Plant-produced Ebola Immune Complex as an Ebola Vaccine Candidate

by

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ABSTRACT

Ebola hemorrhagic fever (EHF) is a severe and often fatal disease in human and nonhuman primates, caused by the Ebola virus. Approximately 30 years after the first epidemic, there is no vaccine or therapeutic medication approved to counter the Ebola virus.

In this dissertation, a geminiviral replicon system was used to produce Ebola immune complex (EIC) in plant leaves and tested it as an Ebola vaccine. The EIC was produced in *Nicotiana benthamiana* leaves by fusing Ebola virus glycoprotein (GP1) to the C-terminus of heavy chain of 6D8 monoclonal antibody (mAb), which is specific to the 6D8 epitope of GP1, and co-expressing the fusion with the light chain of 6D8 mAb. EIC was purified by ammonium sulfate precipitation and protein A or protein G affinity chromatography. EIC was shown to be immunogenic in mice, but the level of antibody against Ebola virus was not sufficient to protect the mice from lethal the Ebola challenge.

Hence, different adjuvants were tested in order to improve the immunogenicity of the EIC. Among several adjuvants that we used, Poly(I:C), which is a synthetic analog of double-stranded ribonucleic acid that can interact with a Toll-like receptor 3, strongly increased the efficacy of our Ebola vaccine. The mice immunized with EIC co-administered with Poly(I:C) produced high levels of neutralizing anti-Ebola IgG, and 80% of the mice were protected from the lethal Ebola virus challenge. Moreover, the EIC induced a predominant T-helper type 1 (Th1) response, whereas

Poly(I:C) co-delivered with the EIC stimulated a mixed Th1/Th2 response. This result suggests that the protection against lethal Ebola challenge requires both Th1 and Th2 responses.

In conclusion, this study demonstrated that the plant-produced EIC co-delivered with Poly(I:C) induced strong and protective immune responses to the Ebola virus in mice. These results support plant-produced EIC as a good vaccine candidate against the Ebola virus. It should be pursued further in primate studies, and eventually in clinical trials.

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TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AlMV	alfalfa mosaic virus
APC	antigen presenting cell
BSL-4	Biosafety Level 4
CD	cluster of differentiation
CDC	U.S. Centers for Disease Control
CMV	cytomegalovirus
CP	capsid protein
CPMV	cowpea mosaic virus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
EHF	Ebola hemorrhagic fever
EIC	Ebola immune complex
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FcR	Fc receptor
FucT	α-1,3-fucosyltransferase
GP	glycoprotein

GMT	geometric mean titer
hEIC	humanized Ebola immune complex
HPIC3	human parainfluenza virus type 3
HRP	horseradish peroxidase
HSA	human serum albumin
IgG	immunoglobulin G
IC	immune complex
IL	interleukin
Ip	intraperitoneal
Kb	kilobase
LIR	long intergenic region
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
mAb	monoclonal antibody
MALDI-TOF/TOF MS	matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry
mEIC	mouse Ebola immune complex
MHC	major histocompatibility complex
MP	movement protein
mRNA	messenger ribonucleic acid
NK	natural killer cell
NP	nucleoprotein

PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing 0.05% Tween 20
PIC	polyinosinic-polycytidylic acid
PVX	potato virus X
Rep	replication initiator protein
rNAPc2	recombinant nematode anticoagulant protein c2
SD	standard variation
sGP	secreted glycoprotein
SIR	short intergenic region
T-DNA	transferred DNA
Th	T helper cell
TMV	tobacco mosaic virus
TNF	tumor necrosis factor
TTFC	tetanus toxin fragment C
VEE	Venezuelan equine encephalitis virus
VLP	virus-like particle
VP	virion protein
VRP	Venezuelan equine encephatlitis virus replicon particle
VSV	Vesicular stomatitis virus
WDV	Wheat Dwarf Virus
WHO	World Health Organization

XylT	β-1,2-xylosyltransferase

Chapter 1

INTRODUCTION

Ebola hemorrhagic fever (EHF) is a severe and often deadly disease that occurs in human and nonhuman primates, such as chimpanzees, monkeys, and gorillas (59). In humans, Ebola virus causes a lethal hemorrhagic disease with very high mortality rates, up to 90%, and with clinical symptoms arising quickly after an incubation period of 2 to 21 days (43). The typical presentation is characterized by flu-like symptoms including high fever, chills, malaise, and myalgia. Taken together, the ensuing symptoms indicate multisystem involvement, including systemic, gastrointestinal, respiratory, vascular, and neurologic manifestations. Abnormalities in blood coagulation appear as petechiae, ecchymoses, mucosal hemorrhages, and uncontrolled bleeding in venipuncture sites. Severe Ebola infections usually progress to shock, convulsions, and diffuse coagulopathy. Within 6 to 9 days after the onset of clinical symptoms, death may occur or recovery begins. The dramatic clinical symptoms and a high fatality rate associated with Ebola virus infection make it an extremely dangerous pathogen. Moreover, all Ebola virus strains have demonstrated the ability to be spread through aerosols under research conditions. Therefore, Ebola virus poses severe threat human populations and is a potential candidate for a biowarfare agent that can be used by terrorists. Approximately 30 years after the first epidemic, there is still no approved vaccine or therapeutic for the Ebola virus.

Epidemiology

The name Ebola originates from the Ebola River in the Democratic Republic of Congo, where the first documented outbreak occurred in 1976 (19). There have been four strains of the Ebola virus identified—the Sudan, Zaire, Reston, and Ivory Coast strains—differing by approximately 30–40% in their nucleotide sequences (83). In the first documented outbreak of Ebola in June 1976, the strain Sudan infected 284 people with a mortality rate of 53%. Several months later, a second Ebola epidemic occurred in the Democratic Republic of Congo (previously known as Zaire); there, the Ebola strain Zaire infected 318 people and had the highest mortality rate of 88% (98). In these two outbreaks, there was significant secondary transmission through reuse of unsterilized needles and syringes and in hospital contacts. The primary transmission in these two outbreaks, however, is not clearly known; it might have resulted from contact with infected animals. The third Ebola strain, Reston, was found in 1989, when infected monkeys were imported to Reston, Virginia, from the Philippines. Hundreds of monkeys were infected with this strain and experienced a high mortality rate. Fortunately, few people were infected in this incident, and no human developed EHF. The fourth strain, known as the Ivory Coast strain, was identified in Côte d'Ivoire in1994 (6). This strain was associated with chimpanzees; the only identified human infection proved to be non-fatal (49). Among these four strains, Zaire has the highest morbidity/mortality rate followed by the Sudan and Ivory Coast

strains, respectively; the Reston strain caused disease only in nonhuman primates. Since the discovery of the virus, the World Health Organization (WHO) has reported approximately 1,850 cases with over 1,200 deaths from Ebola virus infection. Epidemiological history suggests that the countries in the mid-western part of Africa are the epicenter for Ebola virus infections. Although a natural reservoir of the Ebola virus has not yet been identified, based on the evidence, the reservoir is suspected to reside in the rain forest in western Africa.

Clinical Syndrome

The Ebola virus is transmitted in nature through direct contact with infected bodily fluids (e.g., blood, semen, and vaginal fluid) of infected persons or primates (17). When the virus infects a human, it enters the body and starts replicating in mononuclear phagocytic cells such as macrophages and monocytes (26, 29, 63, 76). After infection in these primary target cells in the blood, continued virus production in these cells causes high viremia, which allows the virus to be disseminated throughout the bloodstream and to infect secondary target cells, such as endothelial cells in the liver, spleen, pancreas, lungs, and kidneys. Viral infection of various organs leads to the disease symptoms associated with multi-organ failure, such as prostration, lethargy, anorexia, nausea, vomiting, abdominal pain, diarrhea, chest pain, shortness of breath, cough, conjunctival injection, postural hypotension, edema, headache, confusion, seizure, and coma.

Ebola Virus

Ebola virus is a Filovirus belonging to the Filoviridae family. It is usually 80 nm in diameter and variable in length (28). The 19-kilobase genome of Ebola consists of nonsegmented negative sense RNA and encodes a total of seven proteins: 3' nucleoprotein (NP), virion protein (VP) 35, virion protein 40 (VP40), glycoprotein (GP), virion protein 30 (VP30), virion protein 24 (VP24), and polymerase (L) 5'. The nucleoprotein, virion protein 35, virion protein 30, and polymerase are associated with the genome of Ebola virus as a ribonucleoprotein complex inside the virus, whereas virion protein 40, virion protein 24, and glycoprotein are associated with the membrane (18). These proteins are translated from polyadenylated mRNA, which is transcribed from the viral RNA in the host cell. The ribonucleoprotein complex is surrounded by an envelope that originates from the host cell membrane. VP40 and VP24 form the virion envelope (18, 57, 62). The fourth gene of the Ebola genome encodes a soluble nonstructural secreted glycoprotein (sGP) and a structural transmembrane glycoprotein (GP). GP is expressed by transcriptional editing, resulting in the addition of an extra adenosine within a run of seven adenosines in the coding sequence (86). GP and sGP share roughly 300 amino-terminal amino acids. GP has additional 380 carboxy-terminal amino acids, whereas sGP has 70 additional carboxy-terminal amino acids (79). Previous cell culture studies demonstrated that the majority of GP mRNA is not edited and encodes nonstructural sGP, which accumulates in the

infected cell media (64, 87). Thus, the primary gene product of the Ebola GP gene is not the transmembrane GP, but rather the sGP. The function of sGP is not fully understood, but it has been suggested that sGP might play a role in immune evasion by acting as a decoy molecule that binds to Ebola-specific antibodies and therefore limits the opportunity for antibodies to neutralize the virus (39, 105). Takada and Kawaoka (1998) showed that sGP can bind to neutrophils via CD16b, which is a neutrophil-specific form of Fc gamma receptor III, and inhibit early neutrophil activation (80). Another possible function of sGP is to bind to neutrophils and consequently inhibit their activation. The only viral protein that is detected on the surface of the virus and the infected cell is GP, which forms spikes on the viral envelope (23, 89). The GP spike on the viral envelope is responsible for attaching to the receptors (e.g., dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN), or folate receptor) and entering into the cell by membrane fusion (81, 101). Transmembrane GP is cleaved posttranslationally by furin into N-terminal GP1 and C-terminal GP2 (65, 88). GP1 is linked with GP2 by disulfide bonds and is present as a trimer in the spike of the virus (4, 50). GP expression causes cell rounding and detachment in cultured human endothelial and epithelial cells, which are the target cells for Ebola virus infection (71, 106). This effect requires the mucin-like domain in GP and correlates with the down regulation of

specific molecules on the cell surface such as beta1 integrin and major histocompatibility complex class I (24, 82). This evidence supports the idea that Ebola GP can induce protein down modulation and cytopathic effects.

Pathogenesis

The molecular mechanism of Ebola pathogenesis is difficult to understand due to the rapid onset of disease symptoms and death in nature and also in laboratory animal models. Research on this virus requires Biosafety Level 4 (BSL-4) facilities—the most stringent degree of laboratory protection. BSL-4 labs are designated for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections, cause severe or fatal disease in humans, and for which there are no vaccines or treatments available. The low number of laboratories designated BSL-4 in the world limits Ebola virus research.

The first step of the infection is binding of GP to receptors on the cell surface. The receptor for Ebola virus has not been definitively identified, but previous studies showed that possible receptors are DC-SIGN, L-SIGN, and folate receptor. DC-SIGN was shown to bind with the Ebola glycoproteins and enhance Ebola infection. It is expressed in many cell types such as dendritic cells, macrophages, and endothelial cells in the liver and lymph nodes, which are the target cells for Ebola virus infection (70). L-SIGN is not expressed by dendritic cells, but is expressed on the surface of endothelial cells in the liver, lymph node sinuses, and

placenta villi (5). Wool-Lewis and Bates (101) used the Jurkat cell line, which is an immortalized line of T lymphocyte cells, nonpermissive for Ebola virus infection and considered to be receptor deficient, to identify the Ebola virus receptor. They found that when the Jurkat cells expressed L-SIGN, they could be infected with Ebola virus (1). This result indicated that L-SIGN can mediate an Ebola virus infection. Another possible receptor for GP is the folate receptor. Jurkat cells expressing the folate receptor also showed infection with Ebola virus (11). Macrophages and fibroblasts are examples of cells that express folate receptors, and they are target cells for Ebola virus infection. This result supports the possibility that the folate receptor is a receptor for Ebola infection.

Ebola virus infection in endothelial cells via GP spike/cell receptor interaction shows the destruction of the cells associated with disseminated intravascular coagulation (67). Therefore, the specific binding of GP may play a role in the pathology of Ebola virus infection. All evidence has shown that the Ebola GP is the only protein on the viral cell surface. Moreover, these results suggest that the Ebola GP is the protein that causes viral infection by binding to host cell receptors and also causes the cytopathic effect. Thus, the Ebola GP is one of the target proteins for Ebola vaccine development.

The primary difficulty for patients in recovering from Ebola virus infections results from failure of their immune systems to react to this fast-developing disease. The patients who die from Ebola hemorrhagic fever

are unable to develop an adequate immune response in the face of extreme viremia and multi-organ infection. Previous studies have shown that survivors of Ebola infection must develop early and increasing levels of IgG against Ebola virus, followed by viral antigen clearance and cytotoxic T cell activation. In fatal cases, Ebola-specific IgG and T cell-related mRNA cannot be detected (3). These data imply that both humoral- and cell-mediated immune responses are important for recovery from Ebola virus infection. Thus, the key for Ebola vaccine development is to activate both antibody (Ab) response and cytotoxic T cells against the Ebola virus.

There are different animal models developed for study of the Ebola virus infection including mouse, guinea pig, and nonhuman primates, but the “gold standard” animal models for pathogenesis, treatment, and vaccine studies are rhesus and cynomolgus macaques. Only these animals are lethally infected with non-adapted human isolates, and the resulting pathology is close to the pathology described in humans.

Therapeutic Drugs for Ebola Infection

Currently, there is no approved treatment or cure for Ebola virus infection. However, there are several candidates being researched as applications for Ebola therapeutics, including neutralizing antibodies, Vesicular stomatitis virus-vectored vaccine (post-exposure), and the use of an anticoagulant protein.

Neutralizing antibodies have been studied in the treatment of Ebola-infected animals. The anti-Ebola virus IgG from equine has been shown to protect cynomolgus monkeys and baboons from infection when it is injected before or after infection at a high dose of IgG (40, 45). Wilson et al. (100) found that the passive transfer of mAb against Ebola GP can protect mice from Ebola infection. Moreover, passive transfer of serum from mice that have survived Ebola virus infection can protect naïve mice against lethal challenge (31). Although passive transfer can protect mice from Ebola lethal infection, it is only effective with the presence of a high Ab titer in the serum (41). At similar comparable doses, it has been less successful in protecting non-human primates. Therefore, the passive transfer strategy may not be a promising therapeutic for human treatment.

There are also studies to support Vesicular stomatitis virus (VSV)-based Ebola vaccine as a means of post-exposure prevention. When treated 24 hours after an Ebola intraperitoneal challenge with VSV-expressing Ebola GP, mice showed complete protection from Ebola virus challenge. However, less encouraging results were obtained when this

experiment was repeated in guinea pigs, the survival rate being approximately 50% (22). The most persuasive results show that VSV-expressed Ebola GP post-exposure treatment protected cynomolgus macaques against aerosol exposure of Ebola virus (25). Although VSV-based Ebola vaccine showed promising protection in the macaques, the results are not consistent in different animals.

Another possibility for an Ebola therapeutic is the use of an anticoagulant protein. Previous data showed that Ebola-infected macrophage express a tissue factor, which is a clotting protein on the cell surface that attracts other blood clotting proteins. The accumulation of these blood clotting proteins causes the body to become vulnerable to bleeding, the symptom that leads to fatality (20). Recombinant nematode anticoagulant protein c2 (rNAPc2), which is a potent inhibitor of tissue factor-initiated blood coagulation, has been studied as an Ebola therapeutic. The treatment of monkeys with rNAPc2 immediately or within 24 hours after infection and continuing for eight days saved three out of nine monkeys and delayed the death of the other six monkeys (27). Although this protein can reduce the mortality rate only by about 30%, the reduction is significant because the infection of rhesus monkeys is almost always lethal.

Ebola Vaccine Development

Not only is there no approved Ebola virus therapeutic, but there is no licensed Ebola vaccine for human use at this time. Much research is focused on developing an Ebola vaccine, but the results have not been promising. There are two major types of Ebola vaccine being researched, DNA vaccine and viral-based vector. The viral-based vector includes different strategies such as the use of a virus-like particle or a viral replicon that can replicate in cells but is not infectious.

The first Ebola vaccine tested was a DNA vaccine, which used a eukaryotic expression vector with the cytomegalovirus (CMV) immediate early region enhancer to stimulate transcription for a high level of gene expression in muscle (51). DNA vaccine expressing Ebola GP or NP genes showed protection in mice (85, 102). Guinea pigs that were immunized with plasmids encoding Ebola NP, GP, or sGP were also protected against Ebola infection. Both humoral Ab and T cell-mediated responses are detected with this immunization. In a phase I clinical trial, the DNA vaccine also showed safety and immunogenicity (52). To improve the DNA vaccine efficacy, an Adenovirus vector was subsequently used for study in a prime-boost experiment. When priming with the DNA vaccine expressing Ebola GP was followed by boosting with the Adenovirus vector-expressed Ebola GP, cynomolgus macaques were completely protected against a challenge with a lethal dose of Ebola Zaire (77). Another study employing prime-boost strategy for the Marburg virus,

which is another member of Filovirus family, showed that a priming dose of a DNA vaccine expressing the Marburg GP, followed by boosting with a recombinant baculovirus-derived GP protein, provided protective immunity to guinea pigs (36). Nevertheless, a similar prime-boost experiment did not protect against Ebola virus infection in guinea pigs (54). Although it may be safe to use the DNA vaccine in humans, the efficacy of a DNA vaccine needs more study.

Another strategy for vaccine development is to use a viral-based vector. The first type is a virus-like particle (VLP), which contains proteins that form envelope and surface proteins, but without RNA needed for replication. Unable to replicate in cells, the VLP is not infectious. An Ebola VLP is produced by expression of Ebola VP40 and/or GP (53, 92, 95-97). The use of the Ebola VLP showed protection from lethal challenge of Ebola virus in rodents (92, 96) and in guinea pigs (78). The Ebola VLP can induce an inflammatory response, including the production of several inflammatory cytokines such as IL6, IL8, IL12, and TNFα (53). Moreover, the Ebola VLP is able to activate CD4+ and CD8+ T cells in vaccinated mice (92) and enhance the numbers of NK cells in their lymphoid tissues (94). Another study showed that the Ebola VLP can be uptaken by human dendritic cells (DCs) and induce them to upregulate the costimulatory molecules, major histocompatibility complex (MHC) class I and late DC maturation marker CD83 (8). These results confirmed that the Ebola VLP

effectively stimulates antigen-presenting cells and can enhance innate and adaptive immune response.

