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A Prophylactic multivalent vaccine to prevent acute infectious mononucleosis and EBV+ lymphomas

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A Prophylactic Multivalent Vaccine to Prevent Acute Infectious Mononucleosis and EBV+ Lymphomas

Joslyn Foley



A Dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand, Johannesburg
In fulfilment of the requirements for the degree of
Master of Science in Medicine

Johannesburg, June 2021

Life or death for a young child too often depends on whether he or she is born in a country where vaccines are available or not.

Nelson Mandela



DECLARATION

I, Joslyn Foley, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Joslyn Foley

23 day of June 2021

ABSTRACT

Infection with Epstein-Barr virus (EBV) has been associated with a number of diseases and is responsible for causing approximately 200,000 new cases of cancer each year globally. Despite imposing a significant health burden and more than 50 years of research since it's discovery, no effective vaccine has been developed to prevent or treat EBV infection. The majority of proposed prophylactic EBV vaccines have relied on glycoprotein 350 (gp350) as the sole immunogen, but recently studies have demonstrated the importance of other proteins expressed on the surface of EBV, glycoproteins gB, gH, gL and gp42, that play a key role in entry into host cells. This study explored the combination of all five EBV surface glycoproteins implicated in viral entry as possible prophylactic immunogens. The Newcastle disease virus (NDV) virus-like particle (VLP) platform was used to express all of the glycoproteins simultaneously on the surface of a single biologically inert particle devoid of potentially oncogenic viral DNA and incapable of replication. The VLP was produced in a stable Chinese hamster ovary (CHO) cell line expressing gp350, gB, gp42, gL, and gH sequentially from a single DNA transcript and the cells were characterized by flow cytometry to confirm expression of each glycoprotein. These cells were transfected with NDV structural proteins matrix (M) and neucleoprotein (NP) to initiate VLP production and release into the supernatant. The VLP was purified from the supernatant by sucrose gradient centrifugation and characterized by immunoblot and ELISA. Rabbits were immunized with the VLP, TNE buffer, soluble gp350, UV-inactivated EBV, or a similar VLP expressing KSHV surface glycoproteins and antibodies specific to each of the five EBV glycoproteins was measured by ELISA. Animals immunized with the EBV-LP and UV-inactivated EBV (UV-EBV) had measurable antibody titers to all of the EBV glycoproteins, while animals immunized with gp350 only had gp350-specific antibodies. Neutralization activity was measured by incubating pooled serum from immunized rabbits with EBV-eGFP and assessing the infectivity of the serum:virus mixture in HEK-293T cells. Infected cells expressed eGFP and the percent of infected cells was measured by flow cytometry. Both the serum from EBV-LP and UV-inactivated EBV rabbits were able to achieve greater than 50% neutralization in HEK-293T cells. In conclusion, the EBV-LP immunogen designed, purified and characterized in this study was able to elicit binding and neutralizing

antibodies against EBV, albeit at low levels. Thus, future work should focus on optimizing the expression levels of each protein, as well as mapping the neutralizing antibody activity obtained to direct further optimization of the EBV-LP.

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LIST OF ABBREVIATIONS

72A1	EBV gp350-specific antibody
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AIM	acute infectious mononucleosis
ATCC	American Type Culture Collection
ATLL	adult T cell leukemia/lymphoma
BCA	bicinchoninic acid assay
BL	Burkitt lymphoma
BSA	bovine serum albumin
CAEBV	chronic active EBV
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CT	cytoplasmic
DC	dendritic cell
DLBCL	diffuse large B cell lymphoma
DLBCL-NOS	diffuse large B cell lymphoma not otherwise specified
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
eBL	endemic Burkitt lymphoma
EBNA	Epstein–Barr nuclear antigen 1
EBV	Epstein–Barr virus
ED	Ectodomain
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FDA	U.S. food and drug administration
GC	gastric carcinoma
GVHD	graft-versus-host disease
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HHV	human herpes virus
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma
HPV	Human Papillomavirus
HRP	horseradish peroxidase
HTLV-1	Human T-cell Lymphotropic virus
HVEM	herpesvirus entry mediator
IFN	Interferon
KS	Kaposi sarcoma

KSHV	Kaposi sarcoma-associated herpesvirus
LANA1	latency-associated nuclear antigen
MALT	mucosa-associated lymphoid tissue
MCC	Merkel cell carcinoma
MCV	Merkel cell virus
MHC	major histocompatibility complex
MPLA	monophosphoryl lipid A
nAb	neutralizing antibody
NDV	Newcastle disease virus
NPC	nasopharyngeal carcinoma
OD	optical density
PEI	Polyethylenimine
PTLD	post-transplant lymphoproliferative disorder
rcf	relative centrifugal force
SD	standard deviation
TM	Transmembrane
TPA	tetradecanoyl phorbol acetate
UV	Ultraviolet
VLP	virus-like particle

Chapter 1 INTRODUCTION

1.1. Oncogenic pathogens

1.1.1. Global Burden of Oncogenic Pathogens

In 2018 there were 17.0 million new cancer cases worldwide and 9.5 million cancer deaths (1). Globally, cancer is responsible for 1 in every 6 deaths, second only to cardiovascular diseases (1). In 2012 it was shown that approximately 16% of new cancer cases each year were associated with infectious agents (2). This number is significantly higher in low- and middle-income countries where it is estimated that 20-30% of cancers are attributed to infectious agents, whereas this number is thought to be <5-10% in high-income countries (3). Additionally, people in lower-income countries are more likely to die of these infection-attributable malignancies. The attributable fraction is the number of cases of each type of cancer believed to be caused by the infectious agent. This number varies widely, from 100% of cervical carcinoma cases attributed to human papilloma virus (HPV) infection, to only 1.6% of all cases of urinary bladder cancer resulting from *Schistosoma haematobium* infection (1).

There are 10 infectious agents considered to be carcinogenic in humans (1) (Table 1.1). These include one bacterium, two groups of parasites, and seven viruses, although many people also consider human immunodeficiency virus (HIV) to be an oncogenic virus because immune-suppressed HIV infected individuals have a much higher risk of developing other pathogen-driven cancers (4).

Table 1.1: Cancers attributed to infectious agents globally. A summary of known oncogenic pathogens, their associated diseases and the number of new cases believed to be caused by each pathogen in 2018 (1).

PATHOGEN	ASSOCIATED MALIGNANCIES	# OF CASES ATTRIBUTABLE TO INFECTION (2018)	% OF TOTAL CASES ATTRIBUTABLE TO INFECTION (2018)
VIRUSES			
Human Papillomavirus (HPV)	Cervical carcinoma Squamous cell head and neck cancer Squamous cell anal cancer Vulvar cancer	694,000	3.83%
Hepatitis B virus (HBV)	Hepatocellular carcinoma	360,000	1.99%
Hepatitis C virus (HCV)	Hepatocellular carcinoma Rare lymphomas?	156,000	0.86%
Epstein-Barr virus (EBV)	Burkitt lymphoma Diffuse large B-cell lymphoma Hodgkin lymphoma Nasopharyngeal carcinoma Gastric adenocarcinoma Leiomyosarcoma Post-transplant lymphoproliferative disorder	156,600	0.87%
Kaposi sarcoma herpesvirus (KSHV)	Kaposi sarcoma Primary effusion lymphoma Multicentric Castleman disorder	42,000	0.23%
Human T-lymphotropic virus-1 (HTLV-1)	Adult T-cell leukemia/lymphoma	3,600	0.02%
Merkel cell polyomavirus (MCV)	Merkel cell carcinoma	Approx. 2,000/year in the US, but the number is rising sharply	0.01%
MACRO-PARASITES			
Schistosoma haematobium	Bladder carcinoma	6,000	0.03%
Opisthorchis viverrini and *Clonorchis sinensis*	Bile duct cancer	3,500	0.02%
BACTERIA			
Helicobacter pylori	Non-cardia gastric carcinoma Gastric cardia carcinoma Gastric non-Hodgkin lymphoma	812,000	4.49%

1.1.2. Epidemiology and Mechanisms of Oncogenesis

These infectious agents all use different methods to cause cancer to develop. In general, these are divided into three broad categories: genetic modifications, microenvironment adjustments, and phenotypic alterations (5). These either directly or indirectly result in oncogenesis. Commonly, the direct mechanisms of oncogenesis include alteration of host genes regulating proliferation and cell-cycle, such as p53, a well-known tumor suppressor gene (6). Indirectly, many infections cause chronic inflammation and activation of the host immune system, resulting in an environment favorable for mitogenesis and mutagenesis (7).

Human papillomavirus is transmitted through skin-to-skin or skin-to-mucosa contact. The basal and mucosal epithelial cell tropism is reflected in the types of malignancies that are known to result from infection, including carcinomas of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx and tonsil (2). Oncogenic proteins E6 and E7 promote proliferation of infected cells, prevent apoptosis, and evade immune system detection. The viral genome is preserved, but in the host genome crucial cell-cycle checkpoint inactivation results in genomic instability and the accumulation of mutations in the proliferating cells of the lower epithelium (8).

Generally, Hepatitis B virus (HBV) is transmitted through permucosal and percutaneous exposure to blood or other body fluids. There is an increased risk of HBV infection in children with infected family members, particularly infected mothers, as well as with intravenous drug users and through unprotected sexual contact. There has been a decline in chronic infections with the implementation of a vaccination program, particularly in children in South-Eastern Asia (2). HBV is both directly and indirectly oncogenic. Infected individuals have a 25-37 times higher risk of developing hepatocellular carcinoma (HCC) than uninfected individuals (9). Directly, integration of HBV into the host genome has been shown to cause genetic instability in the host genome, and mutagenesis of a number of cancer-related genes. Prolonged exposure to the viral Hbx and preS/S proteins dysregulate transcription and proliferation in host cells, as well as sensitize them to other carcinogenic factors (10). These effects are thought to act synergistically with indirect mechanisms that cause acute and chronic hepatic inflammation, initiating a

host immune response that results in rapid degradation and regeneration to cells, causing an accumulation of genetic mutations (9).

Hepatitis C virus (HCV) transmission only occurs through direct contact with infected blood. Because HCV has an entirely cytoplasmic life cycle, it is traditionally thought that there is no integration into the host genome, that it acts indirectly to cause HCC through inflammation, fibrosis, and oxidative chromosomal DNA damage. However, the low incidence of HCC in individuals with autoimmune hepatitis, and studies showing the presence of viral DNA in the genome of infected individuals suggest that HCV may have a more direct role in the development of HCC (11). Additionally, viral proteins E1/E2, p7, NS2, NS3, NS4, and NS5 have been shown to directly interact with cell cycle regulators, p53, and other genomic elements and upregulate cell division (2).

Like HBV, Kaposi sarcoma herpesvirus (KSHV) is transmitted through blood and other body fluids, mainly through saliva, and the incidence of infection is higher in individuals with infected family members. Generally, infection with KSHV alone is not sufficient to cause cancer, and HIV is often a co-factor in the development of Kaposi sarcoma (KS) (12). The global incidence of KS rose sharply with the HIV pandemic, but has also sharply declined with the introduction of effective anti-retroviral treatments (13). During lytic replication KSHV produces viral homologs of cellular proteins that are essential to regulating cell function, KSHV vIL-6 can activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) 3 pathway and initiate the activity of DNA Methyltransferase 1 (DNMT1), a transcriptional activator and DNA methyltransferase respectively (14). The majority of KS and primary effusion lymphoma tumor cells, however, only show evidence of latent infection. The viral protein latency associated nuclear antigen 1 (LANA1) is one of the few expressed during latent infection, but it is able to interact with p53 and the retinoblastoma gene, both known tumor suppressor genes implicated in oncogenesis (15).

Human T-lymphotropic virus-1 (HTLV-1) is also transmitted through blood, saliva, sexual contact, and from mother to child through breastfeeding. Adult T-cell

leukemia/lymphoma (ATLL) occurs almost exclusively in areas like the Caribbean, southwestern Japan, and parts of Africa and South America, where HTLV-1 is endemic and can infect more than 10% of the population (16). The viral oncoprotein, Tax, inhibits the host's innate immune system's interferon (IFN) response, while HBX impedes apoptosis while increasing proliferation of infected cells, disrupting their genetic integrity, while simultaneously facilitating immune system evasion (17).

Merkel cell polyomavirus (MCV) was only recently discovered in 2008 using next-generation sequencing, where short sequence tags were used to search for a virus in Merkel cell carcinoma (MCC) tumor samples. MCC is a rare and aggressive cancer that normally develops in elderly or immunosuppressed individuals (18). MCV can infect a number of different cell types, but it is mainly found in the nerve cells of the skin. The precise mode of transmission is unclear, but MCV can be detected in up to 80% of MCC tumors, and in the study by Feng *et al.* (13) 6 out of the 8 MCV positive tumors had the viral DNA monoclonally integrated into the cellular genome, suggesting that infection and integration preceded the expansion of the tumor cells.

There are also parasites that are known to be oncogenic. *Schistosoma haematobium*, a blood fluke, is endemic to sub-Saharan Africa and the Middle East. Seroprevalence in Egypt is between 37% and 48%, in some areas up to 85% (2). Like other schistosomes, freshwater snails serve as an intermediate host. Cercariae released from the snails actively swim through the water and infect humans by penetrating the skin, traveling through the blood to the liver where they mature into adult worms, pair off and then move on to the bladder where they mate and release eggs through the hosts urinary tract for the rest of their life (19). The normal life span is 3-5 years, but they can live up to 30 years, and during this time the eggs that they produce cause constant irritation and inflammation of the bladder. Malignancies form at the site of this inflammation, resulting in bladder cancer, the most prevalent type of cancer in Egyptian men, and the second most common in women (20). The host's inflammatory response to the eggs produces genotoxins which leads to genomic instability that causes oncogenic mutations.

Additionally, host bladder cells at the site of infection are stimulated to proliferate in order to repair tissue damaged by this inflammatory response (2).

The parasites *Opisthorchis viverrini* and *Clonorchis sinensis*, also known as river flukes, are endemic of several Asian countries and are primarily transmitted by eating raw or undercooked fish. Chronic infection is associated with the development of cholangiocarcinoma, a cancer of the bile ducts (2). Similar to *S. haematobium*, liver flukes release severely immunogenic metabolic products causing chronic inflammation which results in major hyperplastic changes to the bile duct (21).

The only bacteria known to cause cancer in humans is also responsible for the greatest fraction of infection-attributed cancers. *Helicobacter pylori*, a helical, gram-negative bacteria which is capable of penetrating the mucosal layer of the stomach (22). Seroprevalence varies from 70% in under-developed areas to 40% in developed countries, and although most infected people do not experience symptoms, *H. pylori* is associated with low-grade B-cell MALT gastric lymphoma and 75% of non-cardia gastric carcinoma (2). Inflammation is induced by the attachment of the bacteria to epithelial cells lining the stomach. The bacteria injects cytotoxin-associated antigen A which disrupts cellular function in infected epithelial cells. In conjunction with *H. pylori cag* Pathogenicity Island, this inflammatory stress activates oncogenic pathways including NF-κB, activator protein-1, PI3K, signal transducers and activators of transcription 3, Wnt/β-catenin and cyclooxygenase 2 along with epigenetic modifications in DNA methylation and histone modification (23).

1.1.3. Herpesviridae

Herpesviruses are a family of large, double-stranded DNA viruses. Since the first one was isolated in 1960, more than 130 herpesviruses have been discovered (24) infecting a range of hosts in addition to humans. Notorious for their ability to evade the host immune system herpesviruses have a biphasic life cycle, undergoing both

latent and lytic replication programs, often enabling them to establish persistent infections.

Table 1.2: Human Herpesvirus classification, target cells, seroprevalence and pathology. Table adapted from Bjørn, 2013 (25).

COMMON NAME	NAME	TYPE	TARGET CELL	LATENCY TARGET CELL	SERO-PREVALENCE	PATHOLOGY
Herpes simplex virus type 1 (HSV-1)	HHV-1	α	Mucoepithelia	Sensory and cranial nerve ganglia	60-90%	Orofacial lesions, keratitis, and encephalitis
Herpes simplex virus type 2 (HSV-2)	HHV-2				15-30%	Genital lesions, encephalitis, and meningitis
Varicella-zoster virus (VZV)	HHV-3				95%	Chicken pox and zoster (shingles)
Cytomegalo-virus (CMV)	HHV-5	β	Monocytes, lymphocytes, and epithelia		30-80%	Mononucleosis, microcephaly
Roseola virus	HHV-6 (A & B)		T cells	Leukocytes	>90%	Roseola
Roseola virus	HHV-7		T cells	T cells, epithelia	>90%	
Epstein-Barr Virus (EBV)	HHV-4	γ	Epithelia, and B cells	Memory B cells	>90%	Acute infectious mononucleosis, Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and post-transplant lymphoproliferative disorder
Kaposi sarcoma-associated virus (KSHV)	HHV-8		Endothelia, epithelia, and fibroblasts	B cells	5-10%	Kaposi sarcoma, Castleman disease, Pleural effusion lymphoma

Nine herpesviruses infect humans (26,27) (Table 1.2) causing a variety of diseases and imposing a considerable worldwide health burden. Cytomegalovirus (CMV) is the leading infectious cause of birth defects in infants, in addition to causing severe disease in transplant patients and other immunocompromised individuals (28) and

being linked to the development of acute lymphoblastic leukemia (29), the most common type of childhood cancer in the US. Numerous CMV vaccine clinical trials are currently underway (28,30), as with the herpes simplex virus type 1 and 2, which were estimated to have infected 118 and 19 million people, respectively, throughout the world in 2012 (31). Little research has been done on HHV-6 and HHV-7, although they both have a global seroprevalence of >90% and are known to cause roseola in infants yet they have only been casually linked with any serious illnesses (32). Varicella-zoster virus (chicken pox) is the only herpes virus to have an effective vaccine publicly available, two doses of this live-attenuated vaccine prevent 95% of all infections, and 99.5% of moderate/severe infections (33).

