amplifying initial infection in the oropharynx as well as providing a means for transmission [18]. Whether EBV gp350, gH/gL, and gB proteins can act additively or synergistically to induce antibodies that block EBV infection remains to be determined, but has important relevance for future EBV vaccine development.

Although the majority of serum neutralizing activity following natural EBV infection has been reported to be specific for EBV gp350 [48], the extent to which natural EBV infection induces gH/gL- and gB-specific neutralizing antibodies remains to be determined. This has clinical relevance since these latter two EBV proteins could also be considered for active boosting of immunity in EBV seropositive individuals for therapeutic purposes, such as prior to undergoing transplantation to prevent PTLD [13], Further, in light of the potential role for active EBV replication in the progression of established undifferentiated nasopharyngeal carcinoma [1], an epithelial malignancy, it remains an intriguing possibility that inducing neutralizing gH/gL- and/or gB-specific antibodies in such patients might slow tumor growth.

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Please cite this article in press as: Cui X et al. Rabbits immunized with Epstein-Barr virus gH/gL or gB recombinant proteins elicit higher serum virus neutralizing activity than gp350. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.06.021