The second type of viral-based vector studied for use as an Ebola vaccine is the Venezuelan equine encephalitis virus (VEE) replicon particle (VRP). In the replicon vector, the VEE structural genes are replaced with a gene of interest. When the replicons are introduced into cells, the replicons are restricted into a single cycle of replication because they lack the VEE structural proteins. Thus, they cannot assemble into particles and spread into uninfected cells. The VRP-expressed Ebola GP showed protection of BALB/C mice and two strains of guinea pigs from lethal Ebola infection (60). The VRP-expressed Ebola NP also protects mice against Ebola challenge by inducing Ab response against Ebola NP and MHC class I restricted cytotoxic T lymphocyte (CTL) response to Ebola NP. The results also showed that the passive transfer of serum-contained antibodies specific to the Ebola NP cannot protect mice from challenge, but adoptive transfer of CTL specific to the Ebola NP can provide protection (99). These results support the VRP as a potential vaccine for Ebola because it can induce both humoral and cell-mediated immune response.

Another viral-based vector that was tested as an Ebola vaccine is the attenuated recombinant VSV vector. VSV vector-expressed Ebola GP completely protected monkeys from Ebola challenge after one intramuscular injection. The vaccine showed induction of humoral and

cellular immune response in the vaccinated monkeys (42). Recently, researchers have tried to develop an Ebola vaccine based on the human parainfluenza virus type 3 (HPIC3) (9, 104). Guinea pigs that were intranasally immunized with a single dose of the HPIC3-expressed Ebola GP showed immunogenic response and complete protection from lethal Ebola challenge. All of these viral-based vaccines showed promising data with respect to activation of both arms of the immune response and provided protection for animals from Ebola challenge. But the greatest concern about this type of vaccine is whether these viral vectors are really safe to be used in humans.

From previous studies, we know that both humoral and cell-mediated immune responses play important roles in protection against Ebola virus infection. Although the study of the Ebola VLP showed protection against Ebola challenge, serum transfer from Ebola VLP-vaccinated mice did not protect the naïve mice (93). This demonstrates that the humoral immune response alone might not be enough for protection. Cell-mediated immune response also plays an important role in the protection against Ebola virus infection. Activation of these two arms of the immune system is vital for developing efficient Ebola vaccines and therapeutics. Many types of Ebola prophylaxis and treatments have been developed, but currently there are no approved vaccines or therapeutics against Ebola virus for human use. The new discovery of an Ebola

vaccine is still necessary. In this thesis, the immune complex is applied as a new strategy for Ebola vaccine development.

Immune Complex

The antigen-antibody immune complex is formed in vivo as a natural process completing a humoral immune response to an antigen. Generally, the immune complex (IC) is effectively engulfed and removed by mononuclear phagocytes (69). Previously, it has been shown that the protein antigen (Ag) contained within the IC is much more effectively processed and presented in the MHC molecule than free Ag by DCs in vitro (2, 68). DCs are the most potent antigen-presenting cells (APC) among all cell types, including macrophages and B cells. The uptake of the IC by cells occurs through two preferential receptors on the surface of APC, complement receptor (61, 103) and Fc receptor (FcR) (15, 35). The IC binds to the Fc receptor and mediates a variety of responses from phagocytosis to Ab-mediated cellular cytotoxicity. The internalization of the IC by the DC, mediated by FcγR, enhances the presentation of both MHC class I and MHC class II binding peptides derived from the antigen presented in the IC. This can lead to DC activation. Previous studies showed that the IC can mediate efficient priming of Th cell response after binding to the FcR on the APC (33). Moreover, the activation of DCs by the IC enables these cells to efficiently prime CD8+ cytotoxic T lymphocyte response both against an exogenously loaded peptide and against endogenously processed peptide from the Ag present in the IC

(68). Due to efficient CD4+ and CD8+ priming activity, the IC is a promising target for Ebola vaccine development.

In addition to FcγR, the complement system is known to interact with the IC. Three pathways of complement activation have been described: the classical pathway, the alternative pathway, and the lectin pathway (90, 91). Each pathway has its own activation and recognition mechanism, resulting in the formation of C3-convertase that cleaves the central complement component C3 into the fragments C3a and C3b. Binding of C3b enables clearance of pathogens and the IC, as well as the generation of the lytic membrane attack complex.

C1q is one of the activator compounds of the classical complement pathway and plays an important role in the activation of the pathway and the clearance of the IC from the circulation (56, 72). C1q contains a collagen-like tail region to which serine protease C1r and C1s are bound, connected to a globular head region responsible for ligand binding. The C1q head can recognize a defined region within the C_H2 domain of the Ag-complexed IgG molecule, thereby opsonizing the IC. C1q recognizes the complexed Ag with higher affinity than the free Ag. After binding to its ligand, C1q changes conformation, leading to the activation of its associated serine proteases, C1r and C1a. A study showed that mice deficient for C1q displayed a substantially reduced ability to present immune-complexed Ag to CD8+ T cells (84).

Published evidence confirms that Ag presented in the IC can induce stronger humoral and cell-mediated immune responses than Ag alone. Thus, the IC is a target backbone used for vaccine development.

Immune Complex Production in Plants

A previous study showed the production of the IC in *Nicotiana tabacum* (12) and in that work, the complex was created by expressing tetanus toxin fragment C (TTFC) fused to the C-terminus of TTFC-specific monoclonal antibody. The IC without the addition of adjuvant proved to be highly immunogenic in mice, with IgG titer more than 10,000 times higher than the titer observed in mice immunized with antigen alone. It was also shown that the IC binds to a macrophage cell line, one of the APC, whereas the TTFC-specific monoclonal antibody does not. This indicates that antigen presented in the IC can enhance the binding to antigen-presenting cells, leading to an increase in the process and antigen presentation via the MHC molecule. This result indicates that the IC expressed in plants are highly immunogenic in mice and can act as a self-adjuvant. These studies point to possibilities for the development of a plant-based Ebola vaccine.

Plant-produced Recombinant Protein

Historically, bacteria were often used as protein expression systems because they are an inexpensive and convenient system. A major drawback to their use, however, is that they are incapable of many of the post-translational modifications necessary for the activity of

numerous mammalian proteins. Other protein expression systems include yeast cells, baculovirus-infected insect cells, mammalian cells, transgenic animals, and transgenic plants. Among these systems, plants have many advantages over the other systems. First, plants have a higher eukaryote protein synthesis pathway that is similar to the pathway in animal cells, but with minor differences in protein glycosylation (10). The post-translational modifications in plants are required for protein activity and suitable pharmacokinetics properties (30). Second, plants can produce recombinant proteins at high levels comparable to those produced in hybridomas (37). Third, using plants as the bioreactor avoids the contamination of expressed proteins with any human or animal pathogens. Importantly, plants are an inexpensive source to produce recombinant proteins. Mammalian cell cultivation requires sophisticated equipment and expensive media supplements, such as fetal bovine serum. It is estimated that protein production in plants is 10 to 50 times cheaper than production in *E.coli* (46). In addition to these advantages over other expression systems, plants have been shown to produce functional forms of complex mammalian proteins, including hemoglobin (16), collagen (73), and antibodies (37).

A wide variety of plant-based expression systems, such as tobacco, potato, tomato, and maize, are now available to produce recombinant proteins. Thus, there are many different candidate host species, and different systems. There is also a choice of several gene delivery and

expression mechanisms. The classical method is to create stably transgenic plants, which have a stable genetic modification either in the nuclear or chloroplast genomes. There are many advantages of this approach. The transformation is a fairly routine procedure in many plant species. A stable transgenic line can be used as a permanent genetic resource. Among the various plant systems, this method is the simplest to maintain and is the most scalable. Although the establishment of permanent transgenic plants has many advantages, the impediments include the long development time (required for transformation, regeneration, and analysis); the unpredictable impact of epigenetic events on transgenic expression; and concerns about transgene escape into the environment.

An alternative strategy is transient gene expression, usually accomplished by vacuum infiltration of leaves with *Agrobacterium tumefaciens*: transferred DNA (T-DNA) is transferred to a very high proportion of the cells, where it can integrate or remain as an episome, and in either case, will express high amounts of protein. This transient somatic transformation or agroinfection allows very high levels of expression without the uncertainties associated with the generation and propagation of transgenic plants.

To improve the protein expression level in transient expression, plant viral vectors can be applied. Plant viruses are very efficient pathogens, using very compact genomes to force host cells to produce

very large amounts of viral proteins. Consequently, plant viruses allow the rapid and convenient production of recombinant protein in plants. Compared with stable transformation, viral vectors are easier to manipulate, and recombinant proteins can be produced more quickly and in greater yield. Several types of RNA viruses have been used to create plant viral expression vectors, including tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), and alfalfa mosaic virus (AIMV) (107).

Geminivirus

Plant viral expression is not limited to RNA viruses; some plant DNA viruses also can be used. Geminiviruses are plant viruses containing a single-stranded circular DNA genome varying in size from 2.5 kilobases (kb) to 3.0 kb, depending on the virus. The genomic DNA is replicated in the nucleus of the host cell by a rolling circle replication mechanism, utilizing double-stranded DNA intermediates (58). There are four different genera— Mastrevirus, Curtovirus, Topovirus, and Begomovirus. Mastreviruses have a monopartite genome and infect both monocot plants (wheat dwarf virus, maize streak virus, and digitalia streak virus) and dicot plants (tobacco yellow dwarf virus and bean yellow dwarf virus). The genome of the Mastreviruses contains two intergenic regions—long intergenic region (LIR) and short intergenic region (SIR)—located at opposite sites of the viral genome. There are four proteins encoded by the genome: the movement protein (MP) and the capsid protein (CP) on the

viral-sense (V) strand, the Replication initiator (Rep) protein, and the RepA protein on the complementary-sense (C) strand. LIR and SIR are the only cis-elements required for replication of the genome. The LIR has bi-directional promoter elements and a stem-loop structure that is essential for initiation of rolling-circle replication of the V-strand. The SIR is the origin of the C-strand synthesis and contains transcription termination signals. Rep is required for replication but it can be supplied *in trans*.

Geminiviruses replicate DNA via a rolling-circle replication mechanism, where Rep is involved with nicking-closing activity (14). Initially, viral single-stranded DNA enters into the cells and is converted into a double-stranded DNA replicative form. The replicative form serves as the template for viral transcription as well as the template for further replication. The next stage is to generate more replicative form and is initiated by the viral Rep protein. Rep can nick the plus strand at a specific sequence and then covalently bind to the 5’ terminus. The 3’ –OH terminus is used as the primer for the synthesis of a nascent plus strand (47). The DNA synthesis is accomplished by host replication proteins. Completion of the nascent plus strand regenerates the origin of replication which is nicked by Rep protein again. This time, Rep protein acts as a terminase to release the displaced plus strand, which is simultaneously ligated to the circular form by the closing activity (48). In the process, Rep is transferred to the newly created 5’ terminus. The third stage, occurring

late in the replication cycle, is responsible for the accumulation of viral genomes for encapsidation (32).

The geminiviral replicon system can be used to enhance foreign protein expression in plants. Previous studies demonstrated that the geminivector system produced recombinant proteins such as GUS protein (55), GFP protein (44), and Staphylococcus enterotoxin B in NT-1 cells (34). Moreover, the geminiviral replicon system can be used to express antibodies in plants (38).

Plant-produced Glycoprotein

More than one-half of all eukaryotic proteins, as well as one-third of the approved biopharmaceuticals, are glycoproteins. There are two types of glycosylation based on the method of carbohydrate attachment to the protein: N-glycan links to the amide group of asparagine, and O-glycan bonds to the hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline residues in the protein. Protein glycosylation occurs in the secretory pathway. N-glycosylation starts in the endoplasmic reticulum (ER), with the cotranslational or post-translational transfer of a preformed lipid-linked oligosaccharide onto the nascent polypeptide. In contrast, O-glycans are synthesized by a stepwise transfer of monosaccharides on the folded protein in the ER and Golgi apparatus.

The biological activity of many therapeutic glycoproteins, such as antibodies, blood factors, and interferons, depends on their glycosylation pattern. This can explain why biopharmaceuticals are often produced in an

expression system with glycosylation capacities. The capacity of plants for glycosylation supports the use of plants as means of therapeutic production over that of *E. coli*. Unfortunately, plants are unable to produce human-type glycosylation patterns for biopharmaceuticals (21, 30); plant-specific glycosylation is considered to be a limitation for the use of plant-made pharmaceuticals in human therapy, although a previous study indicated that plant glycan is not immunogenic in mice (13). Plants synthesize a core complex N-glycan structure similar to that in mammalian cells, but the terminal residues are different. Plant N-glycans are much smaller, lack β1,4-galactose and sialic acid, and carry plant-specific β-1,2-xylose and core α-1,3-fucose residues. Because plant β-1,2-xylosyltransferase and α1,3-fucosyltransferase are responsible for the transfer of β-1,2-linked xylose and α-1,3-linked fucose residues to glycoprotein N-glycans, knockout plants with complete deficiencies of these two enzymes creates plants that have a human N-glycan pattern (7, 66, 74, 75).

Dissertation Summary

Previous studies showed that the IC activates humoral and cell-mediated immune responses. Since evidence indicates that an effective Ebola vaccine should induce both types of the immune responses, it is feasible to use the IC as a candidate vaccine for Ebola virus. In this dissertation, the Ebola immune complex (EIC) produced in *Nicotiana benthamiana* was studied. The EIC was made by fusing the Ebola GP1 at the C-terminus of the heavy chain of the monoclonal antibody 6D8, which binds to an epitope that is close to the C-terminus of the Ebola GP1. When the GP1-heavy chain fusion was co-expressed with the light chain of mAb 6D8, the EIC was formed.

The second chapter of this dissertation describes the transient expression of humanized Ebola immune complex (hEIC) in *N. benthamiana*. After the IC was expressed, it was purified by ammonium sulfate precipitation and protein G affinity chromatography. The purified protein was used to immunize mice and determine the immunological response. The result showed immunogenicity of the plant-expressed hEIC, although the level of anti-Ebola IgG was low. Moreover, the mice produced high levels of the antibody specific to human IgG. Our hypothesis was that the antibody backbone in the IC might affect the immunogenicity of the antigen in the IC. Therefore, when we used humanized antibody backbone in the IC to immunize the mice, the human IgG was highly immunogenic in mice.

In the third chapter, we tested this hypothesis by changing the EIC antibody backbone from human to mouse IgG. The mouse monoclonal antibody 6D8 was produced by using the constant region from mAb 278.02 (12), and the variable region genes for both the heavy chain and the light chain were plant-optimized and synthesized. The results in Chapter 3 indicated that the mouse Ebola immune complex (mEIC) was immunogenic in mice to a similar extent as the hEIC. Changing the antibody backbone to use the one that matches the host did not improve the immune response against the EIC.

The fourth chapter presents the immunological study and challenge study of the EIC vaccine delivered with different adjuvants. The EIC was used to immunize mice with or without adjuvants. The immunized mice were then challenged with lethal Ebola virus to determine whether the EIC could protect the mice. The results showed that mice immunized with four doses of the EIC and the adjuvant polyinosinic-polycytidylic acid (PIC) were protected against the Ebola challenge. This validates the hypothesis that plant-produced EIC may be used as an effective vaccine against Ebola virus.

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Chapter 2

EXPRESSION OF AN IMMUNOGENIC EBOLA IMMUNE COMPLEX IN *NICOTIANA BENTHAMIANA*

ABSTRACT

Filoviruses (Ebola and Marburg viruses) cause severe and often fatal hemorrhagic fever in humans and non-human primates. The U.S. Centers for Disease Control (CDC) identify Ebola and Marburg viruses as "category A" pathogens (defined as posing a risk to national security as bioterrorism agents); this categorization has invigorated a search for vaccines that could prevent the disease. Because the use of such vaccines would be in the service of public health, the cost of production is an important component of their development. The use of plant biotechnology is one possible way to cost-effectively produce subunit vaccines. In this work, a geminiviral replicon system was used to produce an Ebola immune complex (EIC) in *Nicotiana benthamiana*. The Ebola glycoprotein (GP1) was fused at the C-terminus of the heavy chain of the humanized IgG monoclonal antibody 6D8, which specifically binds to a linear epitope on the GP1. Co-expression of the GP1-heavy chain fusion and the 6D8 light chain using a geminiviral vector in the leaves of *Nicotiana benthamiana* produced an assembled immunoglobulin, which was purified by ammonium sulfate precipitation and protein G affinity chromatography. The immune complex formation was confirmed by assays that showed that the recombinant protein bound to the

complement factor C1q. Size measurements of purified recombinant protein by dynamic light scattering and size exclusion chromatography also indicated complex formation. Subcutaneous immunization of BALB/C mice with the purified EIC resulted in anti-Ebola virus antibody production at levels comparable to those obtained with a GP1 virus-like particle. These results show excellent potential for a plant-expressed EIC as a human vaccine.

INTRODUCTION

Recombinant protein expression systems are widely used in the biotechnology industry to produce enzymes, protein hormones, monoclonal antibodies, and antigenic proteins for subunit vaccines. Currently available commercial products are produced by fermentation using bacterial suspensions or cultures of insect, yeast, or mammalian cells. Recently there has been substantial progress in the use of green plants as a platform for production of pharmaceutical proteins (2, 43). Advantages in using plants that facilitate industrial adaptation include rapid time frames for production of gram quantities of new proteins for preclinical testing, the ability of plants to produce large and complex proteins with correct processing and assembly into multi-subunit complexes, and recent advances in plant biotechnology allowing the production of glycoproteins with human-like glycosylation patterns (5, 10, 24, 25, 46). In addition, cost advantages of plant-made pharmaceutical proteins relate to capital cost avoidance (plant production vs.

fermentation) for new manufacturing facilities.