As discussed previously, KSHV is the only other herpes virus that is considered to cause cancer in humans. Like EBV, it is classified as a γ-herpesvirus, and while KSHV has a lower worldwide seroprevalence than the other herpesviruses it poses a significant risk in HIV-infected or otherwise immunocompromised individuals. KSHV was only discovered in 1994, but KS was already gaining attention due to the AIDS pandemic, as AIDS patients have a 10,000 times greater chances of acquiring KS than uninfected individuals (34). Since that time more resources have been directed at KSHV research and vaccine development.

1.2. History and Significance of Epstein-Barr virus

1.2.1. Epstein-Barr virus Discovery and Epidemiology

The first virus ever to be shown to cause cancer in humans was Epstein-Barr virus (EBV). It was discovered by Anthony Epstein, Yvonne Barr, and Burt Achong, who isolated the virus from tumor samples collected by Dr. Denis Burkitt from children in Uganda. After attending a lecture by Dr. Burkitt, Epstein asked for samples to be sent to his laboratory so that he could attempt to prove his theory linking viral infection to certain cancers. Epstein and his colleagues were successful in their endeavor and published their results in Lancet in 1964 (35). In 1968 it was determined that EBV was the causative agent of infectious mononucleosis, a disease that occurs after primary infection in adolescents and young adults, and in

1970 it was found to immortalize B cells resulting in many of the B cell lines that are used in laboratories today (36). In the years following its discovery, EBV was found to be present in all human populations, with a seroprevalence of over 95% worldwide (37). The EBV genome was detected in multiple epithelial and B cell cancers (38,39), and it has been associated with several other lymphatic and autoimmune disorders (40,41) (Table 1.3).

Table 1.3: Epstein-Barr virus (EBV)-associated cancers and diseases (34–36).

TYPE	DISEASE
Acute Infection	Infectious mononucleosis X-linked lymphoproliferative syndrome EBV-associated hemophagocytic syndrome Gianotti-Crosti syndrome
Chronic Infection	Chronic active EBV infection
Epithelial Malignancies	Nasopharyngeal carcinoma Gastric carcinoma Lymphoepithelioma-like carcinoma (salivary glands, thymus, lung)
Mesenchymal malignancies	Follicular dendritic cell tumor/sarcoma Leiomyomas/leiomyosarcomas in immunocompromised patients
Lymphomas and lymphoproliferative disorders	Burkitt lymphoma Lymphomatoid granulomatosis Pyothorax-associated lymphoma Primary effusion lymphoma Extranodal NK cell/T-cell lymphoma, nasal type Hodgkin lymphoma Diffuse large B-cell lymphoma CD30+/Ki-1+ anaplastic large-cell lymphoma T-cell-rich B-cell lymphoma Angioimmunoblastic lymphoma
Lymphoproliferative disorders in immunocompromised patients	Primary immunodeficiency-associated Human immunodeficiency virus-associated Posttransplant condition Methotrexate-induced Senile EBV associated lymphoproliferative disorder
Autoimmune disorders	Systemic lupus erythematosus Multiple sclerosis Rheumatoid arthritis Inflammatory bowel disease Type 1 diabetes Juvenile idiopathic arthritis Celiac disease Sjögren's syndrome

Primary infection normally occurs during childhood when it is typically asymptomatic, however recently studies have shown that the age of primary infection is steadily increasing, particularly in developed countries (42). When primary infection is delayed until adolescence or young adulthood, it has been shown to cause acute infectious mononucleosis (AIM) in approximately 1% of university students in developed countries (43) Individuals who have had AIM have an increased risk of developing Hodgkin lymphoma later in life (44).

1.2.2. EBV-associated Cancers and Diseases

In the absence of other co-factors, the immune system is able to control the virus and it remains latent in the host memory B cells and most healthy adults maintain a life-long asymptomatic infection. In the setting of immunosuppression or co-infection with certain pathogens, EBV can re-activate, leading to the development of EBV-associated B cell lymphomas or epithelial carcinomas, reflecting the tropism of the virus. It is believed that 1.5% of all cancer cases worldwide are attributable to EBV infection (45) and viral genes are expressed in a variety of lymphomas, carcinomas, and mesenchymal malignancies. These viral proteins have known oncogenic functions, including destabilizing the host genome, immune system evasion, avoiding apoptosis, eliciting an inflammatory response, sustaining proliferation, cell migration, and angiogenesis (42). Although the list continues to grow, EBV is most commonly associated with Hodgkin lymphoma (HL), nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), post-transplant lymphoproliferative disorder (PTLD) and Burkitt lymphoma (BL), the original tumor that the virus was isolated from. The role of EBV in these cancers varies, from 100% of endemic Burkitt lymphoma (eBL) being attributed to EBV, compared to only 10% of diffuse large B cell lymphoma not otherwise specified (DLBCL-NOS) (46).

Most people infected with EBV will never develop an EBV-associated cancer. Although some cancers arise as a result of mutations in infected cells, oncogenesis generally requires co-infection with another pathogen or suppression of the host immune system. Co-infection with malaria is believed to be the cause of eBL. The most common type of childhood cancer in equatorial Africa, eBL occurs in malaria

holoendemic areas (47). HIV-infected individuals have a significantly higher risk of developing EBV-associated B cell lymphomas, whereas transplant recipients taking immunosuppressants to prevent rejection and graft-versus-host disease (GVHD) are at risk of developing PTLD (48).

In addition to causing AIM, acute EBV infection is associated with a number of rare diseases, including X-linked lymphoproliferative syndrome, a hereditary disorder characterized by an extreme immune response to EBV infection due to a lack of $CD27^+$ memory B cells (49). Another uncommon consequence of EBV infection is chronic active Epstein-Barr virus (CAEBV) disease, where primary infection of reactivation of EBV can lead to a mononucleosis-like illness that does not resolve. CAEBV can potentially be fatal, usually due to opportunistic infections, multiorgan failure, or EBV-positive lymphomas (50).

1.3. Epstein-Barr virus Structure, Transmission, and Replication

EBV is a 172 kbp, double stranded DNA virus, enclosed in a viral envelope that expresses ten glycoproteins on the surface, five of which have been shown to have a role in entry into host cells, gp350/220 (gp350), gB, gH, gL, and gp42. The linear genome encodes for over 85 genes, however only nine genes are expressed during latency: the Epstein-Barr nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP) and the latent membrane proteins (LMP1, LMP2A, and LMP2B) (51).

Generally, EBV is believed to be transmitted through saliva, initially infecting oropharyngeal epithelial cells, where it undergoes active lytic replication. This leads to the infection of naïve B cells circulating through the lymphoid tissues of the throat, triggering activation and proliferation as the B cells progress into B cell blasts. These infected lymphoblasts express all latent proteins (latency III) and are targeted by CD8+ cytotoxic killer cells. Some of these B cells are thought to migrate to the germinal centers where latent gene expression is downregulated to only the less immunogenic EBNA1, LMP1, and LMP2 (latency II) (52). These proteins provide survival signals and the infected B cells leave the germinal center as memory B cells and transcription is further reduced to no viral protein expression

(latency 0) or only EBNA1 (latency I), allowing the virus to establish persistent life-long infection in these cells (53). Each of these latency stages correspond with certain EBV cancers, characterized by their viral protein expression (Table 1.4). At times the virus reactivates, initiating proliferation and viral shedding, resulting in the infection of new cells.

However, there is an alternate theory, that in the absence of underlying conditions or malignancy, B cells are the target of EBV infection (54). Patients with X-linked agammaglobulinemia, a disease characterized by the lack of immunoglobulin due to a genetic defect preventing the growth of normal, mature B cells, cannot be infected with EBV, indicating the critical and possibly exclusive role of mature B cells (55). Another study examining normal nasopharyngeal tissues from 23 individuals showed evidence of lytic replication in circulating mucosal lymphocytes rather than nasopharyngeal epithelial cells (56). While this evidence is intriguing, the role of epithelial cells in EBV infection remains unclear. It is undeniable that EBV is capable of infecting epithelial cells *in vitro,* indicating that they likely have some role in natural infection.

Table 1.4: Epstein-Barr Virus latency stages and associated expressed proteins and cancers (38).

	LATENCY III	LATENCY II	LATENCY I	LATENCY 0
Cell	B Cell Blast	Germinal Center B Cell	Memory B Cell	
Viral Proteins	All EBNA1s and LMPs	EBNA1, LMP1, and LMP2	EBNA1	None
Cancers	HIV-Related DLBCL (Immunoblastic), Polymorphic PTLD, Plasmablastic Peripheral T cell Lymphoma	Hodgkin Lymphoma, T/NK Cell Lymphoma, Nasopharyngeal Carcinoma, DLBCL (elderly)	Burkitt Lymphoma, HIV-Related DLBCL, Gastric carcinoma, Monomorphic PTLD, Primary Effusion	None

1.4. EBV glycoproteins and viral entry

Previously, one study showed that primary B cells can be infected with recombinant EBV lacking gp350, indicating that viral glycoproteins other than gp350 may mediate EBV infection of host cells (57). Further investigation into the mechanism

of EBV entry demonstrates that in addition to gp350, at least four other EBV glycoproteins: gB, gp42, and the gH/gL complex are critical for EBV entry and infection of host cells, making them attractive targets for developing a prophylactic vaccine (Figure 1.1) (38,58).

Entry into B cells begins when EBV gp350 binds to complement receptor type 1 (CD35) (59) or type 2 (CD21) (Figure 1.1) (60). This interaction enhances infection, but is not necessary, possibly explaining the failure of all gp350-based clinical trials to date. It is possible that gp350 brings the virus into close proximity of target cells, allowing for the series of events leading to viral entry to take place. Viral tropism for B cells and epithelial cells is determined by gp42 and gH/gL, respectively (Figure 1.1) (61). Like the CMV receptor-binding proteins, gp42 forms a complex with gH/gL and binds to the major histocompatibility complex (MHC) class II on B cells, activating gH/gL to interact with gB causing a conformational change which triggers fusion (62).

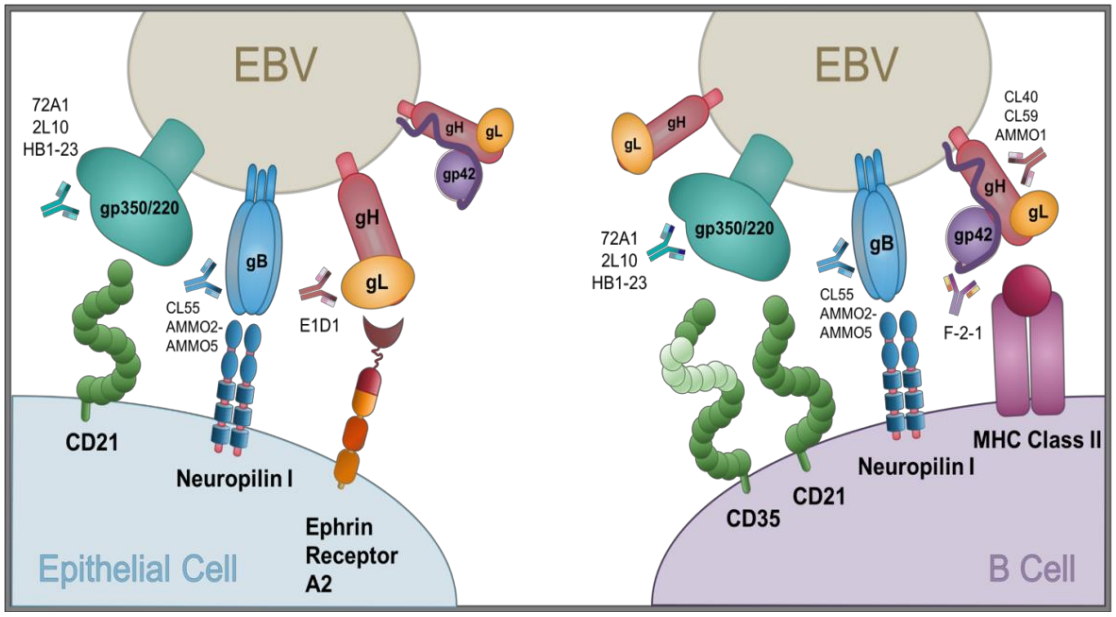


Figure 1.1: Epstein-Barr virus (EBV) surface glycoproteins, their corresponding host cell receptors on epithelial and B cells, and known monoclonal antibodies targeting the specific EBV receptors.

Infection of epithelial cells is dictated by gH/gL, originally it was believed that the virus bound integrins αvβ5, αvβ6, and αvβ8 on host cells, but a study by Chen *et al.* in 2018 dispelled this theory (63). Using a knockout epithelial cell line lacking these integrins, they showed that Chinese hamster ovary (CHO) cells transfected with gB

and gH/gL are still able to fuse with the knockout cells. They further went on to conclude that gH binds ephrin receptor tyrosine kinase A2 (EphA2) on epithelial cells (63).

The core fusion machinery, gH/gL and gB, are generally conserved in herpesviruses, suggesting that they might use a similar mechanism for fusion and entry into host cells. On the other hand, the receptor-binding proteins are specific to the virus, and confer host and cell specificity. The receptor-binding protein for HSV 1 and 2 is gD, which binds to herpesvirus entry mediator (HVEM), nectin-1, and 3-O sulfated heparan sulfate (64). The proteins gO and UL128/130/131A are specific to CMV, gO binds platelet-derived growth factor receptor alpha (PDGFRα) on fibroblasts, although it has been found to be needed for infection into all cell types (65). Together with gH/gL, UL128/130/131A form what is known as the pentameric complex, which binds neuropilin2 (Nrp2) and is required for viral entry in epithelial, endothelial and myeloid cells (66). The ability of anti-gB and -gH/gL antibodies to neutralize viral infection is well conserved in HSV-1 and CMV (67,68), making a strong case that a combination of gp350, gB, gp42, and gH/gL may potentially be capable of blocking EBV infection of epithelial and B cells.

1.5. Previous Vaccine Approaches, Clinical Trials and Progress

In over 50 years of EBV research many potential vaccines have been proposed and tested, targeting both latent and lytic viral proteins and varying widely in complexity. The simplest vaccine design consisted of an EBNA3A epitope peptide adjuvanted with tetanus toxin, intended to induce an EBV-specific CD8+ cytotoxic T cell response to infected B cells expressing EBNA3A (69). Although the desired T cell response was observed in approximately 90% of the subjects, this vaccine also failed to curtail infection.

Despite the known link between EBV and cancer, few prophylactic and therapeutic vaccine candidates have demonstrated any clinical efficacy (70). For nearly three decades after EBV was discovered the vaccination approach of choice was to elicit neutralizing antibodies (nAbs) directed against glycoprotein gp350, the most abundant protein expressed on the surface of the virus. However, when a

recombinant subunit gp350 vaccine was tested in a phase II clinical trial it failed to prevent infection in healthy, seronegative adults, despite inducing nAbs (71). Later vaccine designs reasoned that the weak immune response to the monomeric subunit may be improved upon by repetitively arranging multiple proteins together. This is generally accomplished by using multimerization domains, like the T4 bacteriophage foldon. Cui, *et al.* discovered that rabbits immunized with multimeric gp350 and gH/gL glycoproteins had nAb titers higher than the monomeric form of the same proteins (72). Interestingly, they also showed that compared to monomeric gp350, rabbits immunized with trimeric and monomeric gH/gL, trimeric gB, and tetrameric gp350 produced nAb titers greater than 100-fold, 20-fold, 18-fold, and 4-fold higher, respectively (73), suggesting that both gH/gL and gB may be more optimal targets for a prophylactic vaccine.

Another vaccine platform tested was antigen-armed antibodies, where a latent EBV antigen was conjugated to a dendritic cell (DC) or B cell antibody, aimed at increasing the efficiency of antigen presentation thus increasing the number of antigen-specific CD4 and CD8 cells. Previous studies have shown that antigens conjugated to a DC-specific antibody increased the efficiency of antigen presentation up to 100-fold (74). A vaccine using the immunogenic C-terminus of EBNA1 conjugated to αDEC-205, which recognizes a receptor expressed on DCs was shown to increase EBNA1-specific T cells *in vitro* and prime EBNA1-specific T cells and antibody responses *in vivo* (75). Similarly, antigens fused to antibodies targeting B cell markers, CD19, CD20, CD21, and CD22, were ~4,000 times more immunogenic than peptide alone (76).

Self-assembling vaccine-like particles (VLPs) and nanoparticles have also been used as a platform for delivering EBV antigens. Both are a closer approximation to the structure of the actual virus than monomeric and multimeric subunit vaccines, as both are capable of expressing viral proteins on their surface without containing any viral DNA, making them incapable of replicating. In their simplest form, VLPs and nanoparticles can express many epitopes or monomeric antigens on their surface. A nanoparticle-based vaccine displaying different domains of gp350 was shown to be 10-100 times better at inducing nAbs in mice than soluble gp350 alone (77). These nanoparticles were produced by fusing gp350 to *H. pylori*-bullfrog hybrid ferritin and showed enhanced presentation and recognition of the gp350

CD21-binding site. Another study tested multimeric gp350 presented on the surface of a VLP using the multimerization domain heptad repeat 2 (HR2) fused to the gp350 ectodomain. However, mice immunized with the multimeric gp350 VLP actually showed a similar or slightly reduced antibody titers and neutralization ability compared to animals immunized with soluble gp350, possibly because the HR2 domain altered the conformation of gp350 or concealed important immunogenic epitopes (78).

The VLP vaccine platform was then used to include other EBV surface glycoproteins, gB and gH/gL, as well as a way of packaging latent EBV antigens EBNA1 and LMP2 (79). A combination of VLPs displaying surface protein gB and containing latent antigen LMP2, as well as another VLP expressing gH/gL on the surface and containing EBNA1 generated EBV-specific antibodies in mice. Mice immunized with all three VLPs, gp350, gB-LMP2, and gH/gL-EBNA1 had titers similar to those immunized with UV-inactivated EBV. Additionally, serum from animals immunized with gB-LMP2 and gH/gL-EBNA1 achieved slightly better neutralization of the virus and preventing infection in HEK-293T (epithelial) cells, although this difference was not statistically significant (79).

The most structurally complex vaccines that have been proposed are VLPs that contain numerous EBV proteins, from surface glycoproteins and latent antigens to the viral envelope, capsid, and tegument, allowing for the vaccinee to generate an immune response to many different viral elements. One group engineered a cell line that produced a virus where all the viral oncogenes had been deleted (80). BALB/c mice immunized with these VLPs produced antibodies to 12 viral proteins that were able to neutralize the virus and reduce infection of human primary B cells at levels comparable to the known neutralizing α-gp350 antibody 72A1. Furthermore, these VLPs were able to expand EBV-specific CD4+ and CD8+ T cells in PBMCs from four different EBV-positive donors (81). Another group took a different approach and deleted the terminal repeats in the viral genome that act as a packaging signal for DNA (82). This was meant to produce VLPs devoid of viral DNA, but there was some recombination with the helper virus and the particles retained some of the EBV genome although none of the oncogenes remained, making the use of these VLPs in a clinical setting problematic. The group went on to modify this design, further manipulating the EBV genome to create a series of

mutants defective in various genes needed for maturation (83). They identified two mutants that produced DNA-free VLPs that were capable of inducing a CD4+ T cell response equal to that of the wild-type virus.

1.6. Virus-like Particles

Thus far, the most effective way of generating nAbs to EBV has been vaccination with UV-inactivated virus, often referred to as the “gold standard” and used as a positive control in animal studies and *in vitro* experiments (78,84). However, because EBV is oncogenic, creating a vaccine is not as simple as using the inactivated or dead virus. Instead, an EBV vaccine must be deliberately designed to contain specific humoral and cellular immune response targets devoid of viral DNA. VLPs are self-assembling structures of varying complexity composed of viral proteins, without containing any genetic material. Studies have shown that VLPs can be produced using components of a variety of viruses including *parvoviridae*, *retroviridae* , *flaviviridae*, *paramyxoviridae* and bacteriophages (85). Additionally, a number of cell culture systems can be used to produce VLPs: bacterial, yeast, insect and mammalian cells (86). VLP size and structure closely resemble those of native virus, but VLPs lack genetic material, rendering them non-infectious. These qualities make VLPs a highly immunogenic and safe vaccine platform for oncogenic viruses such as EBV. Several VLP-based, FDA-approved vaccines for human papillomavirus, influenza, hepatitis B virus, and malaria are currently available, and there are many more currently being developed (87). However, all of these vaccines only express a single protein on their surface.

In this study the Newcastle disease virus (NDV)-LP platform was utilized, NDV is an avian paramyxovirus known to spontaneously produces “defective” virus particles that lack viral DNA, making them unable to replicate. In prior studies, it was determined that only a single protein was required for their production, the NDV matrix protein (NDV-M) (88). However, addition of nucleoprotein (NDV-NP) increases the efficiency of VLP production, and the incorporation of NDV fusion protein (NDV-F) and hemagglutinin-neuraminidase (NDV-HN) allows for expression of other proteins on the virion envelope.

Chimeric proteins can be designed containing the ectodomain of a viral glycoprotein fused to the transmembrane domain of NDV-F, resulting in the glycoprotein being expressed on the surface of the VLP (89). Expression vectors encoding the four key NDV structural proteins are co-transfected into mammalian host cells along with expression vectors for the desired viral proteins, and VLPs containing the target proteins are released into the cell culture media. They serve as delivery systems to present antigens from various types of pathogens, including viruses.

The NDV-LP provides a valuable tool for creating a vaccine devoid of viral DNA, as well as studying the function of different viral proteins. Multiple proteins can be expressed on the surface of the VLP in conformations similar to those on the wild-type virus, as compared to subunit vaccines which consist of single immunogenic fragment and have been shown to have lower immunogenicity than live-attenuated or inactivated vaccines (90).

1.7. Rationale for Study, Aims, and Objectives

The study hypothesis was that incorporating all five EBV glycoproteins (gp350, gB, gH, gL, and gp42) required for entry on the surface of a VLP will help to overcome poor immunogenicity reported in previously tested vaccine candidates in small animals, and result in a safe vaccine that is effective in blocking EBV infection. Thus, the overall study aim was to design a multivalent EBV-LP and test the immunogenicity in New Zealand white rabbits.

Study Objectives

1. *To construct, generate, and characterize the structural and functional properties of a multivalent EBV-LP.* A multicistronic expression vector was used to express EBV gp350, gB, gp42, gL and gH from a single transcript in CHO cells. The VLP was purified and assessed for total protein content and glycoprotein composition. Expression of EBV gp350, gB, gp42, gL and gH was confirmed and quantified using western blot and ELISA.
2. *To test the efficacy of EBV-LP vaccine in eliciting nAbs in immunized New Zealand white rabbits.* Animals were immunized three times with either the EBV-LP, gp350 soluble protein, UV-inactivated EBV, or buffer alone adjuvanted alum and MPLA. Serum from immunized animals were collected at days 0, 14, 35, 49, 70, and 90 and tested for EBV glycoprotein-specific antibodies by ELISA. *In vitro* neutralization was performed to determine EBV-specific neutralizing antibody titers.

Chapter 2 METHODS

2.1. General Information

In this study all DNA concentrations were measured using the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All water used, unless otherwise stated, was purified using Millipore Milli-Q water filtration system (MilliporeSigma, Burlington, MA, USA). Water used in DNA extraction was UltraPure™ DNase/RNase-Free Distilled Water (10977015, Thermo Fisher Scientific, Waltham, MA, USA). Purified EBV virus, VLPs, or cells were lysed in RIPA buffer (BP-115, Boston Bioproducts, Ashland, MA, USA). Lysates were incubated for 30 min on ice, centrifuged for 5 min at 11,000 rcf and the protein content of each lysate determined by BCA protein assay (PI23235, Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Plasmids

The EBV-LP constructs were designed using the pCAGGS vector (Belgian Co-Ordinated Collection of Micro-Organisms, Brussels, Belgium), which utilizes the chicken β-actin/rabbit β-globin hybrid promoter (AG), human cytomegalovirus immediate early promoter (CMV-IE); enhancer only, and *Escherichia coli* lac operon promoter. Chimeric fragments of EBV glycoproteins gp350 (nucleotides 1-864), gB (1-735), and gH (1-679) were constructed by fusing the ectodomain (ED) of the viral proteins to the NDV-F transmembrane (TM) and cytoplasmic (CT) domains in order to direct them to be expressed on the surface of the VLP. The glycoproteins (gp350, gB, and gH) that normally have a transmembrane domain, had that domain replaced with NDV-F to anchor them in the same way on the surface of the VLP. The other glycoproteins (gL and gp42) do not naturally have a transmembrane domain, and are only attached through their interaction with gH, so they are expressed in their native form. This increases the likelihood that the gH/gL and gH/gL/gp42 complexes will form and be expressed as they are on the wild type virus. To allow each of the viral proteins to be expressed separately from a single DNA sequence, the transcript included short unique porcine 2A peptide sequences (2A), which act as cleavage signals, separating each of the viral proteins (91).

Since being discovered in foot-and-mouth disease virus, 2A-peptides have been found in a wide range of other viruses. These 2A-peptides induce the cleavage of recombinant proteins in the cell using a novel ribosomal-skip mechanism (92,93). The peptide sequences are commonly used to discretely express multiple proteins from a single transcript under a single promoter increasing the likelihood that each protein will be expressed in essentially equimolar amounts (94,95).

In a study by Ray *et al.* it was demonstrated that a shortened version of NDV-NP consisting of only 15 amino acid residues from the C-terminal end of the protein was sufficient for VLP formation and packaging of proteins within the VLP (96). This study used an NP fragment comprised of 26 amino acids from the C-terminal domain. The smaller plasmid size was meant to increase transfection efficiency, thus increasing VLP production. The plasmid pCAGGS-NP_{26AA}-tEBNA1-LMP2 utilized this shortened NP_{26AA} to package latent EBV antigens inside the VLP. The plasmid encoded a truncated EBNA1 (tEBNA1) protein (nucleotides 326-641) and full length LMP2. The tEBNA1 protein was designed to exclude the N-terminal portion of the protein, including the oncogenic glycine-alanine (gly-ala) repeat region which modulates the interaction of EBNA1 with the cellular DNA of the host and prevents proteasome-dependent processing for presentation on MHC class I (97,98).

To produce the KSHV-LP the pCAGGS-KSHV-K8.1-gB-gL-gH-F plasmid was designed in a similar way to pCAGGS-EBV-gp350-gB-gp42-gL-gH-F. K8,1, gB, gH, and gL. The pCAGGS vector contained sequences encoding K8.1 ED fused to NDV-F, gB ED fused to NDV-F, full-length gL WT, and gH ED fused to NDV-F. Each of the KSHV glycoproteins was separated by a 2A-peptide sequence, which again results in the expression of each sequence as discrete proteins. The pCAGGS-NP_{26AA}-LANA1 plasmid was also used in the production of the KSHV-LP, although this latent KSHV antigen is not discussed in this study.

The final plasmid used for VLP production was pCAGGS-NDV-M, encoding the full-length NDV matrix protein. The NDV-M protein is essential for the production of VLPs, and expression of NDV-M alone is sufficient for the release of membranous particles (99). The sequences for all of the plasmid constructs used in the production of EBV- and KSHV-LPs were then sent to be synthesized by GeneWiz

(MA, USA) where they were also sequenced to confirm the fidelity of the final plasmid products.

Other plasmids used in this study included the pCI-puro, p509, p2670, and p2130. The pCI vector employs a CMV enhancer/promotor (Genbank # U47119.2) to express a puromycin resistance gene used for selection in the production of the stable CHO cell line expressing pCAGGS-EBV-gp350-gB-gp42-gL-gH-F. The pCI-puro plasmid was constructed from pCI-neo and pPUR (Clontech) backbones (100). The plasmids used in virus production from B95-8/F EBV-eGFP 293 cells, p509 encoding EBV-Zta (ZEBRA), p2670 encoding EBV-gB, and p2130 encoding Rta (replication and transcription activator), were provided by Henri-Jacques Delecluse (German Cancer Research Center).

2.3. Cell Lines

A gift from Dr. Liisa Selin (University of Massachusetts Medical School), AGS-EBV is a human gastric adenocarcinoma cell line superinfected with a recombinant Akata strain of EBV modified to impart G418 resistance and expression of enhanced fluorescent green protein (eGFP) as a reporter at the thymidine kinase locus (101). The VLP was produced in Chinese hamster ovary (CHO, ATCC® CCL-61™) cells, an epithelial cell line. CHO cells, along with human embryonic kidney cells expressing SV-40 T antigen (HEK-293T, ATCC® CRL-11268™), and the EBV^+ Burkitt lymphoma B cell line (Raji, ATCC® CCL-86™) were obtained from the American Type Culture Collection (ATCC). The B95-8/F EBV-eGFP 293 cell line was provided by Henri-Jacques Delecluse (German Cancer Research Center).

All cells were cultured in media containing 10% heat-inactivated fetal bovine serum (FBS) (35015CV, Corning, NY, USA), 2% penicillin-streptomycin (15140122, Thermo Fisher Scientific, Waltham, MA, USA), and 1% L-glutamine (25030081, Thermo Fisher Scientific, Waltham, MA, USA). Adherent cells, CHO and HEK-293T, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (MT15017CV, Thermo Fisher Scientific, Waltham, MA, USA), Raji suspension cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (11875093, Thermo Fisher Scientific, Waltham, MA, USA). AGS-EBV-eGFP cells were grown in 50:50 Ham's F-12:DMEM media (11320033, Thermo Fisher Scientific, Waltham, MA, USA),

containing 500 μg/mL of neomycin (G418) (30234CR, Corning, NY, USA). The B95-8/F EBV-eGFP 293 was cultured in RPMI 1640 medium containing 10% FBS, 2% penicillin-streptomycin, 1% L-glutamine and 100 μg/ml Hygromycin B (MT30240CR, Corning, NY, USA).

Raji and HEK293T cells used for neutralization assays were thawed and allowed to rest overnight before being plated for the experiment. The CHO cell line used for VLP production was expanded for no more than ten passages before being plated for transfection. Similarly, AGS-EBV-eGFP cells used for virus production were expanded for no more than ten passages before being treated to induce lytic replication and release of the virus.

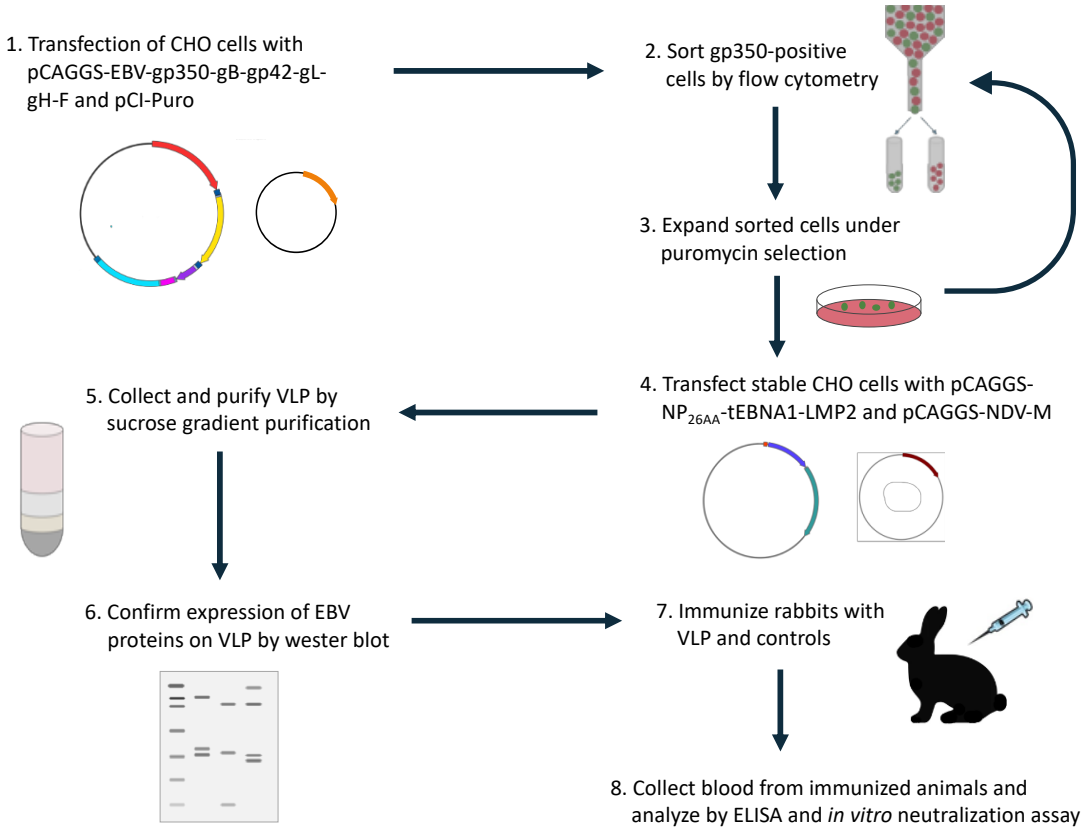


Figure 2.1: A flow diagram of the methods used in this study to create, characterize and test the EBV-LP vaccine.

2.4. Production of Virus

2.4.1. Induction of AGS-EBV-eGFP cells and EBV-eGFP virus purification

The EBV-eGFP virus used in this study was produced from the AGS-EBV-eGFP cell line. Cells were cultured in T75 flasks in Ham's F-12:DMEM containing G418 antibiotic to select for cells retaining the EBV episome. When the cells were 90% confluent, the media was replaced with Ham's F-12:DMEM medium containing 33ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (P8139-5MG, MilliporeSigma, St. Louis MO, USA) and 3mM sodium butyrate (NaBut) (B5887-1G, MilliporeSigma, St. Louis MO, USA) to induce lytic reactivation. Following a 24 hour incubation, the culture induction media was replaced with complete Ham's F-12:DMEM without G418, TPA, or NaBut. The cells were then incubated at 37°C for 5 days without changing the culture media. The resulting supernatant containing the produced virus was collected and centrifuged briefly for 20 minutes at 4,000 rcf to remove cell debris. In order to pellet the virus the supernatant was centrifuged for 70 min at 16,000 rcf . The virus pellet was resuspended in TNE buffer (100 mM Tris; 2.0 M NaCl; 10 mM EDTA; pH 7.4) and stored at -80°C.