Recombinant protein production in plants can be achieved utilizing two different systems: stable genetic transformation or transient gene expression. The establishment of stable transgenic plants is time-consuming, with the major limitation being relatively low levels of protein expression (17, 38). For transient expression, the plant viral vectors can be used to amplify gene copy number, leading to much higher protein expression in comparison to stable transformation. Various types of RNA viruses have been used to create plant viral expression vectors, such as tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), and alfalfa mosaic virus (AIMV) (56). In an effort to improve the efficiency of the traditional viral vector, a deconstructed tobacco mosaic virus (TMV)-based system called magnICON was created (18, 19, 33). The magnICON vector system yielded excellent results with different proteins including enzymes, antibodies, antigens, cytokines, and hormones (17).

DNA viruses such as the geminiviruses can be used as plant viral vectors to express recombinant protein. Geminiviruses have a single-stranded DNA genome that replicates in the nucleus of host cells by a rolling circle replication mechanism using a double-stranded DNA intermediate (20, 47). The viral replication initiation protein (Rep) initiates the replication cycle by binding to a specific viral DNA sequence in the long intergenic region (LIR) and cleaving it, which promotes rolling circle

replication mediated by host cell DNA polymerase and other factors. Rep then ligates the single-stranded DNA products to form a circular viral DNA (29). For recombinant protein expression, the geminiviral coat protein and movement protein can be replaced with the gene of interest. After Rep protein is expressed, the replicon containing the gene of interest is amplified, and the double-stranded DNA intermediates act as transcription templates, leading to high amounts of mRNA and subsequent recombinant protein (24, 25, 37).

Ebola virus, the causative agent of Ebola hemorrhagic fever, is a negative sense, single-stranded RNA virus belonging to family Filoviridae. The shape of the virus is filamentous in form and approximately 80 nm in diameter, but it demonstrates no uniform length (11). There are four distinct species of Ebola virus: Zaire, Sudan, Ivory Coast, and Reston, with the Zaire Ebola virus causing the highest mortality rate among all Ebola viruses, up to 90% (52). Currently, there are no vaccines or antiviral drugs approved for prevention or treatment of this disease in humans. However, there are some new vaccine candidates, which have demonstrated protection in nonhuman primates against lethal challenge with Ebola virus, including Ebola Venezuelan equine encephalitis virus replicon particles (VRP) (39), Ebola virus-like particle (51), recombinant vesicular stomatitis virus-based Ebola vaccine (14, 15, 40), adenovirus-based vaccine (42) and a prime-boost strategy with DNA encoding glycoprotein of the Zaire strain followed with recombinant adenovirus

encoding the same antigen (22, 49). Moreover, a DNA vaccine encoding for the Ebola glycoprotein (GP) has been shown to be both safe and immunogenic in humans (34).

Ebola GP is a glycoprotein containing both N- and O-linked carbohydrate (12, 13). GP is a type I transmembrane protein that forms homotrimers, which create a spike on the viral envelope (31, 54). It mediates viral binding and entry into host cell (30). GP consists of two subunits, the extracellular GP1 and the membrane-anchored GP2, linked by disulfide bonds (32, 44). GP1 is responsible for receptor binding, and GP2 mediates membrane fusion. Transient expression of GP in cultured mammalian cells causes cytopathic effects including cell rounding and detachment (6, 45). GP can down-regulate several cell surface molecules such as major histocompatibility complex class I (48) and integrin (45). Since Ebola GP is the protein that mediates infection and is the major antigen (53), it is a critical target for vaccine development. Ebola GP, or segments thereof, can be expressed in recombinant systems such as *E. coli* (8), insect cells (36, 55), and mammalian cells (35). However, these systems are not optimal, and in order to reduce its toxicity on the host cell, GP1 expression in mammalian cells was regulated by an ecdysone inducible system (35).

Recombinant immune complexes were originally expressed in tobacco plants via fusion of tetanus toxin fragment C (TTFC) to the heavy chain of a TTFC-binding IgG and co-expression with its light chain (7). The

TTFC immune complexes were shown to bind to C1q, Fc receptor gamma RIIa (FcγRIIa), and antigen-presenting cells. Mice immunized with the recombinant TTFC immune complexes showed much higher antibody titers than those immunized with TTFC alone. This study demonstrated the recombinant immune complex as a strong vaccine candidate and led us to pursue a similar strategy with Ebola GP1.

In this study, we used the geminiviral replicon system derived from bean yellow dwarf virus (24, 25, 37) to produce the EIC in *Nicotiana benthamiana*. We fused Ebola GP1 at the C-terminus of the heavy chain of the humanized monoclonal antibody (mAb) 6D8 (25), which specifically binds to a linear epitope on GP1. When the fusion protein was co-expressed with the light chain of mAb 6D8, IgG molecules assembled, leading to IC formation. The EIC were expressed, purified, and used to immunize mice, showing that the plant-expressed EIC represent a viable vaccine candidate for humans.

MATERIALS AND METHODS

Design of the construct for producing the Ebola immune complex

The geminiviral vector pBYK3R was previously described (25). We designed a plant-optimized DNA sequence encoding the Ebola GP1 based upon the GenBank Accession AY354458, using codons that are preferred in tobacco, and removing spurious mRNA processing signals (16). The designed sequence is deposited in the GenBank (Accession HM136775). The GP1 coding sequence was fused via a $(G_4S)_3$ linker to

the C-terminus of a gene encoding the humanized monoclonal antibody 6D8 H2 (25).The linker was added to the 3' end, and an XbaI site was created at the 5' end of the 6D8-H2 gene by end-tailoring PCR using the primers H2-Xba-F (5'-GGTCTAGAACaATGGGATGGTCTTGCATC) and 6D8H2-G4S-Bam (5'-GGGGATCCACCTCCGCCTGAACCGCCTCCACCTGATCCGCCACCTC CTTTACCCGGAGACAAGGAGAG). A BamHI site was created at the 5' end of the GP1 gene by PCR using the primer GP1-Bam (5'-GGGGATCCATCCCACTTGGAGTTATTC), and the 6D8-H2-$((G_4S)_3$ and GP1 genes fused via the BamHI site. The 6D8-H2-$((G_4S)_3$-GP1 fusion was inserted into the geminiviral vector pBYR1 (a derivative of pBYK3R that has unique XbaI, KpnI, and SacI sites for insert cloning) via XbaI and SacI sites. The hexapeptide SEKDEL was added to the C-terminus of the GP1 sequence by end-tailoring with the reverse primer GP1-SEKDEL-Kpn-R (5'-CCGGTACCTTAAAGCTCATCCTTCTCTGAACGCCTAGTTCTTCGTCC) and the modified gene inserted into pBYR1 via XbaI and KpnI sites. H2GP1 is inserted in the geminivector between the LIR and SIR. The light chain of mAb 6D8 or K3 is expressed in another geminivector with Rep protein. When H2GP1 and K3 were expressed in tobacco, the IC was formed because the variable region of mAb 6D8 can specifically bind to the 6D8 epitope of GP1. The dual replicon vector pBYRH2GP1kdK3, containing the fused replicons from pBYH2GP1kd and pBYK3R, was

constructed by ligating three fragments: pBY-HL(6D8).R (25) XbaI-SacI, pBYH2GP1kd/XbaI-HindIII, and pBYH2GP1kd/HindIII-SacI.

Ebola GP1 alone was expressed by co-infiltrating with pBYR6HGP1kd and pPSP19. pBYR6HGP1kd was constructed by fusing the GP1-SEKDEL coding sequence from pBYH2GP1kd (BamHI-SacI) with the soybean vspA N-terminal signal peptide (NcoI-BamHI) from pBTI201.4 (26), which had been modified by addition of a 5' sequence to encode the amino acids M-A-S-S and the 3' sequence encoding a 6-His tag, by PCR using the mutagenic primers aS-MASS (5'-GATCCATGGCTTCCTCTAAGGTCCTTGTTTTCTTCG) and 6H-Bam-R (5'- CGGGGATCCgTGaTGATGGTGATGGTGTC). The two fragments were ligated with pBYR1 (digested NcoI-SacI) to make pBYR6HGP1kd. GFP was expressed by using pBYGFP.R (24), co-infiltrating with pPSp19.

Plant inoculation and protein expression

Nicotiana benthamiana plants were inoculated with *Agrobacterium* by needle infiltration on the lower surface of leaves. The agroinfiltration procedure was performed as previously described (24). For geminivector constructs, final OD600 is 0.25. Plants were maintained in a growth chamber. The leaves were harvested on day 2, 4, 6, and 8 after infiltration for expression time-course experiments. For other experiments, the leaves were harvested on day 4 after infiltration. Soluble proteins were extracted by grinding the leaves with the Fastprep (Bio101) machine in 1ml of extraction buffer (phosphate-buffered saline (PBS) pH7.5, leupeptin, and

0.1% Tween-20) per 0.1mg of leaves. After centrifugation at 13,000 rpm for 5 min, the supernatant was retained for subsequent analysis by ELISA and Western blot.

Sandwich ELISA protocol for Ebola immune complex quantification

The EIC in the plant extract was quantified by ELISA. Goat anti-human IgG was diluted 1:1000 in phosphate-buffered saline (PBS pH7.5; 50µl per well) and was bound to 96-well polyvinylchrolide microtiter plates overnight at 4℃. Each plate was blocked with 5% skim mil k in PBS for 2 hours at 37℃. After washing the wells one time with P BS containing 0.05% Tween 20 (PBST), samples (50µl per well) diluted in 1% skim milk in PBST were added to the wells and incubated 1 hour at 37℃. The wells were washed three times with PBST and incubated with goat anti-human kappa-horseradish peroxidase (HRP) conjugate diluted 1:4000 in 1% skim milk in PBST for 1 hour at 37℃. Plates were develop ed with TMB substrate (Pierce, Rockford, IL) for 5 min at 23℃. Th e reaction was ended by addition of an equal volume of 1M H_2SO_4, and the absorbance was read at 450 nm.

SDS-PAGE and Western blot

Plant protein crude extracts and human IgG were denatured by boiling in SDS-PAGE sample buffer and separated on 4–15% gradient polyacrylamide gels. Proteins were either visualized by Coomassie blue staining or electrophoretically transferred to a polyvinlidene difluoride (PVDF) membrane (Amersham, NJ). To detect the human heavy chain, the membrane was probed with goat anti-human IgG-HRP conjugate (Southern Biotech, AL) diluted at 1:5000 in 1% skim milk in PBST. To detect the human light chain, the membrane was probed with goat anti-human kappa-HRP conjugate (Southern Biotech, AL), diluted at 1:10000 in 1% skim milk in PBST. To detect Ebola GP1, the membrane was incubated with mouse anti-6D8 (antibody against the linear 6D8 epitope in GP1) or mouse anti-13C6 (antibody against a conformational epitope) diluted 1:10000 in 1% skim milk in PBST and goat anti-mouse IgG-HRP conjugate diluted 1:10000 in 1% skim milk in PBST. The membranes were developed by chemiluminescence using ECL plus detection reagent (Amersham, NJ).

C1q binding assay

50µl of 7.5µg/ml human complement C1q in PBS was coated on the wells of a polyvinylchrolide microtiter plate overnight at 4℃. After washing with PBST for 2 times, the plate was blocked with 5% skim milk in PBST for 2 hours at 37℃. The plate was washed with PB ST 1 time. Plant extracts containing 25ng equivalent amounts of human IgG from leaves

expressing the mAb 6D8 or EIC, (as determined by ELISA), were serially diluted, added into the plate, and incubated overnight at 4℃. After washing for 3 times with PBST, the plate was incubated with 1:4,000 goat anti-human kappa-HRP in 1% skim milk in PBST for 1 hour at 37℃. The plate was developed with TMB substrate and read OD450.

Protein purification

Infiltrated tobacco leaves were homogenized by using a blender with extraction buffer (PBS + protease inhibitor tablet from Sigma, MO). Crude extract was filtered through Miracloth and centrifuged at 17700g for 15 min. Ammonium sulfate was added into the supernatant to 35% saturation (194g/l of solution) and mixed with a stirrer at 4^{o}C for 1 hour. The solution was centrifuged at 17700g for 15 min, and the pellet was discarded. Then, ammonium sulfate was added to the supernatant to 60% saturation (151g/l of the solution) and mixed with a stirrer at 4^{o}C for 1 hour. The solution was centrifuged at 17700g for 15 min, and the pellet was dissolved with the extraction buffer. The solution was filtered with a 0.2-micron filter, and Protein G beads (Pierce, Rockford, IL) was added into the filtered solution. The protein extract and the Protein G beads were rotated at 4^{o}C for 1 hour, and then loaded into the column, and the resin beads were allowed to settle. The protein G column was washed with PBS, pH7.5, and eluted with 50mM citric acid, pH2.5. After the protein was eluted from the column, 1M Tris-base was added to neutralize to a final pH of 7.5. The purified protein was filtered through a 0.2 micron filter and

concentrated with Amicon ultra-4 Centrifugal Filter Units-30kDa (Millipore, MA).

Estimation of complex size by dynamic light scattering

The diameter size was measured by Dynamic Light Scattering using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK). The purified plant-made EIC, purified plant-made mAb 6D8, and human IgG (Southern Biotech, AL) were diluted in PBS to 0.1mg/ml concentration and added into the disposable polystyrene cuvette for Zetasizer measurement.

Size exclusion chromatography

A sample volume of 20µl was loaded onto a BioSep SEC-S4000 column, 600X7.8 mm (Phenomenex, CA). The proteins were eluted with PBS, pH 7.3, at a 1ml/min flow rate. A chromatogram was recorded, measuring the UV absorbance at 280 nm. Bovine thyroglobulin (Sigma, MO) and mouse IgG (Southern Biotech, AL) were used as protein markers.

Mice immunization

Female BALB/C mice were subcutaneously immunized with the purified EIC from tobacco leaves. The EIC (10µg dosage, equivalent to ~5µg of Ebola GP1) was injected into the mice. VRP-GP1 (1.6×10^8 replicons per mouse) was used as the positive control (provided by William Pratt, USAMRIID), and PBS was used as the negative control. The mice were immunized on days 0, 21, 42, and 63. Individual

preimmune blood samples were collected before the first immunization, on day 0. Blood samples were obtained 3 weeks after the last immunization.

Serum antibody analysis

The specific serum IgG response was determined by end point titer ELISA. Polyvinyl chloride 96-well ELISA plates were coated with 50µl of irradiated Ebola virus (provided by John Dye, USAMRIID) diluted 1:1000 in PBS and incubated at 4℃ overnight. Plates were bl ocked with 5% skim milk in PBST at 23℃ for 2 hours. Subsequently, the pl ates were incubated with the serum diluted in 1% skim milk in PBST for 1 hour at 37℃ and then HRP-conjugated goat anti-mouse IgG for 1 hour at 37℃. The plates were developed with TMB substrate and read OD450. Endpoint titer was reported as the reciprocal of the highest dilution that had an absorbance value ≥ 0.02 (two times the OD value for pre-immune serum) above the background (absorbance of the well lacking the serum).

RESULTS

Transient expression of the recombinant Ebola immune complex in leaves

We produced the EIC by co-expression of a heavy chain-GP1 fusion protein (H2GP1) with the light chain (K3). We examined EIC expression in *N. benthamiana* leaves using geminiviral replicons. The expression of the viral Rep protein (C1/C2 gene) is required for amplification of the replicon (29). The Rep cassette is contained in the complimentary sense orientation of the light chain vector pBYK3R (Fig. 1). The expression cassettes, driven by the dual-enhancer CaMV 35S promoter, are placed between the long intergenic region (LIR) and the short intergenic region (SIR) in the viral-sense orientation, replacing the viral movement and coat protein genes. In the case of dual replicon vector pBYRH2GP1kdK3, the heavy chain-GP1 fusion and light chain cassettes are placed within different replicons oriented in tandem. Ebola GP1 protein was expressed in plants by using pBYR6HGP1kd, which has a 6His tag at the N-terminus (Fig. 1). We also co-expressed the gene silencing inhibitor p19 from the tomato bushy stunt virus using the non-replicating expression vector pPSp19.

Ebola GP1 expressed from pBYR6HGP1kd produced strong necrosis in leaves. However, fusing the GP1 to the mAb 6D8 reduced the toxicity of the GP1 to the plant (Fig. 2). We compared the protein expression levels using pBYK3R co-delivered with pBYH2GP1 or

pBYH2GP1kd (encoding H2GP1 with SEKDEL at C-terminus), and extracting four days after agroinfiltration. We assayed by ELISA to measure human IgG and found that the SEKDEL construct yielded ~3-fold higher expression than the construct withouth SEKDEL (Fig. 3a). Thus, we used the SEKDEL construct in the dual replicon vector pBYRH2GP1kdK3, which provided somewhat higher expression than co-delivery of the two separate vectors, up to ~50µg IgG per g leaf mass (Fig. 3a). Therefore, we used pBYRH2GP1kdK3+p19 in subsequent experiments to produce and characterize the EIC. A time course of the EIC expression on different days after infiltration showed that the optimal harvest time was 4 days after infiltration (Fig. 3b).

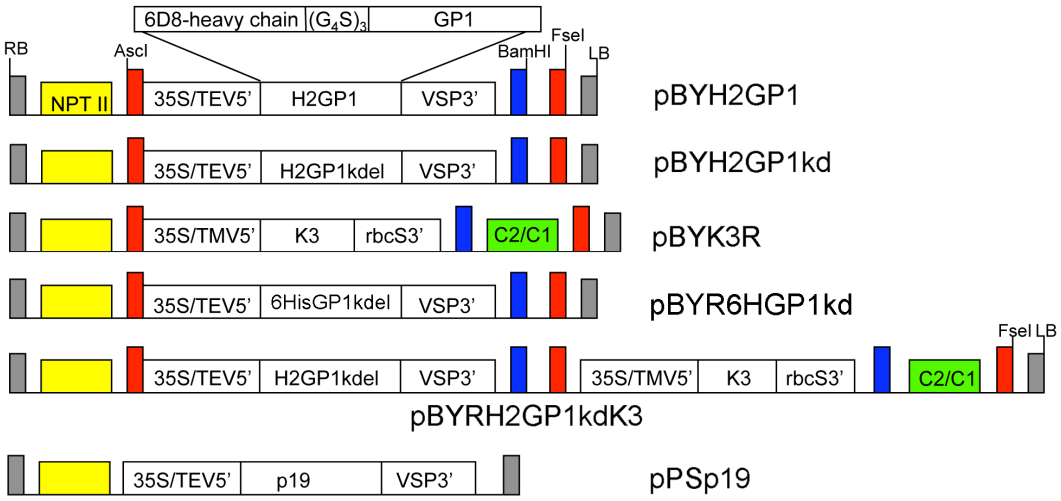


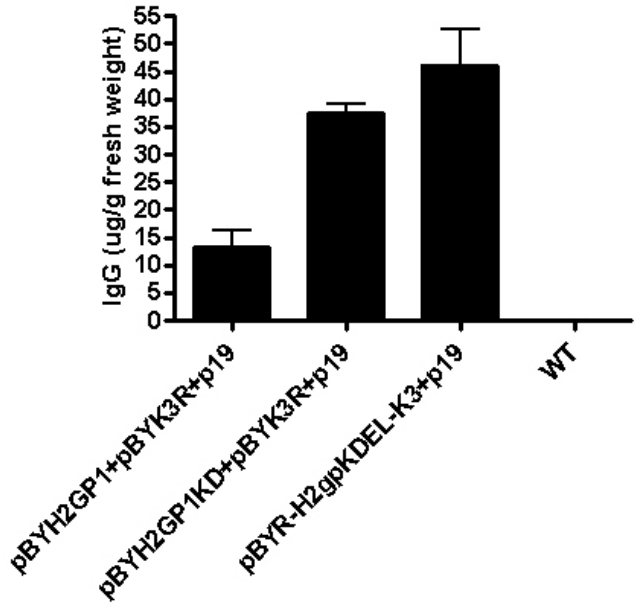
Fig. 1. Schematic representation of the T-DNA region of the vectors used in this study.