2.4.2. Optimization of EBV-eGFP production in B95-8/F EBV-eGFP 293

An alternative method for producing EBV-eGFP was explored using a modified HEK-293 cell line, B95-8/F EBV-eGFP 293. The cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS), 2% penicillin-streptomycin, 1% L-glutamine, and 100 μg/ml hygromycin at 37°C and 5% CO_2. A day prior to transfection cells were seeded in 6-well plates containing medium without hygromycin. The following day, when the cells had reached approximately 75% confluency, they were co-transfected with three plasmids: p509 (BZLF1), p2670 (BALF4), and p2130 (BRLF1). These 3 plasmids encoded EBV proteins absent from the mutant virus, needed for efficient production and release of the virus.

This transfection was optimized by comparing 3 different transfection reagents: Metafectene® (T020-1.0, Biontex Laboratories GmbH, München, Germany),

Lipofectamine™ 3000 (L3000001, Thermo Fisher Scientific, Waltham, MA, USA), and polyethylenimine (PEI) (764965-1G, MilliporeSigma, St. Louis MO, USA). Six different conditions of varying amounts of DNA and transfection reagent were repeated in triplicate for each of the reagents (Table 2.1). The DNA and reagent amounts were based on the range recommended by the manufacturer of that reagent. The three plasmids were used at a ratio of 3:3:1, p509 (BZLF1):p2670 (BALF4):p2130 (BRLF1). The p2130 plasmid was present in a smaller proportion because it is not necessary for induction of lytic activation or release of the virus, but it can increase the efficiency.

Table 2.1: Transfection conditions for production of EBV-eGFP from B95-8/F EBV-eGFP 293 cells.

		CONDITIONS					
		#1	**#2**	**#3**	**#4**	**#5**	**#6**
Metafectene	Total DNA (μg)	1	1	1	2.5	2.5	2.5
	Metafectene Reagent (μL)	2	3	5	5	7.5	12.5
Lipofectamine 3000	Total DNA (μg)	2.5	2.5	2.5	5	5	5
	Lipofectamine 3000 Reagent (μL)	3.75	5	7.5	3.75	5	7.5
	P300 Reagent (μL)	5	5	5	5	5	5
PEI	Total DNA (μg)	2.5	2.5	2.5	5	5	5
	PEI Reagent (μL)	5	7.5	12.5	10	15	25

DNA and reagents were mixed in serum-free Opti-MEM (31985062, Thermo Fisher Scientific, Waltham, MA, USA) at a total volume of 500 μl and incubated at room temperature for 30 min, the mixture was added to cells with an additional 3 ml serum-free Opti-MEM. The supernatant from transfected cells was collected and the virus was isolated as described above. The transfected cells were washed and assessed by flow cytometry to determine the number of eGFP-positive cells. Cells not expressing eGFP served as a negative control for the flow cytometry assessment.

2.4.3. Titration of EBV-eGFP

The infectivity of the EBV-eGFP virus produced from the AGS-EBV-eGFP cells was determined in both B cells (Raji) and epithelial cells (HEK-293T). The cells were seeded in a 48-well plate with 5 x 10^4 cells per well in either complete RPMI (Raji) or DMEM (HEK-293T). HEK-293T cells were incubated overnight at 37°C to allow them to adhere to the plate, while Raji cells were seeded the same day the assay was performed. Before infection the culture media was replaced with 200 µl/well serum-free Opti-MEM media. The cells were then infected by adding either 1, 2, 5, 10, 25, or 50 µl of purified EBV-eGFP to the wells. Each infection was performed in triplicate, and mixed by gentle pipetting. The plates were incubated at 37°C for 24 hours. Virus titer was determined by washing the cells twice with PBS containing 1% FBS, then analyzed by flow cytometry. Uninfected cells were used as a negative control and the percent of eGFP positive cells for the different volumes of virus added was used to determine the amount of virus required for subsequent experiments.

2.5. Multivalent VLP-based EBV Vaccine Production and Characterization

2.5.1. Generation of gp350-gB-gp42-gL-gH-F stable cell lines

Stable cell lines were produced by seeding HEK-293T and CHO cells in a 6-well plate 24 hours prior to transfection. The following day, when the cells were 90% confluence, the media was replaced with 1.5 ml serum-free DMEM without pen/strep. The cells were transfected using PEI with 0.5 µg/well pCAGGS EBV gp350-gB-gp42-gL-gH-F and 0.1 µg/well of pCI vector containing the puromycin resistance gene, at a 3:1 ratio of PEI to DNA, as per the manufacturer's instructions. All transfection reagents were brought to room temperature, the appropriate volume of DNA was determined and added to a tube containing 125 µl of serum-free Opti-MEM media and mixed by gentle vortexing. A separate tube of 125 µl Opti-MEM containing 1.8 µl PEI was mixed completely, and both tubes were allowed to equilibrate 5 minutes at room temperature. The two tubes were then

combined and mixed via gentle pipetting, and incubated at room temperature for a further 20 minutes, then added to each well.

After 48 hours the cells were trypsinized and washed with 1 ml PBS containing 1% FBS (staining buffer) and centrifuged for 5 min at 250 rcf, the wash was repeated twice and cells were then resuspended in 1 ml staining buffer and divided into 6 aliquots. Each aliquot was stained with either 72A1, E1D1, CL40, CL55, CL59, or F-2-1 (Table 2.2) for one hour on ice to determine the percent of cells positive for gp350, gL, gH, gB, gH/gL or gp42, respectively. For some rounds of sorting the cells were only stained with 72A1, as the other antibodies are not commercially available and could only be obtained by request from the groups that produced them. Cells were washed twice with staining buffer then stained with anti-mouse IgG conjugated to AlexaFluor488 (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 1:2,000. The cells were incubated on ice for 30 minutes and washed twice more, then sorted via flow cytometry (S3^{TM} Cell Sorter, Bio-rad, Hercules, CA, USA). Unstained transfected cells and an isotype control were used as negative controls in order to properly gate on AlexaFluor488 positive cells. Gating using FSC and SSC separated cells from debris, and this population was further gated to collect cells that were positive for AlexaFluor488 compared to negative controls. The positive cells were sorted into 5 ml tubes with DMEM media containing 50% FBS, then transferred to a 24-well plate and allowed to recover overnight in DMEM media containing a final concentration of 20% FBS. The following day the media was replaced with DMEM containing 10% FBS and 1 μg/ml (HEK-293T) or 10 μg/ml (CHO) puromycin (selection media). The cells were cultured and expanded in the selection media and the staining process was repeated until cells were >90% positive during sorting. The cells were then aliquoted in FBS containing 10% dimethyl sulfoxide (DMSO) and frozen in liquid nitrogen for use in future VLP production. Following long term storage, cells were thawed and passaged 10 times and determined to have retained expression of all of the surface glycoproteins.

Table 2.2: Antibodies targeting the various Epstein-Barr virus recombinant proteins used in this study.

ANTIBODY	TARGET PROTEIN	SOURCE (CAT. #)	SPECIES	ASSAY	REF.
72A1	gp350/220	Millipore (MAB10219)	Mouse	Immunoblot, Flow	(102)
2L10	gp350/220	Millipore (MAB8183)	Mouse	Flow	(103)
E1D1	gL	Dr. Hutt-Fletcher (LSU)	Mouse	Flow	(104)
CL40	gH	Dr. Hutt-Fletcher (LSU)	Mouse	Flow	(105)
CL55	gB	Dr. Hutt-Fletcher (LSU)	Mouse	Flow	
CL59	gH/gL	Dr. Hutt-Fletcher (LSU)	Mouse	Flow	
F-2-1	gp42	Dr. Hutt-Fletcher (LSU)	Mouse	Flow	(106)
Anti-2A	2A peptide	Millipore (ABS31)	Rabbit	Immunoblot, Flow	(107)

2.6. Production of EBV gp350-gB-gp42-gL-gH-tEBNA1-LMP2 VLPs

2.6.1 Transfection of gp350-gB-gp42-gL-gH-F stable cell lines for production of EBV-LPs

VLP production required the co-transfection of three plasmids, pCAGGS-EBV-gp350-gB-gp42-gL-gH-F (13,539 bp), pCAGGS-NDV-NP_{26AA}-tEBNA1-LMP2 (7,363 bp), and pCAGGS-NDV-M (5,943 bp). Initial transfections resulted in poor yields of VLP, totaling only 5-10 μg as determined by the NanoDrop™ Lite Spectrophotometer, likely due to the long length of these plasmids leading to very low transfection efficiency. To overcome this problem a stable cell line was created to increase the efficiency of VLP production. The stable CHO EBV- gp350-gB-gp42-gL-gH-F cells were expanded in selection media and seeded into T175 cm^2 flasks. Before transfection the cells were analyzed by flow cytometry to ensure that they were still >90% positive for expression of EBV glycoproteins. When the cells reached 80-90% confluent, 24 hours later, they were co-transfected by incubating 8.0 μg of both pCAGGS-NDV-M and pCAGGS-NP_{26AA}-tEBNA1-LMP2 expressing plasmids with PEI as described previously. The supernatant containing expressed VLPs was collected every 24 hours post-transfection, for a total of 5 days. Collected supernatant was centrifuged at 4,000 rcf for 15 minutes to remove cell debris, then stored in sterile bottles at 4°C until purification. This process was

repeated a total of 5 times to obtain the amount of VLP needed for animal immunizations and characterization.

2.6.2. Sucrose gradient purification of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs

The VLPS in the supernatant from the transfected cells was purified by sucrose gradient purification (108). The collected supernatant from VLP transfections was pooled and centrifuged at 38,146 rcf overnight at 4°C. The resulting pellet was resuspended in 4 ml of buffer containing Tris-HCl 10mM, EDTA 0.1mM, and NaCl 50mM (TNE) for every 100 T175 flasks. Clean, sterile tubes were prepared with a sucrose gradient of 4 ml 20% sucrose in TNE layered on top of 2 ml 65% sucrose (Figure 2.1A). The resuspended VLPs were then layered on top of the gradient. The tubes were centrifuged in an SW28Ti rotor at 103,586 rcf for 6 hours at 4°C. The following day the VLPs were collected from the fluffy layer sandwiched between the 20% and 65% sucrose layers. The VLPs were thoroughly mixed with the residual sucrose collected with it, and enough 80% sucrose for the resulting mixture to have a final concentration of 60% sucrose. In another clean tube 1 ml 80% sucrose was added, with 4.5 ml of the 60% VLP layered on top. Another 3.5 ml 50% sucrose was then added on top of the VLP, and 10% sucrose was used to fill the remaining volume (Figure 2.1B). The tube was centrifuged at 209,491 rcf overnight at 4°C. The VLP separated into 2 fluffy layers, which were collected and mixed thoroughly with TNE buffer, added to a new centrifuge tube, then centrifuged at 209,491 rcf in order to pellet the concentrated VLP at the bottom of the tube. The supernatant was discarded and the purified VLPs were resuspended in TNE buffer and stored at -20°C.

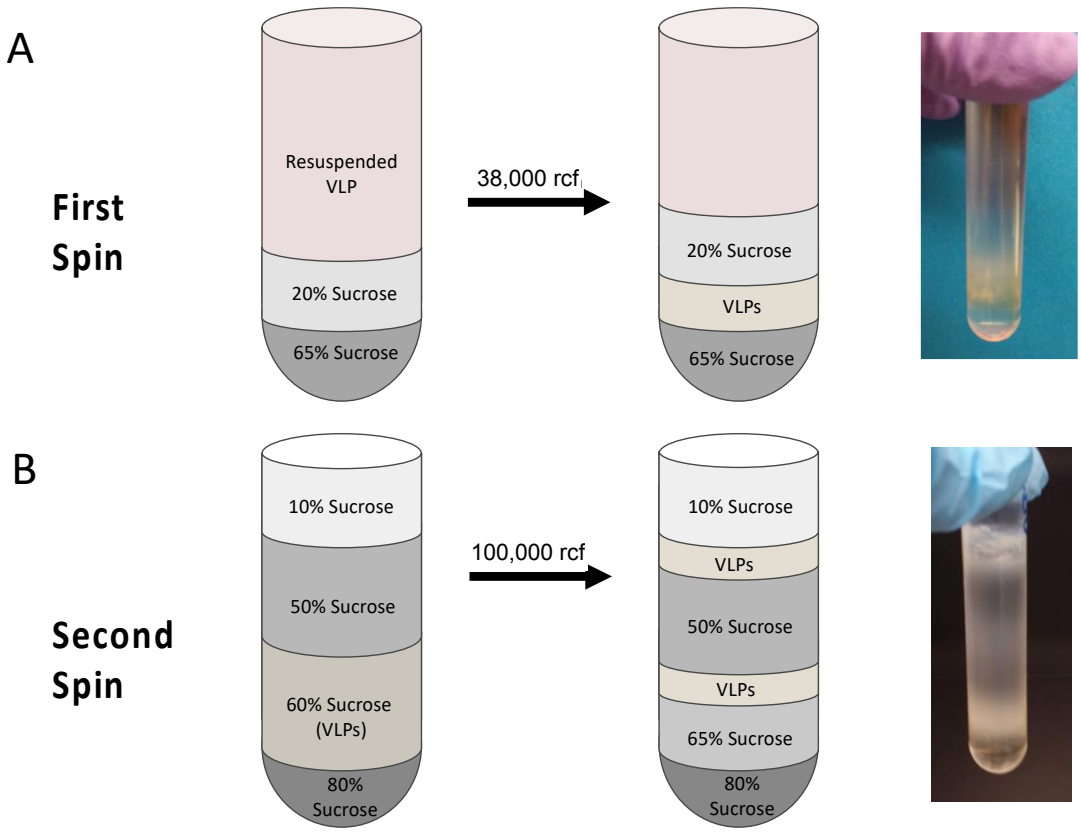


Figure 2.2: Sucrose gradient purification of the EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP. VLPs were purified by layering supernatant from transfected CHO cells with TNE buffer containing different concentrations of sucrose.

2.7. EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP Characterization

2.7.1. Determination of total protein concentration of VLPs and virus

The total protein of the EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP and UV-inactivated EBV, produced from EBV-eGFP UV-inactivated using the Stratalinker® UV Crosslinker (Stratagene, La Jolla, CA, USA) was quantified using a Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) per the manufacturer's instructions. A standard curve was made from a 1:2 serial dilution of purified BSA protein in TNE at an initial concentration of 40 µg/ml down to 0.5 µg/ml. EBV-LP and UV-EBV concentrations were initially checked by Nanodrop to determine the approximate dilution needed to have them within the range of the BSA standard curve, 1:20, 1:50, and 1:100 (EBV-LPs) and 1:200, 1:400, 1:800

(virus). A working reagent solution was prepared by mixing 25 parts of Micro BCA Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). Protein concentration was determined by adding 150 µl of all samples, standards, and blanks (TNE buffer) in triplicate to a 96-well, flat-bottomed, optically clear Nunc-Immuno Plate Maxisorp assay plate (44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA), 150 µl of working reagent was also added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered with sealing tape and incubated at 37°C for 2 hours. After incubation plates were cooled to room temperature and the absorbance read at 562 nm using a Filermax® F3 plate reader (Molecular Devices LLC, CA, USA). The replicates of all samples and standards were averaged. The background averaged from the blank wells was subtracted from the experimental samples and BSA standards, then the absorbance of each of the standards was plotted against the known BSA protein concentration. The resulting standard curve was used to calculate the protein concentration of the VLPs and virus, the concentration at each dilution was multiplied by the dilution factor to determine the concentration of the undiluted stock. For the VLPs, the 1:20 dilution was not used because it fell outside of the range of the standard curve.

2.7.2. Determination of gp350 concentration of VLPs and virus

Due to the VLPs and virus containing a number of proteins other than viral glycoproteins they were both further analyzed to determine the concentration of gp350 by ELISA; gp350 was the only EBV glycoprotein assessed by this method because it was the only purified protein available commercially with a known concentration to use as a standard. A 96-well Nunc-Immuno Plate Maxisorp microtiter plate (44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA) was coated with 50 µl serially diluted VLPs, UV-inactivated EBV, and known concentrations of soluble recombinant gp350 (IT-005-035p, Immune Technology Corp., NY, USA) in PBS, covered with sealing tape, and incubated overnight at 4°C. Each dilution was repeated in quadruplicate (Figure 2.1). The following day the liquid was discarded and plates were blocked by adding 100 µl PBS containing 2% BSA (blocking buffer) and incubated for 1 hour at room temperature on a plate

shaker. The plates were then washed 3 times with 200 µl of wash buffer containing 1% Tween-20 (P1379, Sigma Aldrich, St. Louis, MO, USA) in PBS for 5 minutes per wash while shaking. The wells were then rinsed briefly with 100 µl PBS and blotted on paper towels to remove any additional detergent. After washing, 50 µl/well of a single concentration (10 ng/ml) of anti-gp350 mAb 72A1 (ATCC, Manassas, VA, USA), was added to the first 3 wells of each replicate, reserving the final well as a blank control containing 50 µl of PBS. The plate was covered and incubated for 2 hours on a shaker at room temperature then washed 3 additional times following the incubation, and wells were rinsed with PBS before adding 50 µl of horseradish peroxidase (HRP)-labeled goat anti-mouse IgG secondary antibody (32430, Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:2,000 in PBS and incubated for 1 hour on a shaker at room temperature. The plate was washed once again 5 times with wash buffer, then rinsed with PBS and blotted on a paper towel. The plate was developed with 100 µl/well ABTS peroxidase substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (5120-0032, Sera Care, Milford, MA, USA), prepared by mixing equal parts ABTS solution A and ABTS solution B. The plate was mixed well, then incubated in the dark for 5 minutes at room temperature and the reaction was stopped by adding 100 µl/well 1X ABTS Stop solution (2% sodium dodecyl sulfate) (5150-0017, Sera Care, Milford, MA, USA), and mixing carefully so as not to create any bubbles. The plate was read on a Filermax® F3 plate reader (Molecular Devices LLC, San Jose, CA, USA) by measuring the absorbance at 405 nm. The replicates of all samples and standards were averaged, and the absorbance from the blank well corresponding to each coating condition was subtracted from the average. The resulting absorbance from the wells coated with known amounts of recombinant gp350 was plotted against the protein concentration to create a standard curve which was used to calculate the gp350 protein concentration of the VLPs and virus. The concentration at each dilution was multiplied by the dilution factor to find the concentration of the undiluted stock.