35S/TEV5': CaMV 35S promoter with tobacco etch virus 5'UTR; VSP3': soybean vspB gene 3' element, 35S/TMV5': CaMV 35S promoter with tobacco mosaic virus 5'UTR; VSP3': soybean vspB gene 3' element, rbcS3': tobacco Rubisco small subunit gene 3' element, NPTII: expression cassette encoding nptII gene for kanamycin resistance (yellow box), LIR (red box): long intergenic region of BeYDV genome, SIR (blue box): short intergenic region of BeYDV genome, C2/C1 : BeYDV ORFs C1 and C2 which encode for the replication initiation proteins (Rep) and RepA, LB, and RB : the left and right borders of the T-DNA region.

Fig. 2. Typical phenotype of leaves on day 5 expressing the mAb 6D8 (1), Ebola GP1 (2), Ebola immune complex (EIC) (3), and GFP (4).

N. benthamiana leaves were co-infiltrated with pBYH2kdel+pBYK3R+p19 for the mAb 6D8 expression (1), pBYR6HGP1kdel+p19 for Ebola GP1 expression (2), pBYRH2GP1kdelK3+p19 for EIC expression (3), and pBYGFP.R+p19 for GFP expression (4) at a final OD_{600} = 0.25. The leaves were photographed on day 5 after infiltration.

A



B

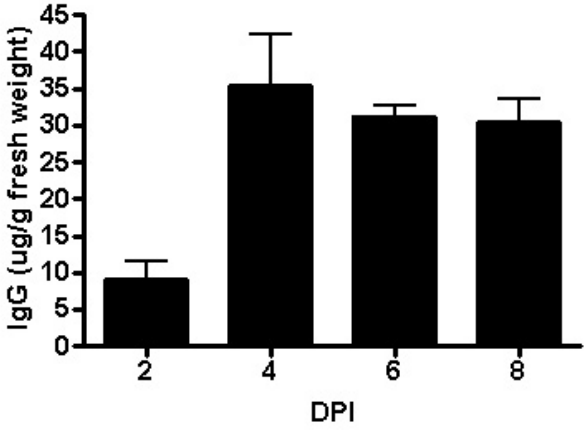


Fig. 3. Expression of the EIC in *N. benthamiana* plants.

A. Protein expression of the EIC compared among different constructs. *N. benthamiana* leaves were co-infiltrated with pBYH2GP1+pBYK3R+p19, pBYH2GP1kdel+pBYK3R+p19, and pBYRH2GP1kdelK3R+p19 at a final OD_{600}=0.25. The leaves were harvested 4 days after infiltration and

extracted to quantify IgG (Experimental Procedures) using ELISA. B. Protein expression levels at different times after agroinfiltration using pBYRH2GP1kdK3 with pPSp19. The leaves were harvested on day 2, 4, 6, and 8 days post-infiltration (DPI). Data are means ±SD of samples from three independent infiltration experiments.

C1q binding assay

C1q is a protein component of the complement cascade of the mammalian innate immune system. C1q binds to the IC with a higher affinity than to antibody alone (28). We used the C1q binding assay to confirm the IC formation in plant leaf extracts. Varying dilutions of leaf extracts expressing the mAb 6D8 (pBYH2kd+pBYK3R) or the EIC (pBYRH2GPkdK3) at equivalent IgG concentrations were incubated with C1q, which was immobilized on the microtiter plate wells. The data (Fig. 4) show that the EIC-expressing leaf extracts produced substantially higher OD signals than the mAb alone, which is consistent with previous observations (28). These data indicate the formation of the IC when the EIC was produced in *N. benthamiana.*

Purification and characterization of the Ebola immune complex

To purify the IC, we used ammonium sulfate precipitation and protein G affinity chromatography. These two steps removed the great majority of the endogenous leaf proteins, including the major plant protein Rubisco, as shown by reducing SDS-PAGE (Fig. 5, lane 3). We estimate that the EIC preparation was ~90% pure, based on visual inspection

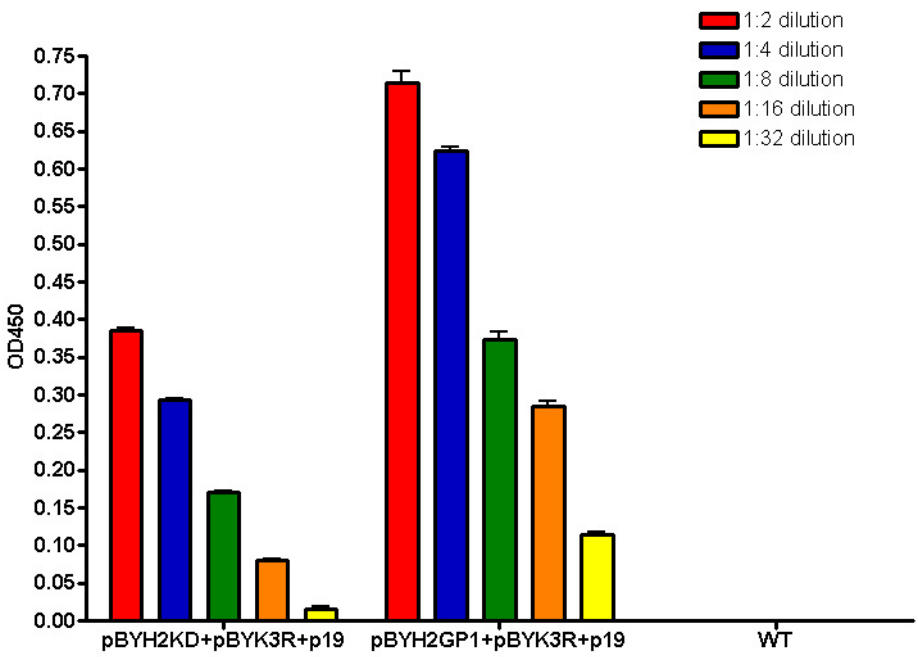


Fig. 4. C1q binding of crude plant extracts.

Crude extracts were quantified for the amount of IgG by ELISA. Crude extracts were diluted to an IgG concentration of 100ng/ml, and then serial 2-fold dilutions were made. The dilutions of crude extracts were added to wells containing immobilized C1q. Detection with HRP-labeled goat anti-human IgG yielded OD450 measurements. Data are means ±SD of samples from three independent infiltration experiments.

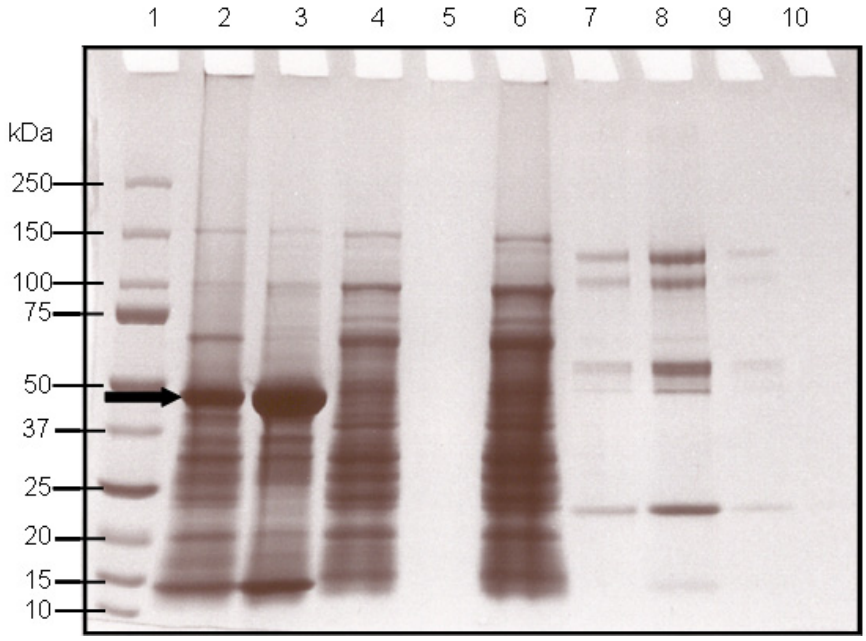


Fig. 5. Purification of the EIC from *N. benthamiana* leaves.

Infiltrated leaves were extracted, and the EIC was purified and analyzed by SDS-PAGE under reducing conditions. Lane 1: Protein ladder; lane 2: clarified crude leaf extract; lane 3: leaf proteins removed by 35% ammonium sulfate precipitation; lane 4: 60% ammonium sulfate precipitate resuspended for Protein G chromatography; lane 5: 100% ammonium sulfate precipitate; lane 6: protein G flow-through fraction; lanes 7-10: sequential elution fractions from Protein G chromatography. The black arrow indicates rbcL protein.

of the Coomassie-stained gels (lane 8 in Fig. 5 and Fig. 6b). We observed that the EIC extracted directly from leaves in SDS sample buffer (reducing conditions) displayed a single band at 130kDa when probed with anti-heavy chain (Fig. 6a). The purified EIC examined on Coomassie-stained gels comprised five different protein species with apparent molecular weights of 130, 110, 55, 50, and 25kDa (Fig. 6b). The Western blot showed that anti-human heavy chain bound to the largest four of the five species (Fig.6c), whereas the anti-GP1 linear epitope 6D8 (53) bound to only the 130kDa protein (Fig. 6e). Anti-human kappa chain antibody bound to the protein at 25kDa (Fig. 6d), which is the expected size of the kappa chain. A gel run under non-reducing conditions and probed with monoclonal antibody 13C6, which binds to a conformational epitope (53), showed a signal at high molecular mass (Fig. 6f), which is consistent with a fully assembled immunoglobulin structure. This confirmed the correct folding of GP1 in the H2-GP1 fusion protein expressed in *N. benthamiana*. The presence of multiple protein species that contain the heavy chain sequence (Fig. 6c) indicates proteolytic degradation during extraction and purification, since the smaller bands were not present when leaves were extracted directly in SDS sample buffer (Fig. 6a). Nonetheless, we conclude that the full length EIC is assembled *in planta* and the GP1 showed correct folding.

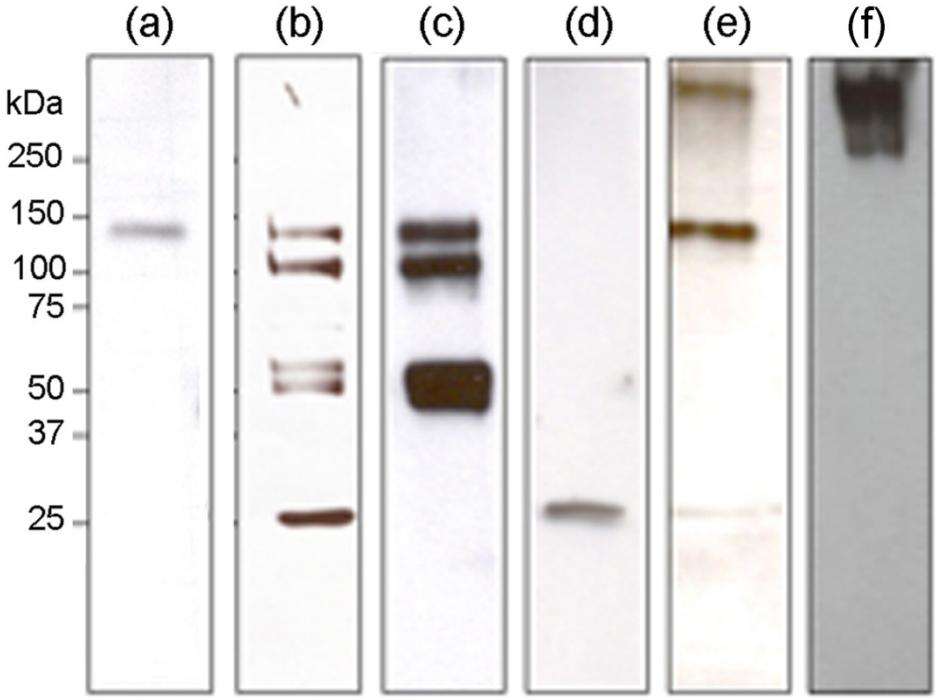


Fig. 6. Western blotting of the EIC.

(a) Crude extract from *N. benthamiana* leaf agroinfiltrated with pBYH2GP1kdel, pBYK3R, and pPS19. The 100mg leaf sample was harvested 4 dpi and extracted with 500µl SDS sample buffer containing 100mM dithiothreitol (DTT), and 20µl of the extract was loaded onto the gel. The Western blot was probed with an anti-human IgG heavy chain. (b-f) Purified EIC from leaves agroinfiltrated with pBYH2GP1kdelK3 and pPS19: (b) Coomassie-stained SDS-PAGE reducing gel; (c) Reducing Western blot detected with anti-human IgG heavy chain; (d) Reducing Western blot detected with anti-human kappa chain; (e) Reducing Western blot detected with anti-linear epitope mAb 6D8; (f) Non-reducing Western blot detected with anti-conformational epitope 13C6.

Molecular sizing of the EIC

We assessed the diameter size of the purified EIC preparation using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK), which measures the hydrodynamic diameter using dynamic light scattering. For these measurements, the purified EIC, the plant-made mAb 6D8, and human IgG (SouthernBiotech, AL) were diluted in PBS, pH7.5, to a concentration of 0.1mg/ml. The data shows that the average diameter of the EIC was ~20nm (Fig. 7), whereas the plant-made mAb 6D8 and standard human IgG were both ~10nm. The diameter of the antibody standard measured by Malvern Instruments was also 10nm (57), which was consistent with our result. Samples were also tested at a lower concentration of 10µg/ml, with the same results (data not shown). Moreover, size exclusion chromatography was used to confirm the complex formation. The mouse IgG and plant-made mAb 6D8 (150 kDa) eluted at 18 min and bovine thyroglobulin (669 kDa) eluted at 15 min (Fig. 8). The EIC eluted over a broad region but had peaks at 11 min and 15–16 min. Therefore, the size of the EIC eluted at 11 min exceeds 669 kDa, which suggests there are more than two molecules in the complex (one molecule is ~310 kDa). The peak at 15–16 min probably represents the dimer, and material eluting between 11–15 min may indicate larger oligomers.

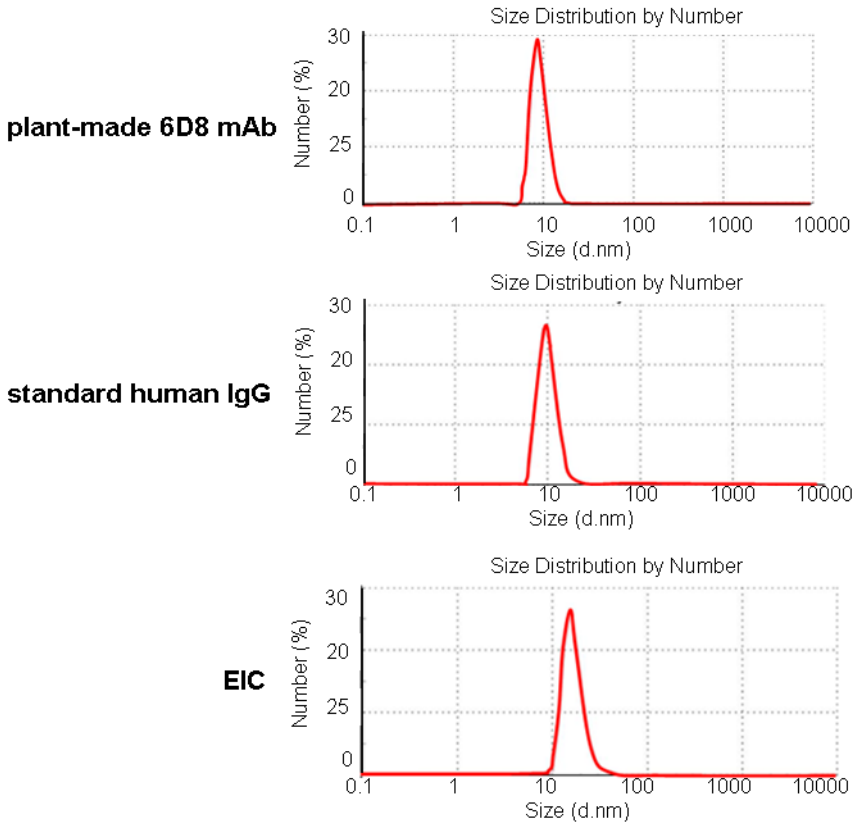


Fig. 7. The size measurement confirmed the complex formation.

The hydrodynamic diameter of the EIC or mAb 6D8 was determined by dynamic light scattering using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK). The abscissa indicates the diameter in nm, and the ordinate indicates the relative number of molecules at that size, comparing human IgG, plant-expressed mAb 6D8 and plant-expressed EIC.

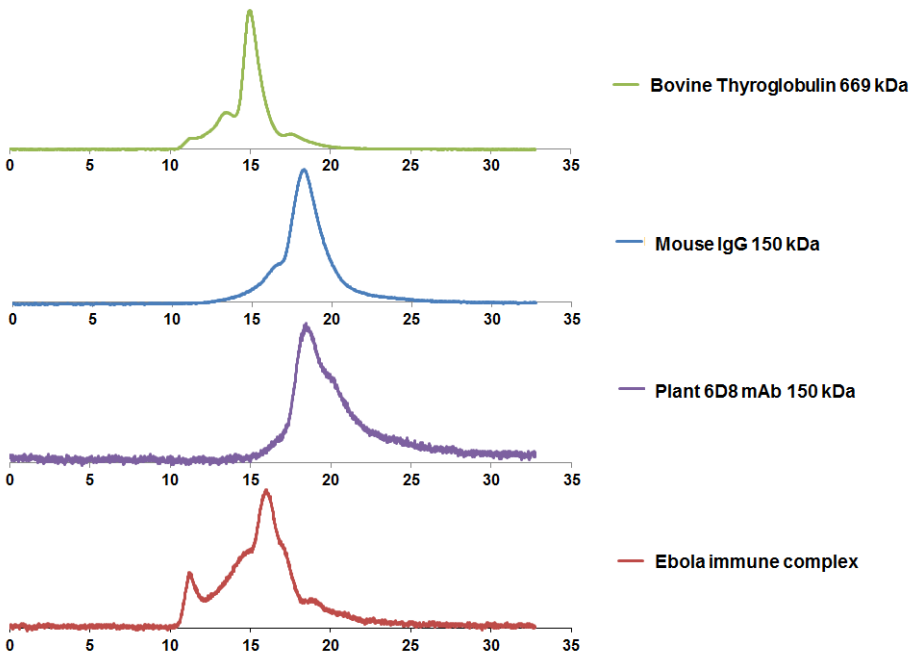


Fig. 8. The size exclusion chromatography confirmed the complex formation.

Size exclusion chromatography was used to determine the size of the IC. The EIC was loaded onto a BioSep SEC-S4000 column (Phenomenex, CA) and eluted with PBS, pH7.3. The elution time in min is shown on the abscissa, and the A_{280} was continuously monitored (colored curves). Bovine thyroglobulin (Sigma, MO), mouse IgG (SouthernBiotech, AL), and plant-expressed mAb 6D8 were used as protein markers.

Immunization study

To examine the immunogenicity of the plant-produced EIC, we immunized mice by subcutaneous injection on days 0, 21, 42, 63, with 10µg purified EIC (measured as IgG equivalent). We used VRP-GP1 (provided by John Dye, USAMRIID) as the positive control (39). Serum antibody responses were evaluated by ELISA using the irradiated Ebola virus as the capture antigen. Three weeks after the last dose, a geometric mean titer (GMT) antibody level in the plant-made EIC group was comparable to the VRP-GP1 group (Fig. 9). A placebo PBS-immunized group showed no anti-Ebola response and was arbitrarily placed at a GMT value of 1.0. These results indicate that plant-made EIC is immunogenic in mice to an extent comparable to VRP-GP1. It should be noted that VRP-GP1 is an alphavirus-based particle and contains a RNA genome that undergoes a single round of replication in the host dendritic cells and macrophages (39).

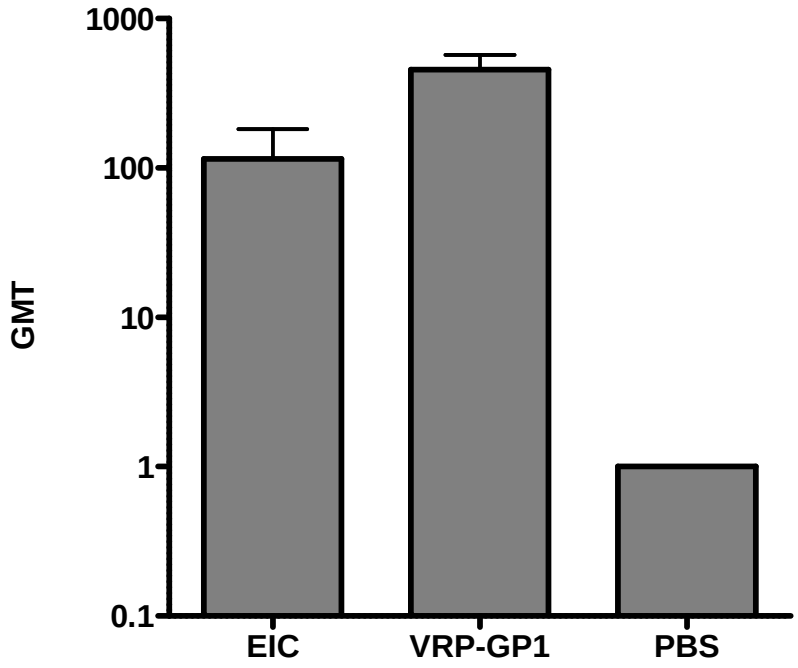


Fig. 9. Anti-Ebola IgG responses in mice immunized with the EIC.

Four groups of seven mice each were immunized subcutaneously with the EIC, VRP-GP1, or PBS. The immunizations were given on days 0, 21, 42, and 63. The serum was collected on day 84 and assayed for anti-Ebola IgG by ELISA, using gamma-irradiated Ebola virus as the capture antigen. The data are presented as Geometric Mean Titer (GMT) $\pm$ 1SD for each group of mice.

DISCUSSION

In this work, *N. benthamiana* plants were used as a bioreactor for the geminiviral replicon system to produce the EIC. The geminiviral replicon system has been used for plant production of several recombinant proteins, including GUS protein (37), GFP (27), staphylococcal enterotoxin (21), and capsid proteins of hepatitis B and Norwalk viruses (24). The system was also shown to produce antibody which is efficiently assembled and retains specific antigen binding properties (25). In this study, the geminiviral replicon system was used to produce a GP1-heavy chain fusion protein, which is approximately 130kDa. The viral Rep protein can be supplied either *in trans* (37) or in the geminiviral vector with the gene of interest (24, 25). In this work, we demonstrate higher protein expression in a single vector system, using pBYRH2GP1kdelK3, than by co-delivery of two separate geminiviral replicons for the H and K chains (Fig. 3). Comparison of the constructs with or without the ER retention signal SEKDEL showed substantially higher expression with those containing SEKDEL. However, there may yet be potential to enhance the yield by optimizing the concentration of *Agrobacterium* in the infiltration, or by inclusion of the p19 expression cassette in pBYRH2GP1kdelK3 in order to avoid the use of two *Agrobacterium* strains for plant inoculation.

The IC resulted from the expression of a fusion protein in which the antigen GP1 was linked to the heavy chain of the mAb 6D8 and co-

expressed with the 6D8 light chain (K3). When the heavy and light chains assemble, the IC can be formed when the 6D8 antigen-combining sites at the N-termini encounter the GP1 6D8 epitope near the C-terminus of another molecule; this process is diagrammatically described in Fig. 10. ICs have several immunological functions, including C1q binding and Fcγ receptor binding, which enhance ability of the antigen to be taken up by antigen-presenting cells (3, 9, 23). Previous studies also demonstrated the binding of IC via the Fc receptor to DC induced DC maturation and promoted the efficient presentation of peptides on major histocompatibility complex classes I and II (4, 41). The enhanced endocytosis, the improved antigen presentation, and thereby the enhanced T cell activation may create the potential for ICs to be used as effective self-adjuvanting vaccines. C1q is a member of the complement cascade, which enhances the ability of the immune system to clear pathogens. It is known to bind to ICs with higher affinity than monomeric IgG (28). In this study, we demonstrated that the EIC binds to C1q significantly better than to the antibody alone. Moreover, the EIC particle size measured by dynamic light scattering indicated IC formation. The diameters of standard human IgG and plant-derived mAb 6D8 were measured at 10nm, which is similar to that reported for IgG by Malvern Instruments, UK (57). The EIC presented a diameter of 20nm, which strongly suggests the formation of the IC.

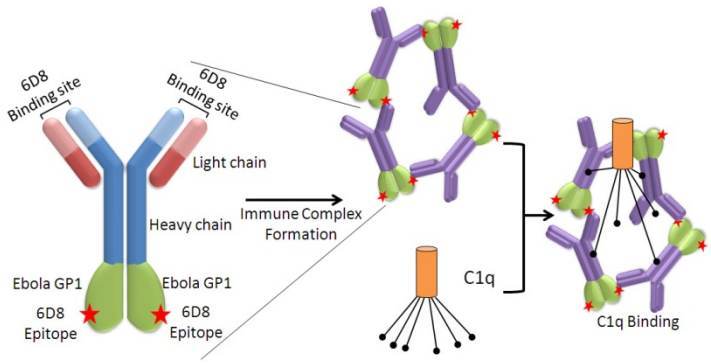


Fig. 10. Diagram illustrating the possible structure of the recombinant IgG and its assembly to form the EIC.

At left, the 6D8 H2-GP1 fusion protein (blue H2 chain, green GP1) is assembled with the light chain (red) to form a chimeric IgG-GP1. The 6D8 epitope on GP1 is shown as a red star. The epitope binding site at the top of the molecule can bind to the 6D8 eptiope displayed on other chimeric molecules, which results in complex assembly (middle, with the IgG component shown in purple). The complement component C1q can bind to the Fc region of IgG molecules that are bound to antigen (right).

Although the structure of Ebola GP1 is unknown, it is reasonable that the 20nm-diameter EIC would accommodate at least four Ag-Ab fusion molecules.

After purification with the protein G affinity chromatography, the material showed five protein species when analyzed under reducing conditions and stained with Coomassie blue dye (Fig. 5, bands at 130, 110, 55, 50, and 25kDa), which indicate proteolytic cleavages in the fusion protein. The calculated approximate size of the full-length glycosylated GP1-heavy chain fusion is ~130kDa, equivalent to the largest band observed. Based on the amino acid sequence, the H2-GP1 fusion protein is expected to be ~100kDa. However, because the GP1 sequence contains 15 potential N-glycosylation sites that may be differentially utilized, the molecular mass could increase by up to ~30kDa. Thus, it is possible that the 110kDa protein represents a glycosylation variant, but it may also represent a fragment resulting from proteolysis at a site proximal to the GP1 C-terminus. The presence of the H-chain species (indicated by the positive reaction in Western blots, Fig. 6) at 55 and 50kDa suggests that some proteolytic degradation of the H2-GP1 occurred during the purification process, despite the use of protease inhibitors. Since extraction of leaves directly in reducing SDS sample buffer yielded only the 130kDa species (Fig. 6a), the full length fusion protein was stable *in planta*, and any degradation occurred only after extraction and processing under non-denaturing conditions. We observed a direct relationship

between the extent of degradation in crude extracts and the time they were kept at 4˚C (data not shown), which further supports the idea of post-extraction instability. Thus, there is potential to increase yields of the EIC by the use of more appropriate protease inhibitors and by decreasing the processing time involved in purification.

It is important to note that the GP1 protein folded correctly to produce authentic antigen. Western blotting with the mAb 13C6, which recognizes a conformational epitope of the Ebola GP1 (53), showed a signal at the expected size under non-reducing conditions (Fig. 6f). This observation indicates that the plant-expressed Ebola GP1 is folded correctly when assembled into a high molecular weight IgG complex.

A further indication that the EIC contains the authentic GP1 antigen is provided by the mouse immunization data. When the EIC was delivered subcutaneously to the mice, it elicited antibodies (Fig. 9) that were specific to the whole Ebola virus. The antibody titers in the mice immunized with the EIC were comparable to those achieved using the VRP-GP1 positive control, which showed complete protection of the mice and guinea pigs from an Ebola virus challenge (39). Since VRP-GP1 is an alphavirus vector, it undergoes limited replication in antigen-presenting cells of the immunized host, thus creating a viral replicon RNA that can act as a powerful adjuvant through Toll-like receptor signaling (1). Thus, the finding that plant-derived EIC produced Ab responses comparable to those obtained with the VRP control is a significant observation that indicates

strong potential for the efficacy of an EIC vaccine. However, the level of Ebola-specific antibody might not correlate with the viral neutralizing activity and protective efficacy. These issues will be addressed in future studies.

Our results are consistent with those of a previous study (7), which showed that a TTFC immune complex produced from *Nicotiana tabacum* enhanced immune response and acted as a “self-adjuvant” when used to immunize mice. In that study, a mouse IgG was fused with TTFC, and the resulting TTFC immune complexes were found to bind to mouse dendritic cells and induce significantly higher anti-TTFC IgG in mice compared to that induced by TTFC antigen alone. We detected substantial levels of antibody against human IgG in the serum of our immunized mice (data not shown). This result is not surprising, since we used the humanized antibody 6D8 in the IC, in order to use the EIC as a vaccine in humans. Therefore, it is possible that the anti-human IgG responses in the mice interfered with optimal anti-GP1 immune responses. We predict that use of the EIC in humans will induce a stronger anti-Ebola immune response, due to the potential for targeting the GP1 to antigen-presenting cells.

Since there is no commercial source of recombinant Ebola GP1, we could not directly determine anti-GP1 specific IgG. Previous studies indicated that transient expression of the Ebola GP caused toxicity in mammalian cells, including cell rounding and detachment (6, 45). When we attempted GP1 expression in *N. benthamiana,* it caused severe

necrosis (Fig. 2), which is consistent with the effects observed in mammalian cells. Interestingly, when the GP1 was fused to an antibody-forming immune complex, the toxicity of the GP1 to the leaves was reduced compared to the unfused GP1 (Fig. 2). Since unfolded protein response can contribute to cell death response (50), fusion of the GP1 to the IgG H-chain may allow more efficient folding in the ER, perhaps by recruitment of ER chaperones and the limitation of an ER stress response.

In conclusion, this study provides evidence that the EIC can be produced in *N. benthamiana* using the geminiviral replicon system, accompanied by folding of GP1 into an immunogenic complex that contains a conformation-specific epitope. The EIC is immunogenic in mice, eliciting antibody levels comparable to the “gold standard” VRP-GP1. Future work will focus on enhancing the vaccine efficacy of plant-produced EIC by optimization of dosage and by use of adjuvants that may enhance cell-mediated as well as humoral immune responses. A challenge study will be performed to determine whether the plant-expressed EIC can protect animals from Ebola virus infection.

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Chapter 3

EXPRESSION OF AN IMMUNOGENIC MOUSE EBOLA IMMUNE COMPLEX IN *NICOTIANA BENTHAMIANA*

ABSTRACT

The use of immune complexes can be applied to vaccine development since they can induce both the cell-mediated and humoral immune responses. In Chapter 2, we demonstrated that plant-expressed humanized Ebola immune complex (hEIC) is immunogenic in mice. However, the antibody responses induced in mice did not reach the levels necessary for complete protection against a lethal Ebola virus challenge. We hypothesized that the use of a mouse IgG backbone in the EIC, instead of the humanized sequence, would improve the immunogenicity of the EIC in mice. In order to test this hypothesis, we produced a mouse Ebola immune complex (mEIC) by replacing the human antibody backbone in hEIC with the mouse mAb 6D8. We reconstructed the mouse 6D8 using the heavy an light chain constant region coding sequences from the mouse mAb 278.02, and fusing them to plant-optimized coding sequences for the mouse heavy and light chain variable regions of 6D8. We expressed the mouse 6D8 in *Nicotiana benthamiana* leaves, purified it, and showed that it binds to irradiated Ebola virus. In the next step, we fused the Ebola GP1 coding sequence to the C-terminus of the heavy chain of the mouse mAb 6D8 to produce the mEIC. After expression and purification, both hEIC and mEIC were compared for their immunogenicity

in mice. The results indicated that the levels of anti-Ebola IgG produced in mice immunized with mEIC is similar to that of the mice immunized with hEIC. Thus, even when the mAb component of the EIC matches with the mouse host, the EIC still does not induce sufficient antibody response for protection against Ebola.

INTRODUCTION

The success of a vaccine in inducing protective immune response is associated with the efficacy of vaccine uptake by antigen-presenting cells (APC), such as dendritic cells (DC) and macrophages. For vaccination to occur, APC must internalize antigens at the vaccination site and transport them to the lymph nodes, where the antigens are processed and the antigenic peptides presented on class I and class II MHC molecules for the activation of CD8+ and CD4+ T cells, respectively (1). A major factor contributing to a vaccine's low immunogenicity can be the inferior capacity of APC to take up the antigen. The immunogenicity of vaccines can be enhanced by administering them in the form of an IC, which combines the antigen and antibody (Ab) specific to that antigen. APC such as DC and macrophages have Fcγ receptors (FcγR) that can bind to the Fc portion of the Ab in the IC (9, 11). This Fc/FcγR interaction induces the internalization of the vaccine by the APC, leading to increased efficiency in processing and presentation of vaccines. The immunogenicity of vaccines can be increased 10- to 1,000-fold when administered as the IC with their corresponding Abs, such as in the case of the vaccines

against tetanus toxoid (3, 12), hepatitis B envelope Ag (2), and Simian immunodeficiency virus (SIV) (13).

In the Chapter 2, a humanized Ebola immune complex (hEIC) was developed by fusing the Ebola GP1 to the C-terminus of the humanized monoclonal antibody (mAb) 6D8. The hEIC was expressed in *Nicotiana benthamiana* leaves, extracted, and purified. After subcutaneous injection into mice, anti-Ebola antibodies were produced, but the antibody titer was not high enough for protection against a lethal Ebola virus challenge. Although the anti-Ebola responses were low, the mice produced high levels of antibodies against human IgG, which suggests that the human IgG backbone of hEIC was immunodominant and could interfere with efficient immune responses against GP1.

Other studies showed that the administration of the IC formed with a xenogeneic antibody was not able to induce both humoral and cell-mediated immune responses, whereas using syngeneic antibody in the IC induced both types of immune responses (6, 7). In these studies, mice immunized with human serum albumin (HSA) bound to rabbit anti-HSA IgG produced lower antibody responses to HSA as compared to the mice immunized with HSA bound with mouse anti-HSA IgG.

The goal of the present study is to test whether plant-derived EIC containing mouse IgG, instead of human IgG, can increase the immunogenicity in mice. We used the mouse mAb 6D8 as the backbone in the IC, and used the mouse Ebola immune complex (mEIC) and hEIC

produced in *Nicotiana benthamiana* leaves to subcutaneously immunize mice. The immunogenicities of hEIC and mEIC were compared by measurement of the antibody responses against Ebola virus present in the serum of the immunized mice.