	1	2	3	4	5	6	7	8	9	10	11	12
	EBV gp350/220gBgp42gLgH VLP				EBV Virus				Recombinant gp350/220			
	Replicate #1	Replicate #2	Replicate #3	Blank	Replicate #1	Replicate #2	Replicate #3	Blank	Replicate #1	Replicate #2	Replicate #3	Blank
A	1:10				1:10				5.0µg/ml			
B	1:30				1:30				2.5µg/ml			
C	1:90				1:90				1.25µg/ml			
D	1:270				1:270				0.63µg/ml			
E	1:810				1:810				0.31µg/ml			
F	1:2430				1:2430				0.16µg/ml			
G	1:7290				1:7290				0.08µg/ml			
H	1:21870				1:21870				0.04µg/ml			

Figure 2.3: ELISA plate setup for EBV gp350 protein quantification in the EBV-LP and UV-inactivated EBV preparations used in animal immunizations.

2.7.3. Immunoblot analysis of VLP

To assess the protein composition of VLPs and stable cell lines, RIPA buffer lysates were boiled for 5 minutes in 4X Laemmli SDS-sample reducing buffer (BP-110R, Boston Bioproducts, Boston, MA, USA). CHO cells transiently transfected to express, gp350-gB-gp42-gL-gH-F, individual EBV glycoproteins, and un-transfected CHO WT cells were used as controls. PageRuler™ Plus (26620, Thermo Fisher Scientific, Waltham, MA, USA) was used as the ladder on all protein gels. The lysates were loaded onto a 4–12% polyacrylamide gel (NP0321PK2, Thermo Fisher Scientific, Waltham, MA, USA) for protein separation.

For immunoblot, proteins were transferred to a PVDF membrane using the iBlot™ 2 Dry Blotting System (Thermo Fisher Scientific, Waltham, MA, USA). The gel was removed from the casing and layered on top of the PVDF blotting membrane in the pre-made, single use iBlot transfer stack (Figure 2.2). A piece of filter paper was layered on top, followed by the top half of the transfer stack. Finally, the iBlot 2 absorbent pad with an electrical contact was placed on top of the assembly and the entire stack was placed in the iBlot 2 instrument and transfer of the proteins proceeded for 7 minutes. Following transfer the membrane was blocked with 1% BSA in Tris-buffered saline (TBS) buffer (blocking buffer) for 1 hour at room temperature on a shaker. After blocking,

proteins were detected with specific antibodies as described in Table 2.2 diluted in 1% BSA in TBS for 2 hours at room temperature on a shaker. The membrane was washed 3X with 0.1% Tween 20 in TBS (wash) buffer, for 5 min each wash. It was then incubated with the corresponding horseradish peroxidase (32430, Thermo Fisher Scientific, Waltham, MA, USA) conjugated secondary antibody diluted in blocking buffer for another hour at room temperature on a shaker. The membrane was then washed 5 times for 5 min with wash buffer under vigorous shaking, and rinsed once with TBS buffer alone. The blot was developed using the SuperSignal West Pico Plus substrate, as per manufacturer's instructions (34579, Thermo Fisher Scientific, Waltham, MA, USA).

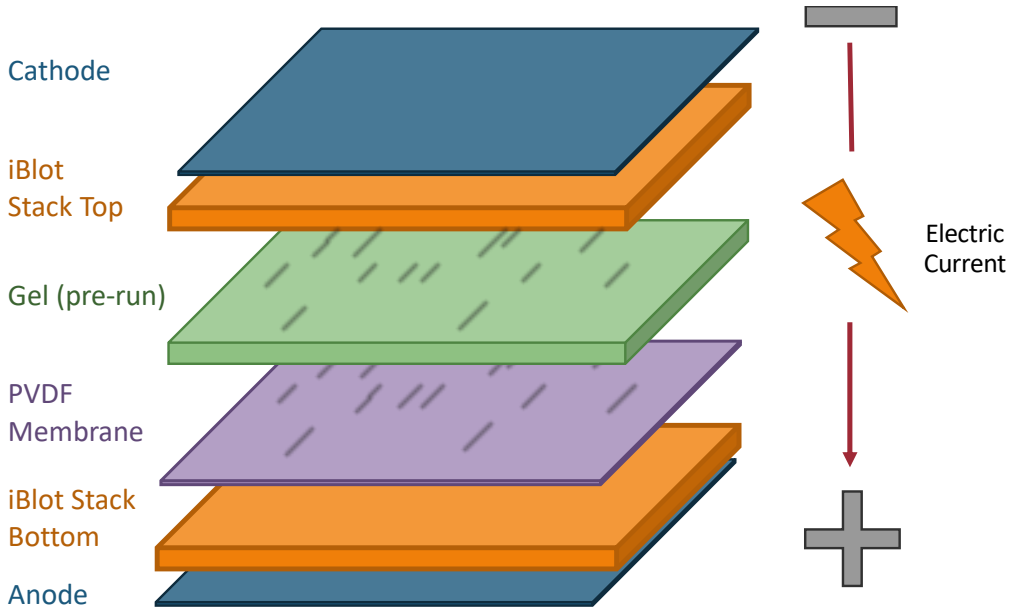


Figure 2.4: Schematic of iBlot™ 2 Transfer Stack showing the flow of current. The components of the iBlot™ 2 transfer stack for the iBlot™ 2 dry transfer device.

2.8. Immune Response in New Zealand White Rabbits

2.8.1. Immunization of rabbits

To test the ability of the EBV-VLP to elicit antibodies in a live animal model, New Zealand White rabbits at Pocono Rabbit Farm & Laboratory, Inc. (Canadensis, PA, USA) were immunized with 50 µg of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs, adjuvanted with 500 µg Alhydrogel adjuvant 2% Aluminum hydroxide gel

(vac-alu-250, InvivoGen, San Diego, CA, USA) mixed with 50 µg monophosphoryl Lipid A (MPLA) (L6895, MilliporeSigma, Burlington, MA, USA). All animal experiments were carried out at the Pocono Rabbit Farm & Laboratory facility by their staff according to the protocol provided to them that was developed as part of this project. Animal ethics clearance was obtained prior to starting the immunizations (Appendix A). Rabbits were divided into four groups of six animals each (n=6) and immunized with: 50 µg gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs (group 1), 50 µg UV-inactivated EBV-eGFP virus (group 2), 25 µg soluble gp350 (group 3) clinical trial vaccine candidate, and TNE buffer (group 4) as a negative control. Dosing was determined based on previous mouse experiments (78) and advice from the Pocono staff. Animals were immunized at day 0 and boosted twice at days 28 and 42. Animals were bled from the ear prior to the first immunization, then at days 14, 35, 49 and 70. A total of 8 mL of blood from each animal was collected at all timepoints except for days 49 and 90, when 15 mL of blood was collected. The final blood sample was taken 90 days after the first immunization, before animals were humanely euthanized by injection of an overdose of anesthesia, ketamine (35 mg/kg) and xyalzine (5 mg/kg) were administered intramuscularly, followed by exsanguination (Figure 2.3).

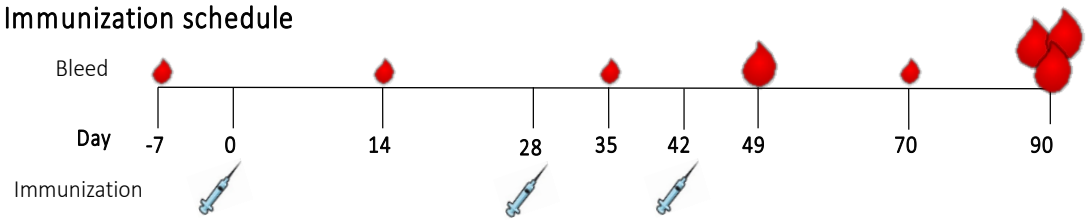


Figure 2.5: Rabbit immunization and bleeding schedule. Rabbits received their first immunization on day 0 followed by were boosts on days 28 and 42. Animals were bled prior to the first immunization, then again on days 14, 35, 49, and 70. The terminal bleed was 90 days after the initial immunization.

2.8.2. Antibody titers in serum from immunized animals

ELISA was used to measure the antibody IgG titers as described previously. Plates were coated with soluble gp350, gH, gL, gp42, and gB as target antigens in order to measure and compare antibody titers to each of the different EBV glycoproteins. Purified soluble gp350 ED (aa 4–863, GenBank: AHA36359) was purchased from Immune Technology Corp, NY, USA, The remaining purified proteins, gH (aa 19–679, GenBank: AFY97969.1), gL (aa 24–137 GenBank: AFY97944.1), gp42 (aa

33–223, GenBank: AFY97939.1), and gB (aa 23–683, Genbank: AFY97983.1), were provided by Dr. Andrew McGuire (Fred Hutchinson Cancer Research Center, WA, USA). The gB protein had further modifications. Residues $WY^{112–113}$ were mutated to HR and $WLIW^{193–196}$ were mutated to RVEA to prevent the formation of rosettes during protein production (109).

First, proteins were diluted to a concentration of 1 µg/ml in PBS and 50 µl was added to each well of a 96-well microtiter plate for a total of 50 ng/well. The plates were sealed and incubated overnight at 4°C. The next day the liquid was discarded and wells were blocked with 1% BSA in PBS. Whole blood from each individual immunized rabbits was centrifuged at 2,000 x g for 15 minutes to separate serum from red blood cells. The serum was then diluted in PBS 1:100, 1:300, and 1:900 then added to the plate with 4 replicates of each sample and dilution. The plate was incubated for 2 hours at room temperature with gentle shaking, then the plate was washed 3 times. Antibody binding was detected with HRP-labeled anti-rabbit secondary antibody (1:1000) after incubation for 1 hour. Plates were washed 5 times and ABTS substrate was added. The reactions were stopped with ABTS stop solution. To determine antibody titer, optical density (OD) was read at 405 nm with a Filermax® F3 plate reader (Molecular Devices LLC, San Jose, CA, USA).

2.8.3. Neutralization of EBV-eGFP with serum from immunized rabbits

Rabbit serum collected on day 49, one week after the third immunization when titers should theoretically be the highest, was used to determine neutralizing antibody titers. The serum was heat inactivated by incubating at 56°C for 30 minutes. Equal volumes of serum from each of the six animals in each group was pooled together and vortexed briefly to mix. The pooled samples were diluted with serum-free Opti-MEM media at dilutions of 1:20, 1:40, 1:80, 1:160, and 1:320. The diluted serum was incubated in quadruplicate with an equal volume of EBV-eGFP virus, prepared as described previously, for 1 hour at 37°C. The virus:serum mixture was then added to HEK-293T (epithelial) cells that were seeded in a 48-well plate in serum-free Opti-MEM media the previous day at a concentration of $5x10^4$ cells per well. Plates were incubated for 1 hour at 37°C. The cells were then washed, and given 500 µl fresh complete DMEM media containing 10% FBS and

incubated at 37°C with 5% CO_2 for 48 hours. After 2 days the cells were transferred from the plate to tubes and washed 3 times with PBS by centrifugation at 200 x g for 5 min to deplete any dead cells. The cells were counted and determined to have >95% viability, then fixed with 4% paraformaldehyde for 10 minutes on ice, protected from light. The samples were stored at 4°C until analysis by flow cytometry. Uninfected cells, and cells incubated with virus without serum were used as negative and positive controls.

Neutralization was defined as the percent different in the infectivity of the virus alone and the virus incubated with serum using the equation:

$$\%\ Neutralization = \frac{(I_V - I_S)}{I_V}$$

Where I_V is the average percent of infected cells in wells incubated with virus alone, and I_S is the average percent of infected cells in wells treated with serum from immunized animals.

2.9. Statistical Analysis

Samples were run in quadruplicate for both ELISA and neutralization assays. All data were expressed as mean ± standard deviation (SD) for replicates. The difference between neutralizing antibody titers of animals immunized with EBV-LP and control animals immunized with TNE buffer, soluble gp350, UV-EBV, and KSHV-LP was determined using the unpaired two-tailed t-test for independent groups. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, San Diego, California USA, www.graphpad.com).

Chapter 3 Results

3.1. Design of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP Plasmid Constructs

Previously the majority of EBV vaccine designs have focused on EBV gp350 as the primary immunogen as it has been shown to induce the highest antibody titers following natural infection. However, recent studies have shown that additional EBV glycoproteins involved in viral attachment and entry such as gH/gL, gB and gp42 are able to elicit neutralizing antibodies and should be considered as possible candidates for EBV vaccine development.

The multivalent plasmid construct used in this study was designed to ensure that all of the five viral glycoproteins known to be involved in viral entry were expressed on the VLP as it would have been presented on the wild type virus. Proteins were expressed using a single transcript to ensure that all the proteins were present in equal amount, increasing the probability that the gH/gL and gp42 complex was formed and the likelihood that the VLP will induce antibodies to each of the proteins. The glycoproteins that are naturally anchored to the surface of the virus via a transmembrane domain, gp350, gH, and gB were fused to the NDV-F protein, anchoring them to the surface of the VLP in order to recapitulate their native conformation. In contrast, gL and gp42 do not naturally have a transmembrane domain, so these proteins were expressed in their wild type (WT) form (Figure 3.1).

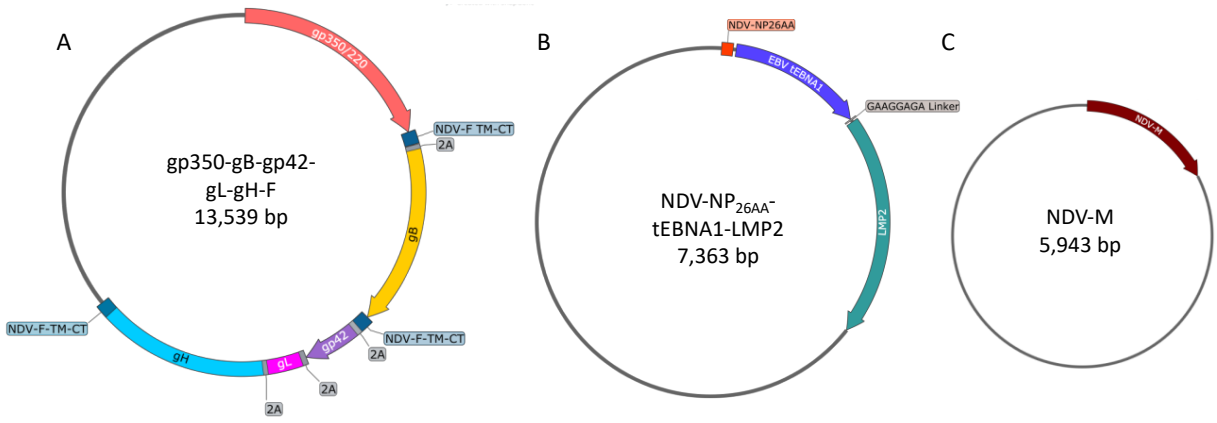


Figure 3.1: Plasmid constructs used in VLP production. Map of the three plasmids used for VLP production: gp350-gB-gp42-gL-gH-F (A), NDV-NP_{26AA}-tEBNA1-LMP2 (B), and NDV-M (C).

In addition to the EBV surface glycoproteins discussed in this study, the EBV-LP was designed to package the latent EBV antigens EBNA1 and LMP2 inside. Both latent antigens have been shown to generate antigen-specific T cell responses (98,110,111). Inclusion of these latent antigens in the EBV-LP design could offer an additional layer of protection and opens up the potential for therapeutic use in already infected or at risk individuals. The scope of this study does not include the characterization of these T cell responses, but rather focuses on the production and characterization of a novel, multivalent prophylactic vaccine.

3.2. Production and purification of EBV-eGFP

3.2.1. Induction of AGS-EBV-eGFP cells

The virus used in this study was produced by induction of AGS-EBV-eGFP cells, an epithelial gastric adenocarcinoma cell line harboring a recombinant Akata strain of EBV engineered to express eGFP and with a G418 (neomycin) resistance gene (112). Following treatment with TPA and NaBut, the cells showed an increase in GFP expression, indicating induction of AGS-EBV-eGFP lytic activation.

3.2.2. Optimization of B95-8/F EBV-eGFP 293 transfection

As an alternative method of virus production B95-8/F EBV-eGFP 293 cells were transfected with p509 (BZLF1), p2670 (BALF4), and p2130 (BRLF1) to induce lytic activation and release of the virus. Three different transfection reagents were tested, each with six variations of DNA to transfection reagent. Induction of lytic replication was measured by eGFP expression 48 hours post-transfection. Cells transfected using Metafectene reagent ranged from 0.81% eGFP-positive in cells transfected with 1 μg DNA and 2 μl Metafectene, but increased to 57.4% using 2.5 μg DNA and 12.5 μl Metafectene (Figure 3.2A). The percent of eGFP-positive cells in cultures transfected with Lipofectamine 3000 had less variation, the lowest being 42.6% in using 5 μg DNA, 3.75 μl Lipofectamine 3000, and 5 μl P300 reagent. The highest was 61.1% positive in cells transfected with 5 μg DNA, 7.5 μl Lipofectamine 3000, and 5 μl P300 reagent (Figure 3.2B). Finally PEI ranged from 14.2% in cells

transfected with 2.5 μg DNA and 5 μl PEI, but increased to 51.2% using 2.5 μg DNA and 12.5 μl PEI (Figure 3.2C).

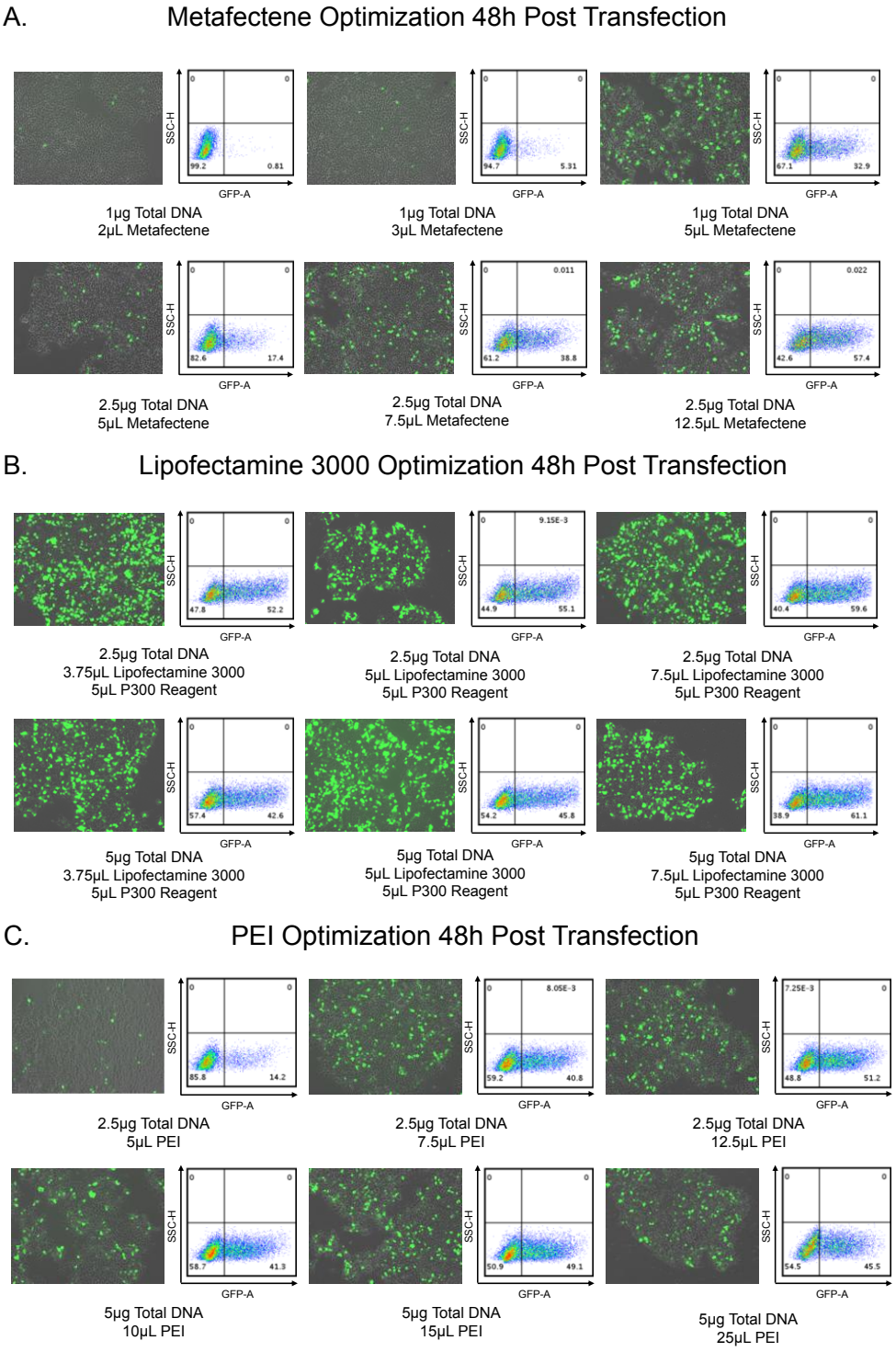


Figure 3.2: Optimization of B95-8/F EBV-eGFP 293 cells for virus production. Three transfection reagents Metafectene (A), Lipofectamine 3000 (B), and PEI (C) were used, the amount of DNA and transfection reagent for each condition is listed below each image. B95-8/F EBV-eGFP 293 cells (40X) and the accompanying flow cytometry plot showing eGFP (x-axis) and SSC-H (y-axis) 48 hours after transfection of p509 (BZLF1), p2670 (BALF4), and p2130 (BRLF1).

3.2.3. EBV-eGFP virus isolation and total protein quantification

The cells were induced, and the released virus was isolated from the supernatant by centrifugation. The resulting pelleted virus was resuspended in approximately 25-50 μL of TNE buffer per T75 flask, based on a visual inspection of the pellet size. The total protein concentration was determined by using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed using the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Prior to this study virus preparations had high titers, but this decreased significantly in subsequent preparations, resulting in difficulty obtaining enough virus to preform neutralization assays.

Because the virus preparations contained proteins other than the surface glycoproteins of interest the virus was further analyzed by ELISA to determine the concentration of gp350 in order to normalize the amount of immunogen delivered in subsequent animal experiments. The virus used for rabbit immunizations was found to have a total protein concentration of 4.87 mg/mL and 57.8 μg/mL gp350 protein, or 1.19% of the total viral protein content was attributed to gp350.

3.2.4. EBV-eGFP infectivity of Raji and HEK-293T cells

EBV is known to mainly infect epithelial and B cells, and previous studies have indicated that virus produced in diverse cell types have variable affinities for different cells (113). The infectivity of the EBV-eGFP virus was assessed for both cells (HEK-293T) by incubating different amounts of the purified virus with pooled serum from rabbits immunized with TNE, EBV-LP, gp350, UV-EBV, or KSHV-LP then evaluating the number of cells positive for eGFP after 48 hours. Prior to this study virus preparations showed high infectivity, with less than 10 μL of virus infecting over 50% of HEK-293T and Raji cells. However, later preparations showed greatly diminished infectivity, with 100 μL of virus infecting less than 7% of HEK-293T cells (Figure 3.3). Due to the very low infectivity of the virus, it was not tested in Raji cells, in order to conserve enough volume of virus for the neutralization assay.

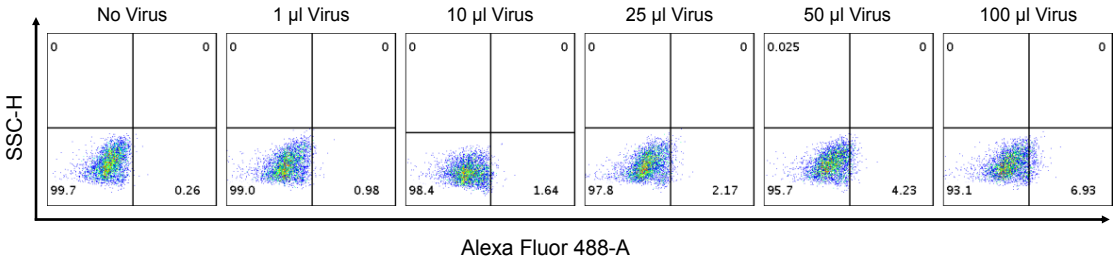


Figure 3.3: Infectivity of EBV-eGFP in HEK-293T cells. Virus isolated from supernatant collected for 5 days after AGS-EBV-eGFP cells were induced with TPA and NaBut. HEK293T cells 48 hours after infection with 100 µl of virus were 6.93% eGFP-positive.

3.3. Production and characterization of EBV VLPs

3.3.1. Generation of stable CHO cell line

In this study we have designed a novel multivalent VLP-based vaccine that incorporates the five glycoproteins, gp350, gB, gp42 and gH/gL implicated in viral entry and infection. Initially, to produce the VLPs, CHO cells were transiently co-transfected with pCAGGS EBV gp350-gB-gp42-gL-gH-F, pCAGGS NDV M, and pCAGGS NDV NP_{26AA}-tEBNA1-LMP2 plasmids. To determine the approximate transfection efficiency, a small sample of the transiently transfected cells were stained with anti-gp350 primary antibody 72A1, followed by an anti-mouse IgG Alexa Fluor 488 secondary antibody and analyzed by flow cytometry. These cells were determined to only be 2.32% gp350 positive (Figure 3.4), possibly due to the large plasmid size, accounting for the low initial yields of VLP production.

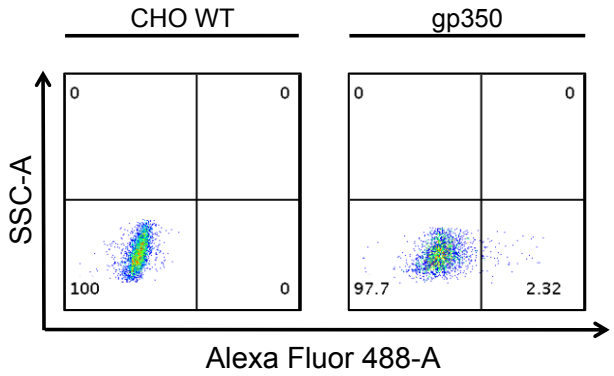


Figure 3.4: Low expression of gp350 in CHO cells transfected with pCAGGS-EBV-gp350-gB-gp42-gL-gH-F. CHO cells co-transfected with pCAGGS-EBV-gp350-gB-gp42-gL-gH-F, pCAGGS-NDV-NP_{26AA}-tEBNA1-LPM2 and pCAGGS-NDV-M.

In an effort to overcome this poor transfection efficiency and increase VLP production, stable cells expressing gp350-gB-gp42-gL-gH-F were generated by co-transfecting pCAGGS EBV gp350-gB-gp42-gL-gH-F with pCI-puro as a selection marker. The cells were cultured in selection media containing puromycin and expanded until they reached 90% confluency in a T75 flask. At this point the cells were stained for gp350 expression and sorted by flow cytometry. Transfected CHO cells rapidly increased the proportion of positive cells following sorting and puromycin selection (Figure 3.5).

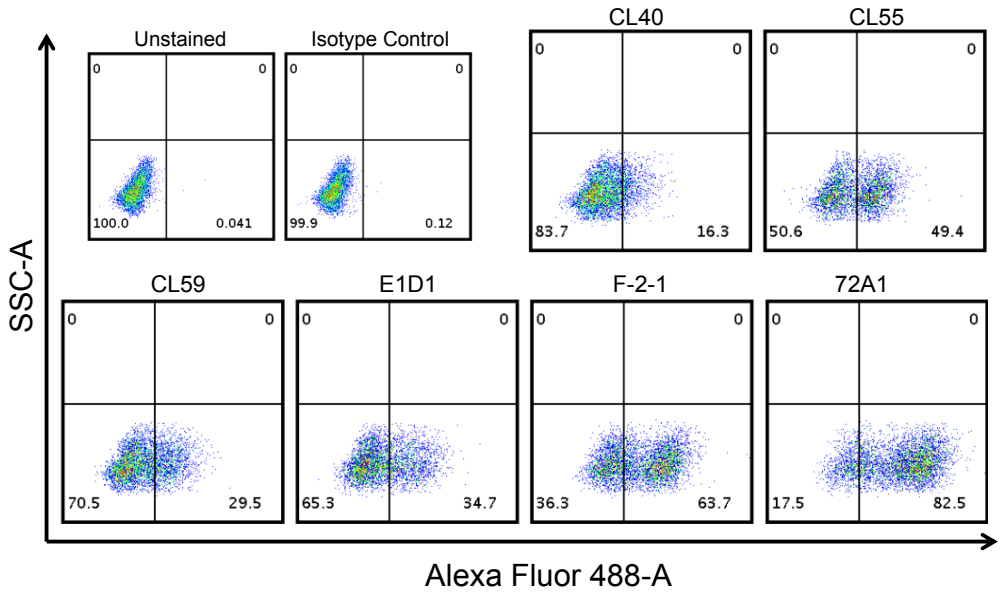


Figure 3.5: Generation of a stable CHO cell line. Transfected CHO cell line expressing pCAGGS-EBV-gp350-gB-gp42-gL-gH-F following two rounds of sorting for gp350-positive cells and puromycin selection. Cells were stained with antibodies directed against EBV glycoproteins: gH/gL (CL40), gB (CL55), gH/gL (CL59), gL (E1D1), gp42 (F-2-1) and gp350 (72A1). Cells were then stained with HRP-labeled goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by flow cytometry.

The EBV gp350 positive cell populations increased progressively with each sort in both HEK-293T and CHO cell lines, however HEK-293T cells showed increased sensitivity to puromycin and required a lower concentration, this reduced selective pressure likely caused the percent of positive cells to diminish over several passages (Figure 3.6). From this point onwards, only CHO stable cells were maintained and expanded. Once the cells were greater than 90% positive for gp350 following multiple passages and continuous culturing in puromycin culture media, they were stained with EBV glycoprotein specific primary antibodies for gH, gL, gB, gp42, and the gH/gL complex and determined to be >90% positive for all 5 EBV

glycoproteins by flow cytometry. These stable CHO cells were frozen and stored in liquid nitrogen for later use in VLP production. Flow cytometry confirmed that they maintained the same stable expression of gp350-gB-gp42-gL-gH-F following thawing and expansion of the banked cells.

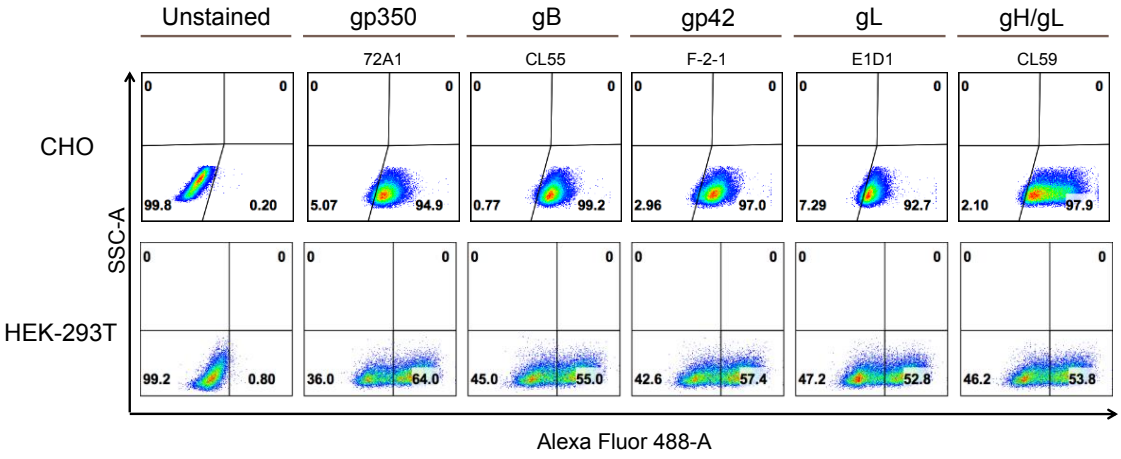


Figure 3.6: Stability of EBV gp350-gB-gp42-gL-gH-F expression in CHO and HEK-293T cell lines. After multiple passages in selection media without sorting cells were stained with antibodies for gp350 (72A1), gB (CL55), gp42 (F-2-1), gL (E1D1) and gH/gL (CL59), followed by Alexa Fluor 488-labeled goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by flow cytometry to determine the number of cells continuing to express each of the EBV glycoproteins.

3.3.2. VLP production using stable EBV gp350-gB-gp42-gL-gH-F CHO cell line

The total protein concentration of the VLP was determined in the same way as the virus, using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed using the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A total of five rounds of transfection of 125-150 T175 flasks of stable CHO cells was required to produce enough EBV-LP for animal experiments and characterization, as each round of transfection yielded 160-230 μg. The final protein concentration of the VLP was 1.68 mg/mL. The gp350 protein concentration was also determined in the same way as the virus, using an ELISA to compare known concentrations of soluble gp350 to dilutions of the VLP. The concentration of gp350 in the VLP was calculated to be 201.3 μg/mL, or 12.0% of the total protein content was attributed to gp350 (results not shown).

3.3.3. EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP protein expression

Where antibodies were available, the EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs were analyzed by western blot to confirm the presence of EBV surface glycoproteins and latent antigens, both in the VLPs or cells transfected with the individual plasmids used in VLP production. Individual surface glycoproteins were verified by western blot using gp350-specific antibody 72A1 (Figure 3.7A). Detection of gH (Figure 3.7B) was accomplished using serum from rabbits immunized with EBV gH provided by Dr. Richard Longnecker.
Rabbit polyclonal anti-2A antibody was used to detect the 2A cleavage signal peptide attached to the end of gp350, gB, gp42, and gL. Glycoprotein gH could not be detected with the anti-2A antibody because cleavage occurs at the c-terminal end, resulting in the peptide remaining attached to the upstream protein, in this instance gL. The use of this antibody resulted in non-specific binding to CHO WT, CHO gp350-gB-gp42-gL-gH, and EBV-LP lysate. The amount of lysate loaded was reduced to try to eliminate these bands, and the concentrations of the primary and secondary antibodies were adjusted to 1:2,000 and 1:10,000 respectively (Figure 3.7C).