MATERIALS AND METHODS

Design of the constructs

Plant-optimized DNA sequences encoding the variable regions for both the heavy and light chains of mouse mAb 6D8 were designed based on the sequences obtained from Mapp Biopharmaceutical, Inc. (San Diego, CA), using codons that are preferred in tobacco, and removing spurious mRNA processing signals. The plant-optimized synthetic genes were obtained from a commercial supplier (Integrated DNA Technologies, Coralville, IA). Fig. 1 shows all the constructs that were used to produce the mouse 6D8 mAb and the mEIC

For pBYHGP1kdel, the constant region was amplified from the DNA encoding the heavy chain of the mAb 278.02 (3) using the primer NheImCHFor (5'-TCAGCTAGCACAACAGCCCCATCGGTCTA), which added a NheI restriction site at the 5' end and the primer BamHImCHBack (5'-GATGGATCCACCTCCGCCTGAACCGCCTCC), which added a BamHI at the 3' end. The synthetic gene encoding the heavy chain variable region was digested with NcoI and NheI.

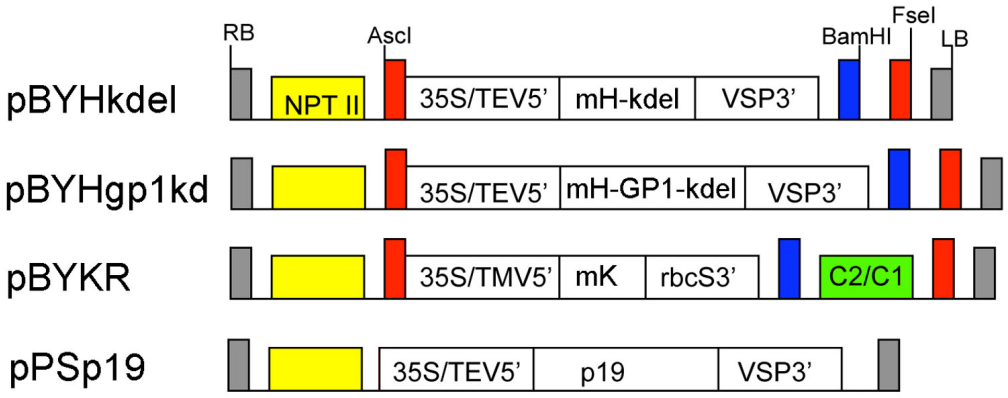

Fig. 1. Schematic representation of the T-DNA region of the vectors used in this study.

35S/TEV5': CaMV 35S promoter with tobacco etch virus 5'UTR; mH-kdel: coding sequence for mouse mAb 6D8 heavy chain with SEKDEL added to C-terminus, VSP3': soybean vspB gene 3' element, mH-GP1-kdel: coding sequence for mouse heavy chain-GP1 fusion, 35S/TMV5': CaMV 35S promoter with tobacco mosaic virus 5'UTR, mK: coding sequence for mouse mAb 6D8 kappa (light) chain, rbcS3': tobacco Rubisco small subunit gene 3' element, NPT II : expression cassette encoding nptII gene for kanamycin resistance (yellow box), LIR (red box) : the long intergenic region of BeYDV genome, SIR (blue box): the short intergenic region of BeYDV genome, C2/C1 : BeYDV ORFs C1 and C2 which encode the replication initiation proteins (Rep) and RepA, LB and RB : the left and right borders of the T-DNA region.

The PCR product encoding the constant region was digested with NheI and BamHI. The geminiviral vector pBYH2GP1kdel (Chapter 2) was digested separately with BamHI/KpnI, SbfI/KpnI, and SbfI/NcoI, and the resulting three fragments were joined together with the NcoI/NheI and NheI/BamHI fragments described above in a five-piece ligation.

For pBYK1R, the gene encoding the constant region of the light chain of mAb 278.02 (3) was amplified using the primer XhoImCLFor (5'-CTGCTCGAGATCAAACGGGCTGATGCTGC), which added a XhoI restriction site at the 5' end, and the primer KpnImCLBack (5'-CTGGGTACCCTAACACTCATTCCTGTTGA), which added a KpnI site at the 3' end, and the resulting product was digested with XhoI/KpnI. The synthetic variable region gene was obtained by digestion with NcoI and XhoI. The geminiviral vector pBYK3R (Chapter 2) was digested separately with SbfI/KpnI and SbfI/NcoI, and the resulting two fragments were joined together with the NcoI/XhoI and XhoI-KpnI fragments described above in a four-piece ligation.

For pBYHkdel, the gene encoding the heavy chain of the mouse mAb 6D8 was amplified from pBYHGP1kdel using the primers H-Forward (5'-CCTTCGCAAGACCCTTCCTC) and H-Backward (5'-CTCGGTACCTTAAAGCTCATCCTTCTCTGAATTCGCGCGCACGTACGGT). The PCR product was digested with SwaI and KpnI and inserted into the pBYGP1kdel that had been digested with SwaI and KpnI.

For pBYK1R, the constant region of the light chain was amplified from the DNA encoding the light chain of mAb 278.02, and XhoI was added at the 5’ end and KpnI at the 3’ end. The variable region was digested with NcoI and XhoI. The constant region was digested with XhoI and KpnI. These two fragments were inserted into the Geminiviral vector pBYK3R, which was digested by NcoI, SbfI, SbfI, and KpnI by four-piece ligation.

For pBYHkdel, the heavy chain of the mouse mAb 6D8 was amplified from pBYHGP1kdel using H-Forward (5’ ccttcgcaagacccttcctc 3’) and H-Backward (5’ ctcggtaccttaaagctcatccttctctgaattcgcgcgcacgtacggt 3'). The PCR product was digested with SwaI and KpnI and inserted into pBYGP1kdel that had been digested with SwaI and KpnI.

Plant inoculation and protein expression

N. benthamiana leaves were infiltrated with *Agrobacterium* by needle infiltration on the lower surface of leaves. The agroinfiltration procedure was performed as previously described in Chapter 2. For geminiviral constructs, the final OD_{600} of *Agrobacterium* was 0.25. Plants were maintained in a growth chamber. The leaves were harvested on day 4 after infiltration. Soluble proteins were extracted by grinding the leaves with a Fastprep machine in 1ml of extraction buffer (phosphate-buffered saline (PBS), pH7.5, leupeptin, and 0.1% Tween-20) per 0.1mg of leaves. After centrifugation at 13,000 rpm for 5 min, the supernatant was retained for later analysis by ELISA and Western blot.

Sandwich ELISA protocol for antibody quantification

The EIC in the plant extract was quantified by ELISA. Goat anti-mouse IgG was diluted 1:1000 in phosphate-buffered saline (PBS, pH 7.5; 50µl per well) and bound to 96-well polyvinylchrolide microtiter plates overnight at 4℃. Each plate was blocked with 5% skim mil k in PBS for 2 hours at 37℃. After washing the wells one time with P BS containing 0.05% Tween 20 (PBST), samples (50µl per well) diluted in 1% skim milk in PBST were added to the wells and incubated 1 hour at 37℃. The wells were washed three times with PBST and incubated with a goat anti-mouse kappa-horseradish peroxidase (HRP) conjugate diluted 1:4000 in 1% skim milk in PBST for 1 hour at 37℃. Plates were develop ed with TMB substrate (Pierce, Rockford, IL) for 5 min at 23℃. The reaction was ended by the addition of an equal volume of 1M H_2SO_4, and the absorbance was read at 450 nm.

SDS-PAGE and Western blot

Plant leaf crude extracts and mouse IgG reference standard (Invivogen, San Diego, CA) were denatured by boiling in SDS-PAGE sample buffer and separated on 4–15% gradient polyacrylamide gels. Proteins were either visualized by Coomassie blue staining or electrophoretically transferred to a polyvinlidene difluoride (PVDF) membrane (Amersham, NJ). To detect the mouse heavy chain, the membrane was probed with goat anti-mouse IgG-HRP conjugate (SouthernBiotech, AL) diluted at 1:5000 in 1% skim milk in PBST. To

detect the mouse light chain, the membrane was probed with goat anti-mouse kappa-HRP conjugate (SouthernBiotech, AL) diluted at 1:10000 in 1% skim milk in PBST. To detect Ebola GP1, the membrane was incubated with human anti-6D8 (antibody against linear 6D8 epitope in GP1) (5) diluted 1:10000 in 1% skim milk in PBST and goat anti-human IgG-HRP conjugate diluted 1:10000 in 1% skim milk in PBST. The membranes were developed by chemiluminescence using ECL plus a detection reagent (Amersham, NJ).

Protein purification

Infiltrated tobacco leaves were homogenized by using a blender with extraction buffer (PBS + protease inhibitor tablet from Sigma, St.Louis, MO). The crude extract was filtered through Miracloth and centrifuged at 17,700g at 4˚C for 15 min. Ammonium sulfate was added into the supernatant to 35% saturation (194 g/l of the solution) and mixed with stirrer at 4^{o}C for 1 hour. The solution was centrifuged at 17,700g for 15 min, and the pellet was discarded. Ammonium sulfate was added to the supernatant to 60% saturation (151 g/l of the solution) and mixed with a stirrer at 4^{o}C for 1 hour. The solution was centrifuged at 17,700g for 15 min, and the pellet was dissolved with the extraction buffer. The solution was filtered with a 0.2 micron filter, and protein G beads (Pierce, Rockford, IL) was added into the filtered solution. The protein extract and the protein A beads were rotated at 4^{o}C for 1 hour, and then loaded into the column and the resin allowed to settle. The protein A column was washed with

PBS, pH 7.5, and eluted with 50mM citric acid, pH 2.5. After the protein was eluted from the column, 1M Tris-base was added to neutralize to a final pH of 7.5. The purified protein was filtered through a 0.2 micron filter and concentrated with Amicon ultra-4 Centrifugal Filter Units-30kDa (Millipore, Billerica, MA).

Ebola binding assay

Polyvinyl chloride 96-well ELISA plates were coated with 50ml irradiated Ebola virus (from Dr.William Pratt, USAMRIID), diluted 1:1,000 in PBS and incubated at 4^{o}C overnight. Plates were blocked with 5% skim milk in PBST at room temperature for 2 hours. The plates were incubated with different concentrations of the plant-derived mouse mAb 6D8, the mouse mAb 6D8, and the mouse IgG (SouthernBiotech, Birmingham, AL) diluted in 1% skim milk in PBST for 1 hour at 37^{o}C. HRP-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) was added and incubated for 1 hour at 37^{o}C. The plates were developed with TMB substrate (KPL, Gaithersburg, MD), and the OD was read at 450 nm.

Mice immunization

Female BALB/C mice were subcutaneously immunized with purified hEIC and mEIC from tobacco leaves. A dose of 10µg of the EIC was injected into each mouse. VRP-GP1 (1.6×10^8 replicons per mouse) injected subcutaneously was used as the positive control (provided by William Pratt, USAMRIID), and PBS was used as the negative control. The mice were immunized on day 0, 21, 42, and 63. Individual preimmune blood samples were collected before the first immunization, on day 0. Blood samples were obtained three weeks after last immunization.

Serum antibody analysis

The specific serum IgG response was determined by end point titer ELISA. Polyvinyl chloride 96-well ELISA plates were coated with 50µl of irradiated Ebola virus (provided by John Dye, USAMRIID) diluted 1:1000 in PBS and incubated at 4℃ overnight. Plates were bl ocked with 5% skim milk in PBST at 23℃ for 2 hours. Subsequently, the pl ates were incubated with the serum diluted in 1% skim milk in PBST for 1 hour at 37℃ and then with the HRP-conjugated goat anti-mouse IgG for 1 hour at 37℃. The plates were developed with TMB substrate and read OD450. Endpoint titer was reported as the reciprocal of the highest dilution that had an absorbance value ≥ 0.02 (two times the OD value for pre-immune serum) above the background (absorbance of the well lacking the serum).

RESULTS

Transient expression, purification, and characterization of mouse mAb 6D8 in plants

N. benthamiana leaves were infiltrated with different cultures of the *A. tumefaciens* strain LBA4404 bearing one of the expression constructs, pBYHkdel, pBYKR, or p19 (Fig. 1). After co-infiltration with all three constructs, the plants were harvested on day 4 after infiltration. The antibody was purified by using ammonium sulfate precipitation and protein A affinity chromatography. Fig. 2 lane 2 shows that most Rubisco protein, the major plant protein, was precipitated at 35% ammonium sulfate. Fig. 2 lane 6 indicates the elution fraction from protein A chromatography, which has the purified antibody composed of the heavy chain and the light chain, was comparable to the mouse IgG standard (lane 7). Mouse mAb 6D8 was purified to >80% purity with intact light and heavy chains (Fig. 2, lane 6). The purified mAb was examined in a binding assay in which different concentrations of the plant-derived mouse mAb 6D8 were incubated with an irradiated Ebola virus coated on an ELISA plate. A generic mouse IgG was used as a negative control for the assay. The mouse mAb 6D8 (a gift from Dr. William Pratt, USAMRIID) was used as a positive control. Fig. 3 shows that OD450 increased as more of the mAb 6D8 was applied in the reaction. In contrast, the OD_{450} for the generic mouse IgG remained at a basal level regardless of the amount of this IgG used in the reaction (Fig. 3).

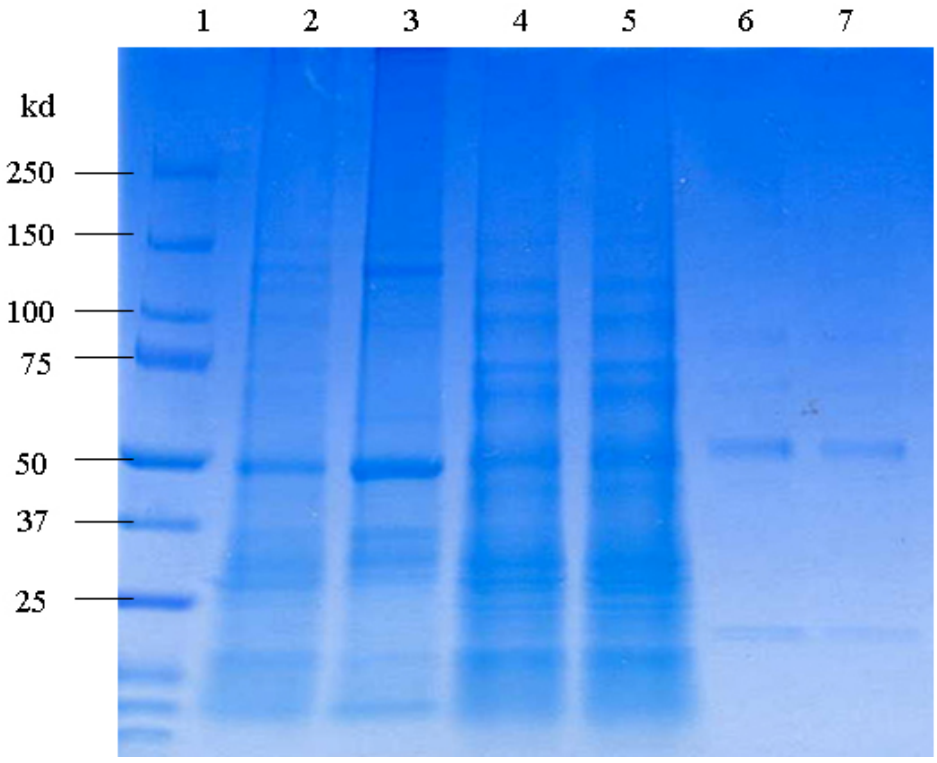

Fig. 2. Purification of mouse mAb 6D8 from *N. benthamiana* leaves.

Infiltrated leaves were extracted, and mouse mAb 6D8 purified and analyzed by SDS-PAGE under reducing conditions (100mM DTT). Lane 1: Protein ladder; lane 2: clarified crude leaf extract; lane 3: leaf proteins removed by 35% ammonium sulfate precipitation; lane 4: 60% ammonium sulfate precipitate resuspended for protein A chromatography; lane 5: protein A flow-through fraction; lane 6: purified mouse mAb 6D8 in the protein A eluate; lane 7: mouse IgG as a reference standard

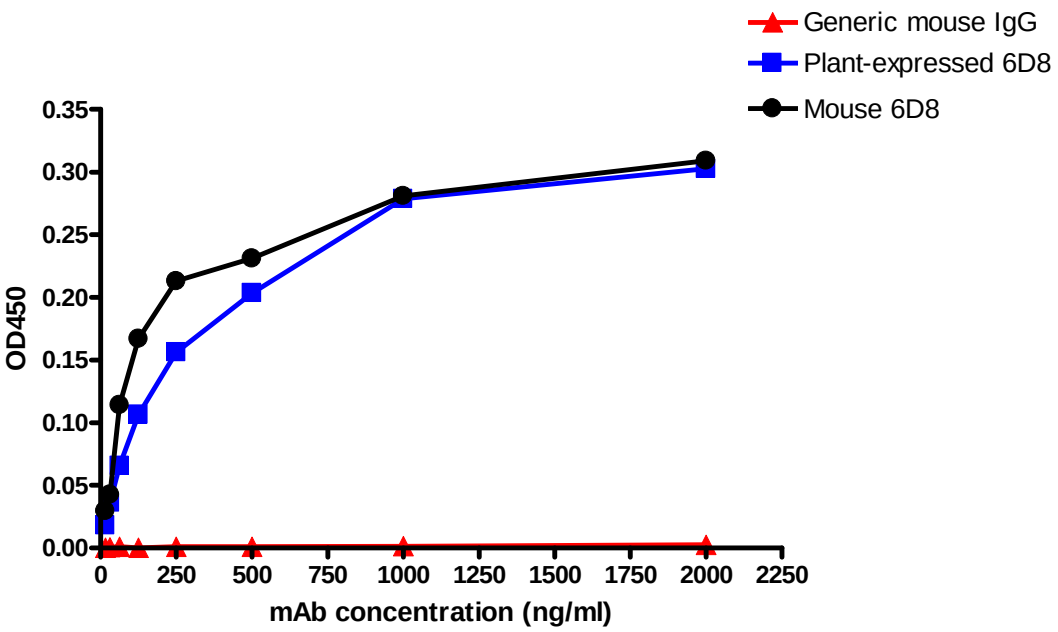

Fig. 3. Plant-derived mouse mAb 6D8 shows specific binding to the Ebola virus.

Plant-derived mAb 6D8, with concentrations between 0 and 2,000ng/ml, was incubated with irradiated Ebola virus immobilized on a 96-well ELISA plate. Mouse mAb 6D8 bound to the Ebola virus was detected by anti-human IgG secondary antibody conjugated with horseradish peroxidases. A generic mouse IgG was used as a negative control and a mouse mAb 6D8 (from Dr. William Pratt, USAMRIID) was used as a positive control. An increase in the OD450 indicates formation of the complex.

This result indicates that plant-produced mouse mAb 6D8 retains its specific affinity for the Ebola virus GP1 protein, which is similar to humanized mAb 6D8 produced from *N. benthamiana* leaves (5).

Purification and characterization of the mouse Ebola immune complex from *N. benthamiana* leaves

N. benthamiana leaves were infiltrated with three different cultures of *A. tumefaciens* strain LBA4404, each bearing one of three different constructs, pBYHGP1kdel, pBYKR, or p19 (Fig. 1). The leaves were harvested on days 2, 4, 6, and 8 after infiltration, and ELISA was used to measure the amount of the antibody. Fig. 4 shows the level of the mEIC expression on different days after infiltration, and the result confirms the highest expression level occurred on day 4 after infiltration. To purify the IC, we used ammonium sulfate precipitation followed by protein A affinity chromatography. SDS–PAGE and Coomassie blue staining of purification fractions showed that the combination of ammonium sulfate precipitation and protein A affinity chromatography effectively removed most plant host proteins, including the most abundant plant protein, Rubisco. Fig. 5 shows that the mEIC was purified from other plant proteins (lanes 6, 7, and 8). Western blot analysis shows that anti-mouse heavy chain, anti-mouse light chain, and anti-GP1 bind to the protein at the same position on a non-reducing gel (Fig. 6a).

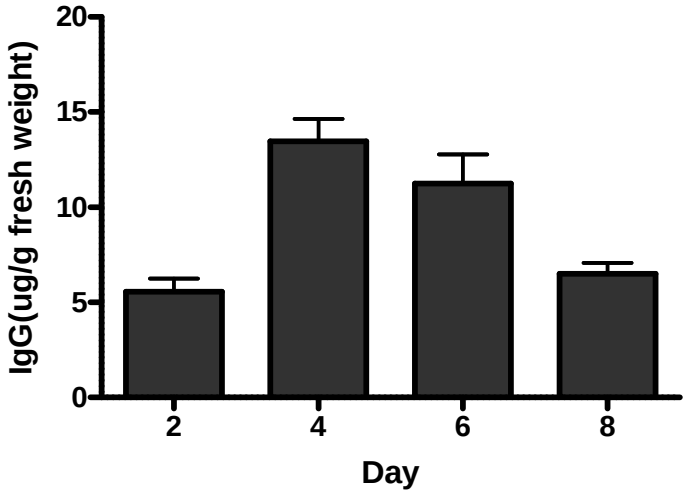

Fig. 4. Expression level of the mEIC in *N. benthamiana* leaves.

The protein expression levels were measured at different times after agroinfiltration with the combination of pBYHGP1kdel, pBYKR, and pPSp19. The leaves were harvested on days 2, 4, 6, and 8 after infiltration. Data are means ±1SD of samples from three independent infiltration experiments.

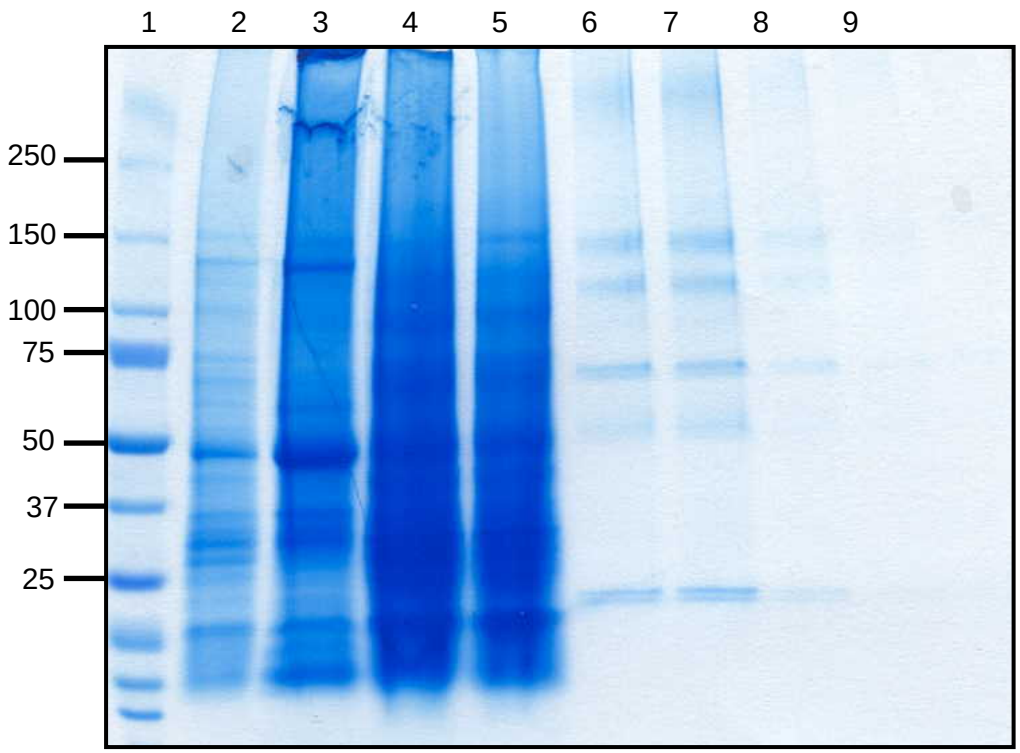

Fig. 5. Purification of the mEIC from *N. benthamiana* leaves.

Lane 1: Protein ladder; lane 2: clarified crude leaf extract; lane 3: leaf proteins removed by 35% ammonium sulfate precipitation; lane 4: 60% ammonium sulfate precipitate resuspended for protein A chromatography; lane 5: protein A flow-through fraction; lanes 6-9: sequential elution fractions from protein A chromatography.

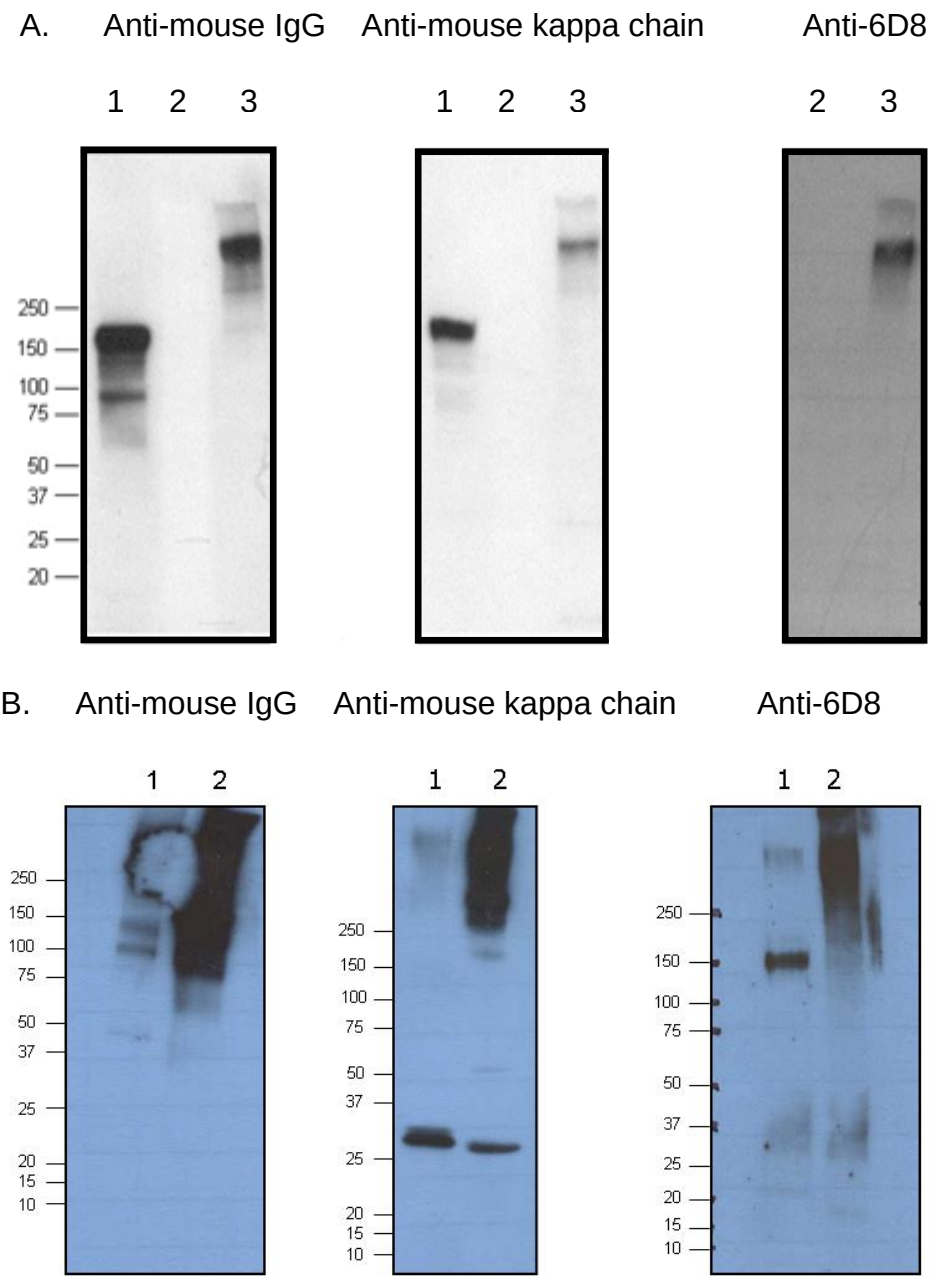

Fig. 6. Western blotting of the mEIC.

Crude extracts from *N. benthamiana* leaves agroinfiltratred with the combination of pBYHGP1kdel, pBYKR, and pPS19. The 100mg leaf samples were harvested 4 dpi and extracted with 500μl SDS sample buffer containing 100mM DTT, and 20μl of the extract was loaded onto the

gel. A. Reducing Western blot detected with anti-mouse IgG, anti-mouse kappa, and anti-6D8. Lane 1: mouse IgG, lane 2 wild type non-infiltrated plant, and lane 3: mEIC. B. Comparing reducing and nonreducing Western blots detected with anti-mouse IgG, anti-mouse kappa, and anti-6D8. Lane 1: reducing sample (100mM DTT), lane2: nonreducing sample.

On the reducing gel, goat-anti mouse IgG detected the bands at 130, 110, 55, and 50 kDa, similar to the hEIC (Chapter 2), whereas goat anti-mouse kappa and mouse anti-GP1 protein bound to the bands at 25 kDa and 130 kDa, respectively. This result confirms the expression of mouse IgG fused with the Ebola GP1.

Molecular sizing of mouse Ebola immune complex

We assessed the diameter size of the purified EIC preparation using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK), which measures the hydrodynamic diameter using dynamic light scattering. For these measurements, the purified mEIC, plant-made mouse mAb 6D8, and mouse IgG (SouthernBiotech, Birmingham, AL) were diluted in PBS, pH 7.5, to a concentration of 0.1mg/ml. The data show that the average diameter of mEIC was ~20nm, whereas plant-made mouse mAb 6D8 and standard mouse IgG were ~10nm (Fig. 7). The diameter of the antibody standard measured by Malvern Instruments (14) was also 10nm, which was consistent with our result. This result confirms that the mEIC is of similar size as the hEIC (Chapter 2).

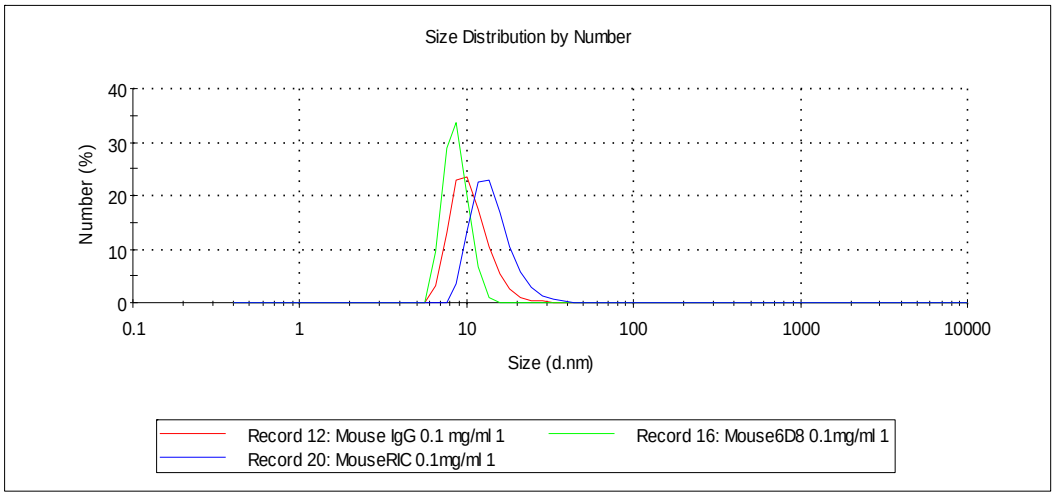

Fig. 7. Hydrodynamic diameter of the mEIC or the mouse mAb 6D8 determined by dynamic light scattering, using a Zetasizer nano-ZS (Malvern Instruments, UK).

The abscissa indicates the diameter in nm, and the ordinate indicates the relative number of molecules at that size. Blue indicates plant-produced mEIC; red indicates generic mouse IgG; and green indicates plant-produced mouse mAb 6D8.

Immunization study

To compare the immunogenicities of the plant-produced mEIC and the hEIC, we immunized mice by subcutaneous injection, on days 0, 21, 42, and 63 with 10µg purified hEIC or mEIC (measured as the IgG equivalent). We used VRP-GP1 (provided by John Dye, USAMRIID) as the positive control (8). Serum antibody responses were evaluated using ELISA with irradiated Ebola virus as the capture antigen. Fig. 8 shows that the mEIC induced anti-Ebola IgG at the same level as the hEIC, which is comparable to that obtained by immunization with VRP-GP1. This result indicates that the mEIC is immunogenic in mice, but the GMT of anti-Ebola IgG in the immunized mice is approximately 200 (Fig. 8), which still did not reach the protection level of GMT equal to or greater than 10,000.

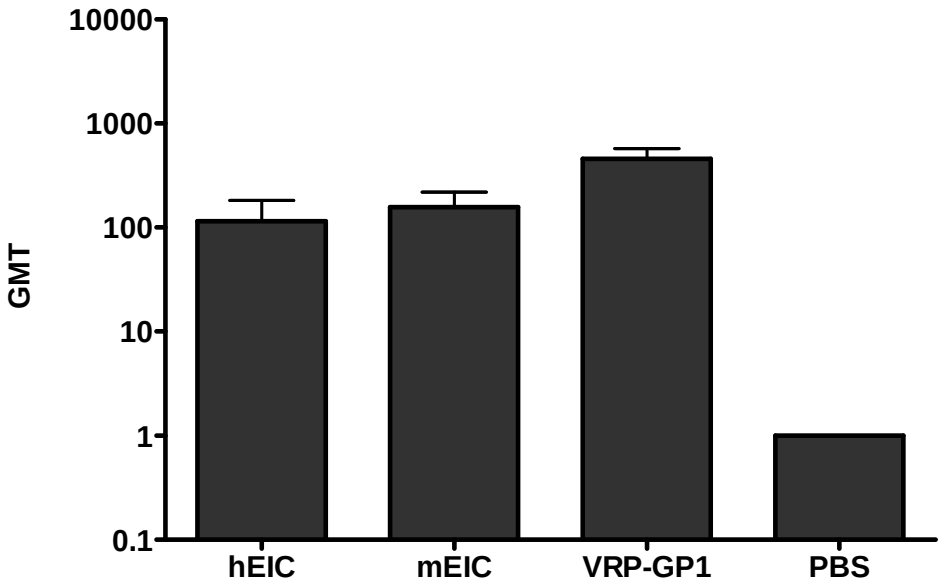

Fig. 8. Anti-Ebola IgG responses in mice immunized with the mEIC or hEIC.

Four groups of seven mice each were immunized subcutaneously with hEIC, mEIC, VRP-GP1, or PBS. The immunizations were given on days 0, 21, 42, and 63. The serum was collected on day 84 and assayed for anti-Ebola IgG by ELISA, using gamma-irradiated Ebola virus as the capture antigen. The data are presented as GMT ±1SD for each group of mice.

DISCUSSION

The results in Chapter 2 demonstrated the expression of hEIC in *N. benthamiana* leaves and its immunogenicity. However, the hEIC was not sufficiently immunogenic in mice. In this chapter, we tested whether the immunogenicity of the IC in mice would be improved if a mouse, rather than human, antibody backbone was used in the IC. The mEIC was expressed and purified in *N. benthamiana* leaves. Mouse mAb 6D8 was constructed and used for fusion with the Ebola GP1. The plant-produced mouse mAb 6D8 bound to irradiated Ebola virus (Fig. 3), which indicates the potential for EIC formation. The measured size of the mEIC (~20nm) is larger than the antibody alone (10nm), which suggests that a complex was formed.

The level of anti-Ebola antibody in mice immunized with the mEIC was not significantly different from the groups immunized with the hEIC or VRP-GP1. Although the antibody backbone used in the mEIC is syngeneic with the mouse host, the mEIC still does not induce a high antibody level, as was observed with hEIC. Thus, the hypothesis that use of a syngeneic antibody in the IC would enhance the immune response is rejected.

Antigen-antibody IC can induce the immune response because the IC can bind to the Fc receptor on APC, thus enhancing the presentation of antigenic epitope to T cells. One study (12) found that anti-Fc receptor antibodies blocked the clearance of the IC from the blood. Thus, the Fc

region on the antibody is implicated as a factor in the immunogenicity of the IC.

For the EIC, because the structure of the Ebola GP1 is unknown, it is also unknown whether the structure of the Ebola GP1 hinders the Fc region on the mAb 6D8. If the structure of the Ebola GP1 affects the Fc region, this might explain why the EIC cannot induce high antibody titers. In this study, $(G_4S)_2$ was used as the linker between the mouse 6D8 heavy chain and the GP1; $(G_4S)_3$ was used as the linker in the hEIC (Chapter 2). Both are short and flexible linkers. For future work, different linkers could be tested for their enhancement of the EIC's immunogenicity. Further experiments should include using the truncated version of the GP1 to fuse to the heavy chain of the mAb 6D8. If the structure of the full-length GP1 protein hinders the Fc region on the mAb 6D8, using a smaller part of the Ebola GP1 protein containing the 6D8 epitope might increase the immunogenicity of the EIC.