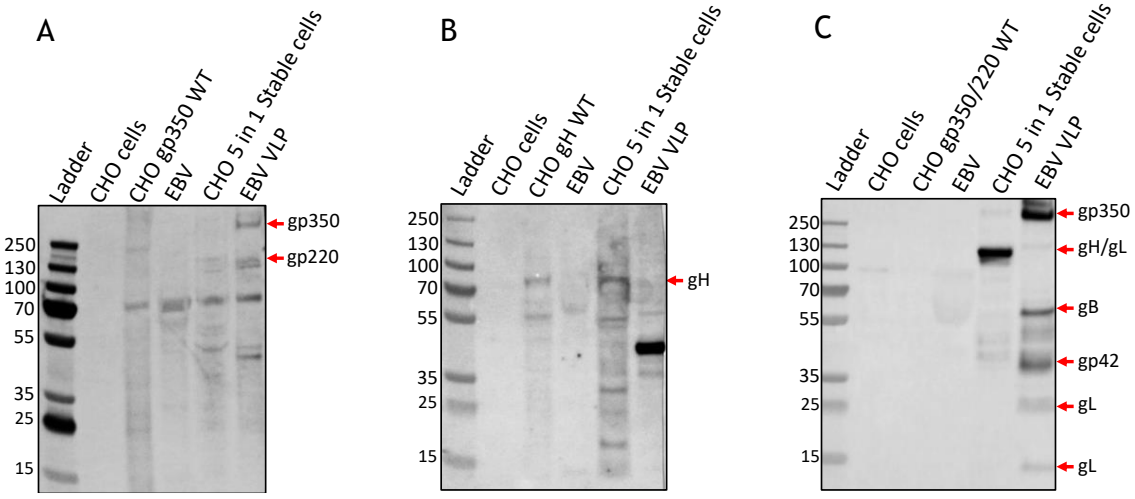


Figure 3.7: Immunoblot analysis of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs. Cell virus and VLP lysates were analysed by Western blot. Proteins were detected using antibodies specific for gp350 (A), gB (B), and 2A peptide (C) and anti-mouse HRP-labeled secondary antibody. PageRuler™ Plus (Thermo Fisher Scientific, Waltham, MA, USA) was used as the ladder. The molecular weight (kDa) of each band is indicated to the left. Blots were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate, an enhanced chemiluminescent (ECL).

3.3.4. Serum antibody titers from rabbits immunized with EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs

To establish the ability of the EBV-LPs to induce an immune response, four groups of six (n=6) New Zealand white rabbits received a primary immunization intramuscularly at Day 0 and boosts on Days 21 and 42. The rabbits showed no signs of adverse reactions to the vaccine, including local or systemic inflammation or changes in behavior during the study.

Production of antibodies specific to gp350, gB, gp42, and gH/gL was assessed by ELISA to determine the antibody titers induced by immunization with EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs. Recombinant EBV glycoproteins gp350, gB, gp42 and gH/gL were used as the target antigens for the ELISAs, eliminating the measurement of potential antibodies directed against cellular or structural components.

Antibody titers directed against gp350/220 increased in animals immunized with EBV-LP, UV-inactivated EBV (UV-EBV), and soluble gp350 above the initial pre-bleed titers, and compared to the TNE immunized animals (Figure 3.8A). In the EBV-LP and gp350 groups titers increased after the first two immunizations then decreased slightly on days 49 and 70. While remaining higher than pre-bleed and TNE controls, the gp350 group titers remained stable at day 90, the EBV-LP group showed a slight increase. The gp350-specific titers in the UV-EBV group increased at day 35, then dropped at day 14. Following the second boost, titers increased slightly at day 49 then gradually decreased on days 70 and 90. Antibody titers specific to gH/gL (Figure 3.8B) were highest in the UV-EBV group, increasing after the first immunization at day 14 and remaining stable for day 35, then increasing significantly at day 49 and 70 before waning slightly at day 90. In the VLP group the titers followed a similar pattern albeit with lower titers, falling close to the levels of the TNE and gp350 groups which remained near baseline as expected. The UV-EBV group also had the highest gp42 titers (Figure 3.8C). Although the titers were overall lower compared to the other antigens, both the UV-EBV and EBV-LP group were perceptibly higher than pre-bleed and TNE and gp350 control groups. However, due to the variations within each group, this observation was not statistically significant. Finally, titers specific to gB (Figure 3.8D) were highest in the

VLP group, followed by UV-EBV. The UV-EBV group again showed a decrease at day 35, and previously high titers dropped back to baseline levels by day 90. Though the titers varied between antigens, all animals immunized with UV-EBV and EBV-LP did show a response to each of the proteins expressed on the EBV-LP.

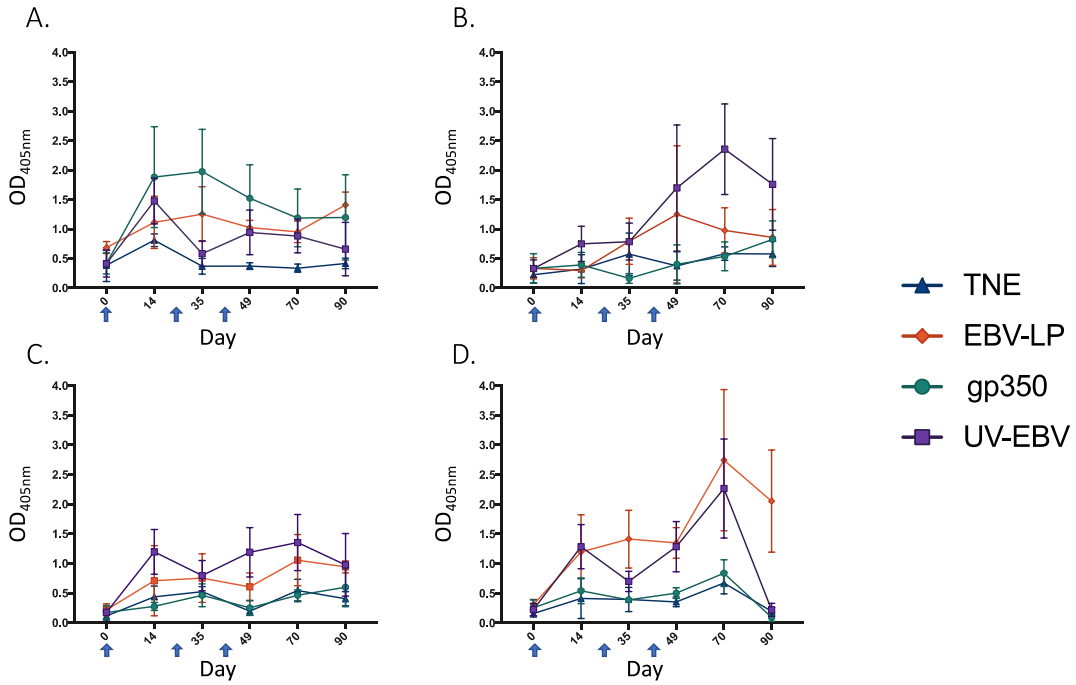


Figure 3.8: EBV-specific antibody titers in rabbits, as determined by ELISA. Plates coated with purified proteins gp350 (A), gH/gL (B), gp42 (C), and gB (D) to detect antibodies specific to these proteins in the serum of New Zealand white rabbits with HRP-labeled anti-rabbit secondary antibody (1:1,000). Four groups of six animals (n=6) were immunized with TNE buffer, EBV-LPs, soluble gp350, or UV-EBV. Animals were bled on days 0, 14, 35, 49, 70, and 90. Blue arrows indicate days of primary immunization and boosts. Plates were developed using ABTS and read at 405nm. Data points represent the average antibody titer of all animals within a group, with the SD within the group indicated by the error bars.

3.3.5. Neutralizing antibody titers in rabbits

The neutralizing antibody activity in rabbits immunized with the multivalent VLPs as well as the controls TNE, EBV-LP, gp350, UV-inactivated EBV, and KSHV-LP were determined (Figure 3.9). The KSHV-LP is a multivalent VLP expressing KSHV-specific surface glycoproteins K8.1, gB, gH. The KSHV-LP was developed in parallel to the EBV-LP in the same lab. Similarly to the EBV-LP, the KSHV-LP uses a stable CHO cell line expressing KSHV K8.1-gB-gL-gH-F transfected with NDV-M and NDV-NP_{26AA}-LANA1, where LANA1 is a latent KSHV antigen. Sera from

animals in each group was pooled from day 49, the timepoint following the second boost, and used to conduct neutralization assays *in vitro* against EBV-eGFP virus in HEK-293T (epithelial) cells. The percent of $eGFP^+$ cells was determined by flow cytometry and 50% neutralization of infection was described as effective neutralization activity. Pooled serum diluted in Opti-MEM medium (1:20, 1:40, 1:80, 1:160 and 1:320) from all four treatment groups and rabbits immunized with a multivalent KSHV-LP which served as an additional negative control was pre-incubated with 50 ul of EBV-eGFP virus then added to HEK-293T cells. In HEK-293T cells incubated with EBV-eGFP virus alone 5.2% of cells were positive for eGFP, indicating that they were infected, with a standard deviation of 0.1% (Figure 3.9).

At the highest concentration of serum (1:20) the TNE group infected only 3.7% of cells, reducing infection by 29.1%. At this same 1:20 dilution the EBV-LP group showed only 0.2%, gp350 1.4%, UV-inactivated EBV 1.2%, and KSHV-LP 3.3%. Lower concentrations of serum reduced infection progressively less, with the EBV-LP serum showing effective neutralizing activity at the 1:80 dilution, with 70.1% of the virus being neutralized. The highest dilution at which the gp350 and UV-EBV serum both showed effective neutralizing activity (preventing at least 50% of infection) was the 1:40 dilution, with percent neutralization of 58.3% and 64.9% respectively compared to 90.8% neutralization in the EBV-LP group at this dilution.

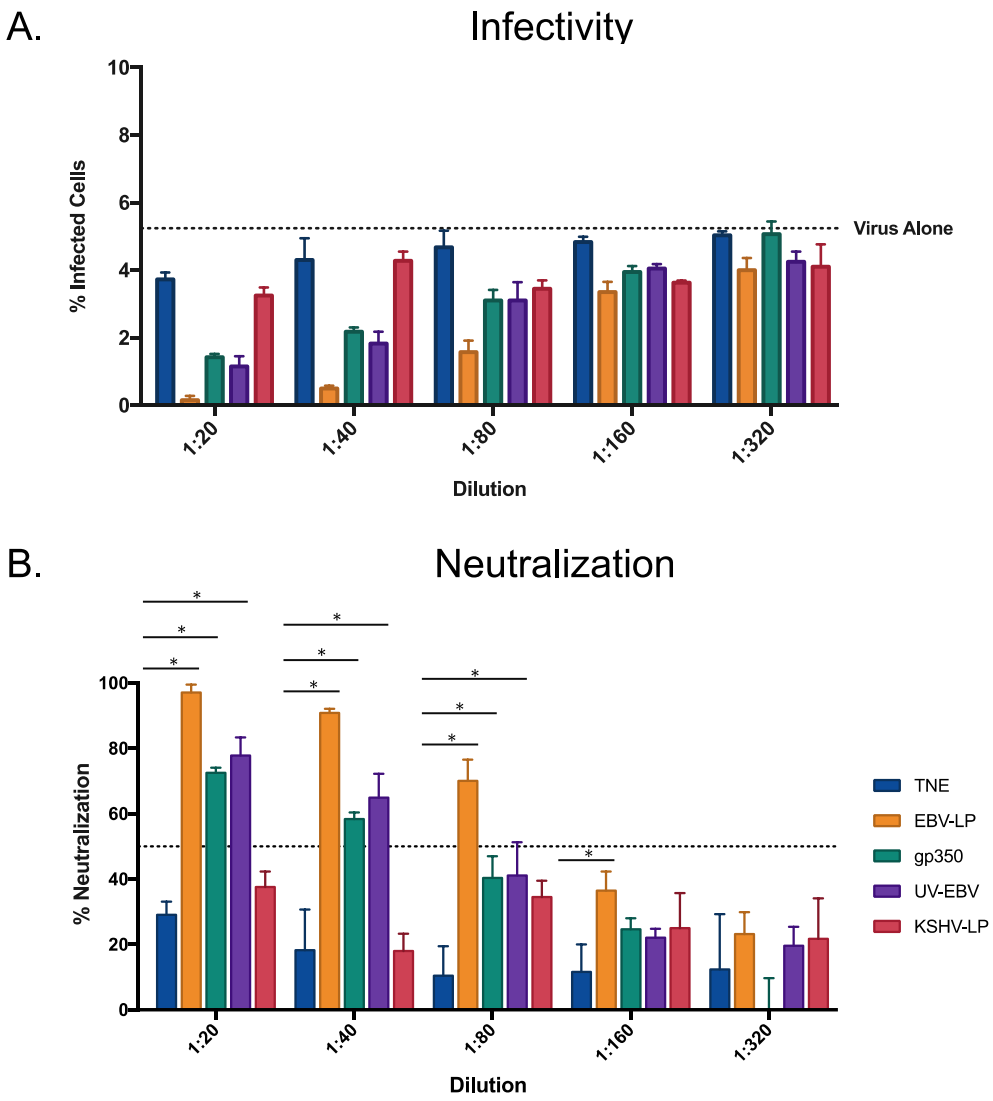


Figure 3.9: Neutralizing antibody titers in rabbits. Pooled serum from animals immunized with TNE, EBV-LP, gp350, UV-inactivated EBV, or KSHV VLP was diluted in serum-free media and incubated with EBV-eGFP for 1 hour at 37°C before the mixture was added to HEK-293T cells. Infectivity was measured by eGFP expression after 48 hours. Dotted line indicates percent of cells infected when cells were incubated with virus alone (A) and 50% neutralization (B). Statistically significant differences with was based on two-sided p-values ≤0.05 are indicated (*) as determined by unpaired two-tailed t test for independent groups.

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To determine if the differences in percent neutralization was statistically significant a two-tailed, nonparametric t-test was used to compare each immunogen as a whole over all serum dilutions to TNE. Only the EBV-LP and UV-EBV groups were significantly different from TNE by this method, with p-values of 0.0159 and 0.0317, respectively. Additionally, when each serum dilution was considered individually EBV-LP was significantly different from TNE at dilutions 1:20, 1:40, 1:80, and 1:160, while soluble gp350 and UV-EBV were significantly different from TNE at dilutions 1:20, 1:40, and 1:80.

Chapter 4 Discussion

Despite being the first virus shown to cause cancer in humans, an effective vaccine for EBV has evaded researchers for over 50 years. This failure is possibly due to the majority of prophylactic vaccines designs focusing exclusively on gp350. Although gp350 is the most abundant protein expressed on the surface of EBV, it is not necessary for infection (57). Studies of the other glycoproteins found on the surface of EBV have led to a better understanding of the mechanism of entry and the glycoproteins involved (62,114). The complexes of gH/gL and gH/gL/gp42 have been shown to determine the tropism of the virus for epithelial and B cells respectively (114), and neutralizing antibodies directed at these proteins can block infection into both cell types (115). Another study isolated antibodies from infected subjects, including several anti-gB antibodies which were determined to neutralize epithelial cell infection (116).

Oncogenic pathogens are responsible for a significant portion of cancer cases globally every year, resulting in a huge health burden and draining resources, particularly in developing countries. An effective vaccine providing protection against these pathogens can greatly reduce the cancer burden in many countries around the world. Recently it has been shown that since the implementation of a vaccine program for HPV in 2006, the number of HPV16/18 CIN2+ cases has steadily declined in the United States (117). HPV is responsible for 99% of cervical cancer cases, and 70% of cases are caused by strains 16 and 18. The original vaccine formulation protected against these strains, and strains 6 and 11, which cause genital warts (118,119). In 2014 a new version of the vaccine was introduced protecting against nine strains of the virus giving it the potential to prevent 90% of all cervical cancer (120).

This dissertation describes the design, production, and characterization of a VLP-based multimeric EBV vaccine expressing the viral glycoproteins gp350, gB, and the complexes gH/gL and gH/gL/gp42, the characterization of this vaccine and its potential pre-clinical efficacy.

4.1. Design and production of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs

4.1.1. Selection of EBV glycoproteins and plasmid design

Although immunization with gp350 has been shown to produce neutralizing antibodies, no gp350-based vaccine has been successful at preventing EBV infection in human clinical trials (70,71,121). Other vaccine strategies directed against latent EBV antigens depend on the immune system recognizing and clearing cells after EBV has established infection (114). Researchers have gained a better understanding of the mechanism of EBV infection and the proteins involved (122), lending insights to alterative vaccine targets.

This study explored the potential use of other glycoproteins expressed on the surface of EBV that studies have shown to be necessary for entry into host cells. These proteins, gB, gH, gL, and gp42, in addition to gp350, were expressed from a single transcript to minimize the chances of differences in protein expression due to varying transfection efficiency. To ensure the proteins were each processed separately the plasmid sequence was designed with a 2A cleavage peptide between the individual viral protein sequences (91,123) Expressing the proteins in equimolar amounts also increases the likelihood that the natural complexes of gH/gL and gH/gL/gp42 will form on the surface of the VLP similarly to WT EBV.

4.1.2. EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLP production

The large size of the plasmid encoding the EBV proteins initially resulted in very low transfection efficiency and a low VLP yield. The creation of a stable EBV gp350-gB-gp42-gL-gH CHO cell line increased yields, as the cells only required transfection with the smaller NDV-NP_{26AA}-tEBNA1-LMP2 and NDV-M plasmids to initiate VLP production. The cell line also enabled the use of flow cytometry to confirm the expression of the all of the 5 viral glycoproteins at the cell level. The binding of gp42-specific antibody F-2-1 and the gH/gL complex-specific E1D1 antibody strongly indicated that the gH/gL and gH/gL/gp42 complexes were being formed and expressed on the surface of the stable cell line, as gL and gp42 have no transmembrane domain anchoring them to the cell (124,125).

Although the generation of stable cells increased the efficiency of VLP production, the process is still labor and resource intensive. Several rounds of transfection and purification were required to obtain the amount of VLP required for this study. For each transfection the stable cells had to be expanded to a large number of flasks while maintaining robust growth, a process requiring several days and huge amounts of medium. Further work will need to be done to continue to increase the efficiency of this process before it is feasible for large-scale production, as would be required for a licensed vaccine.

4.2. Characterization of EBV gp350-gB-gp42-gL-gH-EBNA1-LMP2 VLPs

4.2.1. Quantification of VLP protein content

The BCA protein quantification assay, a widely accepted method of protein quantification, was used to determine the concentration of both the EBV-LP and UV-inactivated EBV preparations used for rabbit immunizations. To gain a better understanding of the amount of immunogen being delivered in the EBV-LP and UV-EBV immunizations In the VLP preparation, gp350 accounted for 11.99% of the total protein content, as compared to 1.19% in the virus. Although no previous comparison of the total protein to gp350 protein in the virus has been published, these numbers are reasonable as the virus is significantly larger, with a greater number of proteins than the EBV-LP. The other viral glycoprotein concentrations were not measured due to the lack of available antibodies and purified proteins to serve as a standard at the time of the assay.

Liu *et al.* (126) found that expression progressively decreased in downstream proteins. In their study, HEK-293T cells were transfected with single DNA sequences encoding four protein transcripts separated by porcine 2A-peptides. When tdTomato (Td) was positioned as the first protein in the transcript, approximately 30% of the transfected cells were Td-positive, with a mean fluorescent intensity (MFI) of approximately 0.8, When Td was the fourth protein in the sequence expression was reduced to approximately half of those values (126). Without having measured the amount of gB, gH, gL, and gp42 in the EBV-LP we

cannot rule out the possibility that protein expression was reduced for these downstream proteins.

4.2.2. Confirmation of individual viral glycoprotein expression

Due to the small size of the VLP, flow cytometry could not be used for verification of the individual surface proteins. Protein expression was instead demonstrated by immunoblot for gp350, gB, gH, and the 2A peptide. The lack of reliable and readily available antibodies for western blot prevented detection of gL and gp42. All of the antibodies used in western blots showed some degree of nonspecific binding, making it difficult to conclusively determine if the specific EBV glycoprotein target was present.

4.3. Humoral Immune responses in rabbits

4.3.1. ELISA antibody titers

A measurable antibody response to all of the 5 glycoproteins was detected in all of the animals in both the EBV-LP and UV-inactivated EBV groups compared to animals immunized with TNE buffer. Rabbits immunized with soluble gp350 only developed gp350-specific antibodies, as expected. The animals in the TNE control group did not react to any of the antigens.

It is generally anticipated that animals immunized with UV-inactivated virus will achieve the highest antibody titers, a trend that was not seen in the ELISA assays measuring gp350 and gB, in which the gp350 group and the EBV-LP group achieved the highest titers, respectively. This could potentially be explained by the difference in the amount of antigen delivered to the animals in the UV-EBV group compared to animals immunized with the EBV-LP and soluble gp350.

Immunizations were calculated based on the total protein concentration. While both the EBV-LP and UV-inactivated EBV immunizations contained 50 μg of total protein, the UV-inactivated EBV contained approximately 10 times less gp350 protein by weight than the EBV-LP. Being that gp350 is the most abundant protein expressed on the surface of EBV it is likely that gB, gH/gL and gp42 are present in

even smaller numbers in the UV-EBV compared to the EBV-LP, which theoretically expresses all of the viral proteins in equal numbers. Compared to the 25 μg of soluble gp350, the UV-EBV group received only 0.59 μg. This large discrepancy in the amount of immunogen delivered to different groups could reasonably account for the antibody titer results. Titers for anti-gH/gL, and gp42-specific antibodies were highest in the UV-EBV group, indicating other possible variations in immunogenicity, antibody binding, and expression levels. It is also possible that gH, gL, and gp42 are being expressed correctly, but the gH/gL and gH/gL/gp42 complexes are not forming correctly.

Because the ELISA assay was not repeated, individual animals and plates that would normally be considered “outliers” were not removed from the analysis so as to not falsely manipulate the results. For example, one animal from both the EBV-LP and UV-EBV groups consistently had titers 2-3 times lower than other animals in the group. Without going back to the original serum samples and repeating the assay, it is unclear whether these animals were low/non-responders or there was an experimental inconsistency such as an error in serum dilution. Excluding non- or low-responding animals from the analysis could greatly inflate the perceived efficacy of the vaccine, depending on the frequency with which these animals occur.

On the other hand, the sensitivity of the ELISA assay is such that a small pipetting error can translate to large variations in OD. Serum dilutions should be performed with the intentions of minimizing errors and variations. A small droplet on the side of the pipette tip or an air bubble can lead to values several times higher or lower than what is accurate if a 1:100 dilution is made by adding 1 μl of serum to 99 μl diluent. Rather, two 1:10 dilutions should be made with dedicated, recently calibrated pipettes. Unless serum is severely limited total volumes should be ≥ 1 ml, pipette tip should be observed for any inconsistencies and tubes should be vortexed and centrifuged briefly a minimum of two times following each dilution to ensure proper mixing and collection of the entire volume. Individual single-use aliquots of each serum dilution should be stored in low protein binding tubes and frozen at -80°C until use.

If appropriate controls are available in future, the assay should be optimized using well-characterized antibodies to determine the optimal assay conditions and the dynamic range of the detection reagents for each antigen. The assay should be repeated at least twice to validate the observed results.

4.3.2. Neutralizing antibody titers

Production of adequate amounts of infectious EBV-eGFP was a significant impediment to this study. Although virus production prior to this study yielded high infectivity this rapidly diminished with subsequent attempts at virus production. It is possible that an early miscommunication, where the protocol provided to us from a colleague indicated that 50 μg/ml of G418 should be used rather than 500 μg/ml, lead to progressive loss of the virus from the AGS cell line. This error was discovered after obtaining the original paper describing the method. It is also possible that further optimization of the cell culturing conditions and virus purification is needed. Ideally, a higher titer virus would have enabled the neutralization assay to be repeated in both HEK-293T and Raji cells both with pooled serum and gp350, gB, gH/gL, and gp42-specific IgG purified antibodies to determine the contribution of each antigen to the neutralizing activity.

4.4. Concluding remarks and future directions

In recent years a number of studies have demonstrated the importance of other EBV surface glycoproteins in addition to gp350 as vaccine immunogens. The first aim of this study was to construct, generate, and characterize the structural and functional properties of a multivalent EBV-LP. The expression of gp350, gB, gH/gL, and gp42 on the surface of a CHO cell line used for VLP production was confirmed by flow cytometry. The expected pellet and bands during purification by sucrose gradient centrifugation indicated that the transfection of this stable EBV gp350-gB-gp42-gL-gH-F CHO cell line with NDV-NP_{26AA}-tEBNA1-LMP2 and NDV-M successfully resulted in the production of VLP.

Western blot results strongly indicate the presence of gp350, gB, and gH in the purified VLP. The gp350 protein content of the UV-EBV and the EBV-LP was also

detected by ELISA. In the future additional probing of the expression of each of the five EBV surface glycoproteins on both the EBV-LP and the UV-inactivated virus used in animal immunizations should be done. Additional well-characterized antibodies and soluble proteins would enable gp350, gB, gH, gL, and gp42 to be all be quantified by ELISA and verified by western blot or immunofluorescence. Quantifying the amount of each antigen being used for immunization would provide a better understanding of the immunogenicity of each individual component. This would inform later studies where additional EBV-LP designs could omit proteins not found to elicit a potent immune response.

Antibodies specific to the complex of gH/gL or gH/gL/gp42 would enable the quantification of both complexes, to determine if the amount or ratio of these complexes influences the production of neutralizing antibodies. In depth characterization of gB, which naturally exists as a trimer, and it's conformation on the VLP would determine if modifications should be made to the gB protein to better recapitulate the expression on wild-type EBV.

The second goal of this project was to test the immunogenicity of the EBV gp350-gB-gp42-gL-gH-tEBNA1-LMP2 VLPs in New Zealand white rabbits. Serum from the EBV-LP and UV-EBV groups showed a measurable antibody titer to each of the specific EBV antigens, although the difference in gH/gL titer for EBV-LP compared to TNE was not statistically significant due to the large variation between samples within these groups. Ideally these ELISAs would be repeated, to confirm these results or, if rabbit antibodies specific to gp350, gB, gH, gL, and gp42 become available, to better quantify the antibody titers by including a standard curve of known antibody concentrations on each plate. This would provide a better control for plate to plate variation.

The results from the neutralization assay were mildly encouraging, despite the low infectivity of the virus used in the assay. The EBV-LP, UV-inactivated EBV, and gp350 serum neutralized over 50% of infection, however none completely prevented infection. As previously stated, further studies should determine the neutralizing antibody titers in both HEK-293T and Raji cells, starting at a serum dilution of 1:10. Column-purified gp350, gB, gH, gL, and gp42 specific antibodies purified from the immunized rabbit serum could be used to determine the

neutralizing ability of antibodies directed at each antigen should they be present in equal concentrations. Again, this would help inform future studies as to which of these EBV glycoproteins alone or in combination provides the most potent neutralizing antibody response.

Due to the limitations of this study, including the lack of commercially available antibodies, and virus, the results do not conclusively indicate that this vaccine design may be clinically effective. However, it does suggest that targeting EBV surface glycoproteins other than gp350 may be worth further exploration. This is supported by several recent studies that have shown that glycoproteins involved in viral fusion are able to elicit neutralizing antibodies in both mice (127) and rabbits (73). This study also presents a novel platform for producing a vaccine devoid of pathogenic DNA with multiple viral proteins expressed on the surface, although significant advancements will be needed in production efficiency.

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APPENDIX A: ETHIC APPROVALS

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-200430-04

30/04/2020

TO WHOM IT MAY CONCERN:

Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Joslyn Foley
Supervisor:	Nancy Meulenberg
Department:	Department of Molecular Medicine and Haematology
Project title:	A prophylactic multivalent vaccine to prevent acute infectious mononucleosis and EBV+ lymphomas
Reason:	*In vitro* bacterial and cell culture research involving the establishment of protein expression systems. No human participants, human data or human tissues will be involved in the study.

CB Penny

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

CITY OF HOPE NATIONAL MEDICAL CENTER
BECKMAN RESEARCH INSTITUTE OF CITY OF HOPE

NOTICE OF INSTITUTIONAL ANIMAL CARE & USE COMMITTEE (IACUC) ACTION

IACUC PROTOCOL #:	17093
IACUC PROTOCOL TITLE:	Unmasking the roles of herpesvirus glycoproteins in virus entry and vaccine development

TO: Javier Ogembo, Ph.D., Principal Investigator
City of Hope - Experimental Therapeutics-BRI

FROM: Susan Kallay, M.S.
Director, Laboratory Research Protections



Signature applied by Susan Kallay on 08/22/2017 01:46:32 PM PDT

DATE OF NOTICE: August 22, 2017

DATE OF REVIEW: August 22, 2017

SUBMISSION: Initial Review Submission

SUBMISSION REFERENCE#: 146014

DECISION: Protocol Approved From 08/22/2017 Until 08/21/2018

As the PI of this study, you are responsible for the following:

1. You are required to make sure that all research staff involved in the conduct of this animal research project have read, and have a clear understanding of the IACUC approved protocol procedures and requirements.
2. You must obtain IACUC approval prior to initiating any changes in the protocol. (No changes can be initiated until IACUC approval has been secured.)
3. You are required to report any unexpected problems involving animals as soon as you are aware of them. This report should be made to the IACUC.

If concordance review of an associated grant application is required, please ask your grant manager in the Office of Sponsored Research to submit a request through iRIS.

PRF&L
Quality Antibody Production Since 1960

Pocono Rabbit Farm & Laboratory Inc.
306 Dutch Hill Road • Canadensis, PA 18325
Phone: (800) 622-6381 • Fax: (570) 595-9277
www.prfal.com • e-mail: antibody@prfal.com

Animal Care and Use Committee

ANIMAL PROTOCOL APPROVAL NOTICE

PRF&L Protocol Form Number: PRF2A

Project Title: Production of Polyclonal Antibodies in Rabbits

Species / Strain: New Zealand White Rabbits

The Pocono Rabbit Farm and Laboratory Animal Care and Use Committee approved this Protocol, as submitted.

Date of IACUC Protocol Approval: January 15, 2018

The Animal Welfare Act requires that all research projects using animals must be reviewed by the Animal Care and Use Committee.

Pocono Rabbit Farm and Laboratory, Inc. has an Animal Welfare Assurance on file with The Office of Laboratory Animal Welfare (OLAW). The Animal Welfare Assurance number is A3886-01 / D16-00508. The effective dates are February 4, 2017 through January 31, 2021.

AAALAC International has continued full accreditation for all programs and facilities of Pocono Rabbit Farm and Laboratory, Inc. under file number 926.

Pocono Rabbit Farm and Laboratory Animal Care and Use Committee is constituted in accordance with U.S. Public Health Service (PHS) Policy and includes a member of the public and a non-scientist.

Scott Conklin
IACUC Chairperson
Pocono Rabbit Farm & Laboratory
Animal Care & Use Committee

Laboratory Research Protections
1500 East Duarte Road
Duarte, CA 91010-3000
Phone: (626) 218-5030
IACUC@coh.org

Institutional Animal Care and Use Committee
Notification of IACUC Protocol Continuation Approval

Date: February 18, 2020

To: Javier Ogembo, Ph.D., Principal Investigator
City of Hope - Immuno-Oncology BRI

From: Alyse DiStefano, Director
Laboratory Research Protections

IACUC #: /Ref #:	16003 / 186511
Protocol Title:	Unmasking the roles of virus proteins in entry, replication and vaccine development
Action Date:	02/18/2020
Action:	Approved
Full Approval Period:	04/15/2016 until 04/14/2022
Annual Approval Period:	04/15/2020 until 04/14/2021
Annual Continuation Due Date:	04/14/2021

This protocol has been reviewed and approved by the City of Hope (COH) Institutional Animal Care and Use Committee (IACUC). Modifications required to secure approval by the IACUC, if any, have been met.

As Principal Investigator, you are responsible for the following:

1. Make sure that all research staff involved in the conduct of this animal research project have read and have a clear understanding of the IACUC approved protocol procedures and requirements.
2. Obtain IACUC approval prior to initiating any changes in the protocol. No changes may be initiated until IACUC approval has been secured.
3. Report to the IACUC any unexpected problems involving animals as soon as you are aware of them.
4. If concordance review of an associated grant application is required, ask your grant manager in the Office of Sponsored Research to submit a request through iRIS.

Post-Approval Monitoring (PAM)

- All approved IACUC protocols will be subject to a PAM visit at least once during each 3-year approval period. Most PAM visits will be arranged with the PI in advance, however in addition to scheduled visits, some PAMs may be conducted unannounced.
- IACUC Policy on Post-Approval Monitoring

If you have questions or concerns about this submission, please contact Rowelle Enriquez, M.S. at IACUC@coh.org or ext. 85030.

Laboratory Research Protections
1500 East Duarte Road
Duarte, CA 91010-3000
Phone: (626) 218-5030
IACUC@coh.org

Institutional Animal Care and Use Committee
Notification of IACUC Protocol Continuation Approval

Date: February 18, 2020

To: Javier Ogembo, Ph.D., Principal Investigator
City of Hope - Immuno-Oncology BRI

From: Alyse DiStefano, Director
Laboratory Research Protections

IACUC #: /Ref #:	16002 / 186340
Protocol Title:	Viral entry and vaccine development
Action Date:	02/18/2020
Action:	Approved
Full Approval Period:	03/11/2016 until 03/10/2022
Annual Approval Period:	03/11/2020 until 03/10/2021
Annual Continuation Due Date:	03/10/2021

This protocol has been reviewed and approved by the City of Hope (COH) Institutional Animal Care and Use Committee (IACUC). Modifications required to secure approval by the IACUC, if any, have been met.

As Principal Investigator, you are responsible for the following:

1. Make sure that all research staff involved in the conduct of this animal research project have read and have a clear understanding of the IACUC approved protocol procedures and requirements.
2. Obtain IACUC approval prior to initiating any changes in the protocol. No changes may be initiated until IACUC approval has been secured.
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- IACUC Policy on Post-Approval Monitoring

If you have questions or concerns about this submission, please contact Rowelle Enriquez, M.S. at IACUC@coh.org or ext. 85030.

APPENDIX B: PLAGIARISM REPORT

Turnitin Originality Report

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David H. Mulama, Lorraine Z. Mutsvunguma, Jennifer Totonchy, Peng Ye et al. "A multivalent Kaposi sarcoma-associated herpesvirus-like particle vaccine capable of eliciting high titers of neutralizing antibodies in immunized rabbits", Vaccine, 2019

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Joost Snijder, Michael S. Ortego, Connor Weidle, Andrew B. Stuart et al. "An Antibody Targeting the Fusion Machinery Neutralizes Dual-Tropic Infection and Defines a Site of Vulnerability on Epstein-Barr Virus", Immunity, 2018

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