In addition, another experiment to avoid the putative effect of the Ebola GP1 structure on the Fc region of the antibody involves mixing the Ebola GP1 protein with the mAb 6D8 *in vitro* before immunization. When the Ebola GP1 is mixed with the mAb 6D8, the variable region of the mAb 6D8 will bind to the 6D8 epitope in the Ebola GP1. The structure of the Ebola GP1 protein will then not affect the Fc region on the mAb 6D8. One potential problem with this experiment is that the Ebola GP1 is highly toxic

to host cells, causing cell rounding and detachment (13, 14). Thus, production of the Ebola GP1 protein is difficult.

Another possibility that might reduce the immunogenicity of the EIC is plant glycosylation. In this study, we used the wild type *N. benthamiana* plants as the expression host. The glycosylation pattern of protein produced in plants is different from mammalian cells (4, 10). For example, plants only produce bi-antennary N-glycans, whereas the N-glycans in mammals can be more branched. In this work, we added the C-terminal hexapeptide SEKDEL, which is an ER retention signal, to GP1. This causes the EIC to be retained in the ER and to have a high mannose N-glycan pattern. To test whether the plant glycosylation affects the immunogenicity of the EIC, the EIC could be produced in a genetically modified plant, so as to have a glycosylation pattern similar to mammalian cells. The down-regulation of the endogenous β-1,2-xylosyltransferase (XylT) and α-1,3-fucosyltransferase (FucT) genes in *N. benthamiana* can produce plants with significantly reduced xylosylated and/or core α-1,3-fucosylated glycan structures, which produces a more human-like N-glycan structure (15). To test whether plant glycosylation affects the immunogenicity of the EIC, the IC can be expressed in such a modified plant with human-like N-glycan and constructed without the SEKDEL sequence. Both XylT and FucT enzymes are in the Golgi apparatus. As we have shown here, if SEKDEL is added at the C-terminus of the Ebola GP1 gene, the protein will be retained in the ER, and the glycosylation

pattern will be a high mannose N-glycan, which is different from mammalian glycan. If the EIC is produced in a genetically modified plant producing human-like N-glycans, it could be used to immunize the mice. If plant glycosylation is a factor that affects the binding to the Fc receptor and the immunogenicity of the EIC, the EIC produced from this modified plant, when used to immunize mice, should result in increased immune response to Ebola virus in the mice.

In summary, the EIC produced in *N. benthamiana* is immunogenic in mice, because it can induce an antibody response against Ebola virus. Although the level of the antibody against Ebola virus still does not reach the titer required for the protection against a lethal Ebola challenge, there are additional studies that can be conducted with the objective of increasing the immunogenicity of EIC. Moreover, adjuvants can be administered along with the EIC to enhance its immunogenicity. Different adjuvants should be studied to test which one works best with the EIC. Ensuring protection against Ebola lethal challenge is crucial for any new vaccine, necessitating further research to improve immunogenicity of recombinant antigens.

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Chapter 4

PLANT-PRODUCED EBOLA IMMUNE COMPLEX CO-DELIVERED WITH TLR3 AGONIST PROTECTS MICE FROM A LETHAL EBOLA CHALLENGE

ABSTRACT

Ebola hemorrhagic fever (EHF) is a fatal disease in humans caused by the Ebola virus. Currently, there are no specific and effective treatments or vaccines for EHF. Hence, there is an urgent need for a safe and effective vaccine. The goal of this study was to evaluate the immunogenicity and efficacy of a novel plant-produced Ebola vaccine candidate, the Ebola immune complex (EIC), because it was shown to be immunogenic in mice. As noted in the studies presented in Chapters 2 and 3, the EIC delivered subcutaneously in mice was not highly immunogenic. Therefore, the present study was designed to evaluate two Toll-like receptor (TLR) agonists as adjuvants co-delivered with the EIC. Mice were immunized subcutaneously with the hEIC (Chapter 2) alone or the hEIC co-administered with a TLR3 agonist [polyinosine-polycytidylic acid (PIC)] and/or a TLR7/8 agonist (CL097). A Venezuelan Equine Encephalitis replicon particle expressing the Ebola GP1 (VRP-GP1) was used as a positive control for immunization, and PBS was used as a negative control. The hEIC co-delivered with PIC elicited a strong antibody response, which was comparable to that elicited by VRP-GP1. PIC and CL097 delivered together with the hEIC did not result in a synergistic

effect with regard to antibody production. To determine whether the hEIC co-administered with an adjuvant could protect the mice against a lethal Ebola challenge, the hEIC was delivered with either PIC or alum followed by a live Ebola virus challenge. Although immunization with the hEIC alone could not protect the mice, the hEIC co-delivered with PIC protected 80% of the mice from a lethal challenge, similar to immunization with VRP-GP1 ($p=0.7630$). In contrast, the hEIC co-delivered with alum could not significantly ($p=0.5772$) protect mice from a lethal Ebola challenge relative to PBS mock-immunized mice. The levels of total anti-Ebola IgG and the neutralizing antibody specific to Ebola virus were directly correlated with the level of protection afforded by the vaccine candidate. Moreover, the titers of each IgG subtype demonstrated that the hEIC alone and the hEIC co-delivered with alum induced a T-helper type 2 (Th2) dominant response, whereas the hEIC co-administered with PIC induced a mixed Th1/Th2 response. These results suggest that a mixed Th1/Th2 response facilitates the protection afforded by this vaccine. The protective efficacy of the hEIC co-administered with PIC against a lethal Ebola virus challenge in mice affirms the need for further study in non-human primates and humans for the development of an effective Ebola vaccine.

INTRODUCTION

Ebola hemorrhagic fever is a severe and often-fatal disease that can occur in human and nonhuman primates, such as chimpanzees, monkeys, and gorillas (45). Ebola virus can cause lethal hemorrhagic disease in humans, with a very high mortality rate, up to 90%, and with clinical symptoms arising quickly after an incubation period from 2 to 21 days (33). The typical presentation is characterized by flu-like symptoms including high fever, chills, malaise, and myalgia. The ensuing symptoms indicate multisystem involvement, including systemic, gastrointestinal, respiratory, vascular, and neurologic manifestations. Abnormalities in blood coagulation appear as petechiae, ecchymoses, mucosal hemorrhages, and uncontrolled bleeding in venipuncture sites. Severe Ebola infection usually progresses to shock, convulsions, and diffuse coagulopathy. Death or recovery typically happens 6–9 days after the onset of clinical symptoms (24, 43). Not only do the dramatic clinical symptoms and high fatality rate of the Ebola virus infection make it extremely dangerous, but additionally, all Ebola virus strains have displayed the ability to be spread through aerosols under research conditions (13, 29, 31). Therefore, Ebola virus is a potential deadly bioweapon that could be used by terrorists, especially given that approximately 30 years after the first epidemic, there is yet no approved vaccine or therapeutic for the Ebola virus.

The primary difficulty for patients in recovering from an Ebola virus infection is the failure of their immune systems to react to this rapidly progressing disease. The patients who die from Ebola hemorrhagic fever are unable to develop an adequate immune response in the face of extreme viremia and multi-organ infection. Previous studies have shown that survivors from an Ebola infection must develop early and increasing levels of IgG against the Ebola virus followed by viral antigen clearance and cytotoxic T cell activation. In fatal cases, Ebola-specific IgG and T cell-related mRNA were not detected (3). These data imply that both humoral and cell-mediated immune responses are important for recovery from an Ebola infection. Thus, the key for Ebola vaccine development is to find a candidate that activates both antibody and cytotoxic T cell responses against the Ebola virus.

Several different types of Ebola vaccine have been developed previously, including the DNA vaccine (16, 38, 61), the Ebola virus-like particle (VLP) (39, 58, 66, 71), the vesicular stomatitis virus-based vaccine (19, 20), the adenovirus vector (34, 63, 64, 70), and the Venezuelian equine encephalitis virus (VEE) replicon particle (VRP) (44, 47). However, there is no approved vaccine for human use at present. In Chapter 2, we showed production of the humanized Ebola immune complex (hEIC) in *Nicotiana benthamiana* as a vaccine candidate, by fusing the Ebola GP1 at the C-terminus of the heavy chain of the humanized monoclonal antibody (mAb) 6D8 (Fig. 1).

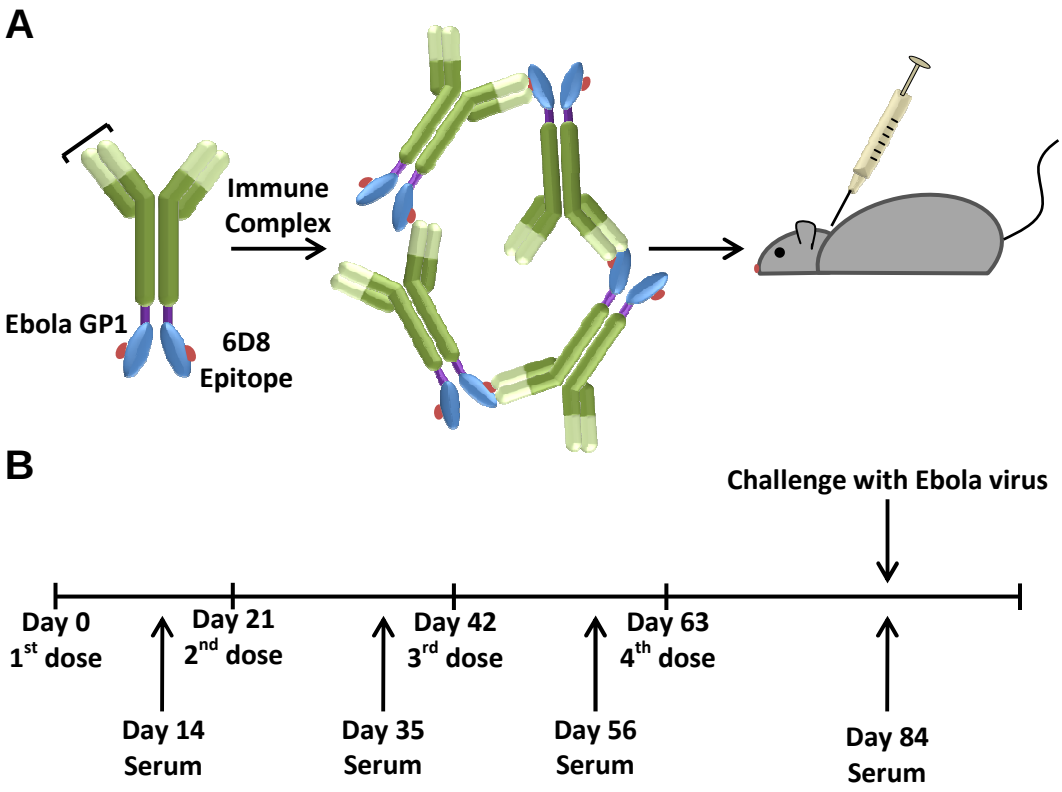

Fig. 1. Diagram illustrating the structure and assembly of the hEIC.

The hEIC was designed and expressed by fusing Ebola GP1 to the C-terminus of the humanized heavy chain of mAb 6D8 (A, left). The recombinant IgG can bind to the Ebola GP1 and form an immune complex (A, center). Female BALB/C mice were immunized with hEIC subcutaneously, with or without candidate adjuvants, following the designed immunization schedule (B). Mice were immunized on days 0, 21, 42, and 63 for the 3-dose groups or on days 21, 42, and 63 for the 4-dose groups. Blood was collected 2 weeks after each immunization (days 14, 35, and 56) and 3 weeks after the last immunization (day 84). For the challenge study, mice were lethally challenged with the Ebola virus 3 three weeks after the last immunization (day 84).

After the fusion protein was co-expressed with the light chain of mAb 6D8, assembly of IgG occurred that allowed formation of the hEIC. We subcutaneously immunized mice with purified hEIC and showed immunogenicity, but the mice did not develop sufficient immune responses to protect them against a lethal Ebola virus challenge.

In this study, we used different TLR agonists and alum as adjuvants with hEIC in order to determine which one produces the best protection of mice from an Ebola lethal challenge.

TLR are a type of pattern recognition receptors of the innate immune system that recognize different molecules broadly shared by pathogen (35). For example, TLR 7/8 recognize single-stranded RNA whereas TLR3 recognizes double-stranded RNA from the virus and can induces the activation of NF-kB to increase production of type I interferons which signal other cells to increase their antiviral defenses (5). TLR ligands, which mimic pathogen-associated molecular patterns and activate immune cells via TLR, can be used in humans as vaccine adjuvants (11, 18, 28, 30, 67, 72, 73). Poly(I:C) (PIC) and CL097, which are TLR3 and TLR7/8 agonists, respectively, were chosen to use in this study because both can mimic viral RNA, and the Ebola virus has a RNA genome. Moreover, we also studied alum as another adjuvant, because it is the only classical adjuvant which is approved by the FDA for use in humans (7, 51).

In this study, we demonstrate that the hEIC delivered with PIC induced a high IgG antibody response against the Ebola virus and protected the immunized mice from an Ebola lethal challenge. The hEIC delivered alone induced a Th2 dominant response against the Ebola virus, and provided no protection in a lethal Ebola virus challenge. When PIC was co-administered with the hEIC, the mice produced a mixed Th1/Th2 response, which correlated with protection against the lethal Ebola virus challenge. These results support the candicacy of plant-expressed hEIC as a vaccine for Ebola virus in humans.

MATERIALS AND METHODS

Ebola immune complex preparation

The binary vectors (see Chapter 2, page 43, and Chapter 3, page 83 were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. The final OD_{600} of *Agrobacterium* infiltrated into the leaves was 0.25. For the hEIC, *N. benthamiana* leaves were co-infiltrated with pBYRH2GP1kdelK3 and pPS19. For the mouse Ebola immune complex (mEIC), the leaves were co-infiltrated with pBYHGP1kdel, pBYKR, and pPS19. The infiltrated leaves were harvested on day 4 after infiltration. The leaves were extracted, and the proteins were purified by protein G or protein A affinity chromatography for the hEIC or the mEIC, respectively, followed by ammonium sulfate precipitation (described in Chapter 2 and Chapter 3).

Preparation of *N*-linked glycans and MALDI-TOF/TOF MS analysis

The preparation and purification of glycans derived from total endogenous leaf proteins were performed as described by Strasser et al. (56). Mass spectrometric analysis of endogenous glycans was carried out with a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflector mode at positive ionization. The matrix was 2,5-dihydroxybenzoic acid (DHB) [2% (w/v) DHB in 50% (v/v) acetonitrile in H_2O].

Immunization

To test the antibody response of the mice immunized with the EIC co-administered with PIC and/or CL097, seven female BALB/C mice in each group were immunized subcutaneously with 10µg EIC alone, 10µg EIC co-administered with PIC, CL097, or the combination of PIC and CL097. The immunizations were performed on days 0, 21, 42, and 63. The pre-immune blood samples were obtained on day 0 before the first immunization, and the sera wereanalyzed for anti-Ebola IgG by ELISA on day 14, 35, 56, and 84 (Fig. 1B). For the challenge study, 15 female BALB/C mice in each group were subcutaneously immunized with 10µg EIC, 10µg EIC co-delivered with alum (Sigma, St. Louis, MO), PIC (Invivogen, San Diego, CA), or with the combination of alum and PIC on days 0, 21, 42, and 63. Two groups were subcutaneously immunized with 10µg EIC co-administered with PIC or 25µg EIC co-delivered with PIC and alum were injected on days 0, 21, and 42. Groups of mice were

immunized with four doses of VRP-GP1 (47), or PBS as positive and controls, respectively. Ten mice in each group were shipped to USAMRIID on day 70 and infected with the Ebola virus on day 84. Five mice from each group were bled on day 84 by cardiac puncture and analyzed for IgG specific for Ebola virus by ELISA.

Ebola virus challenge

Mouse-adapted Ebola virus was obtained from Dr. Mike Bray (6). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. For infection, mice were inoculated intraperitoneally (i.p.) with 1000 PFU (30,000 LD_{50}) of mouse-adapted Ebola virus in a Biosafety Level 4 (BSL-4) laboratory. The survival was recorded for 30 days. The average weight of all surviving mice was measured for 14 days.

Anti-Ebola antibody analysis by ELISA

Anti-Ebola antibody was measured by ELISA as described in Chapters 2 and 3. Briefly, polyvinyl chloride 96-well ELISA plates were coated with 50µl of irradiated Ebola virus diluted 1:1000 in PBS and incubated at $4^{o}C$ overnight. Plates were blocked with 5% skim milk in PBST at $23^{o}C$ for 2 hours. Subsequently, the plates were incubated with

the serum diluted in 1% skim milk in PBST for 1 hour at 37°C and then with HRP-conjugated goat anti-mouse IgG for 1 hour at 37°C. The plates were developed with TMB substrate and read OD_{450}. For IgG1, IgG2a, IgG2b, and IgG3 antibody titers, HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 were used to detect, respectively.

Neutralizing antibody measurement

Four ten-fold serial dilutions of serum were mixed with 100 plaque-forming units of Ebola Zaire at 37℃ for 1 hour and used to infect Vero cell monolayers. Cells were overlaid with agarose, and a second overlay containing 5% neutral red was added 7 days later. Plaques were counted the next day. Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 80% compared with control wells.

Ebola virus competition binding assay

Polyvinyl chloride 96-well ELISA plates were coated with 50µl of irradiated Ebola virus diluted 1:1000 in PBS and incubated at 4˚C overnight. Plates were blocked with 5% skim milk in PBST at 23˚C for 2 hours. Subsequently, the plates were incubated with the mouse serum alone diluted 1:10, 1:100, or 1:1,000 in 1% skim milk in PBST or mouse serum combined with 100µg/ml or 10µg/ml plant-produced humanized mAb 6D8 diluted 1:10 in 1% skim milk in PBST for 1 hour at 37˚C and then with HRP-conjugated goat anti-mouse IgG for 1 hour at 37˚C. The

plates were developed with TMB substrate, and the OD_{450} was determined.

Statistical analysis

The survival data and IgG titers were analyzed by the log rank test and the Kruskal-Wallis Test using GraphPad Prism 5 (GraphPad Software), respectively. Statistical significance was determined at the level of $P < 0.5$.

RESULTS

The mAb 6D8 glycosylation patterns are similar in wild type and humanized N-glycan *N. benthamiana.*

To examine the N-glycan compositions of the mAb 6D8 produced in the wild type and the humanized N-glycan *N. benthamiana*, purified humanized mAb 6D8 and mouse mAb 6D8 from both types of plants were subjected to matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis. The results showed no differences in the N-glycan compositions of antibodies produced from the wild type or the humanized N-glycan *N. benthamiana*. Fig. 2 and Fig. 3 show that both the human IgG and the mouse IgG from

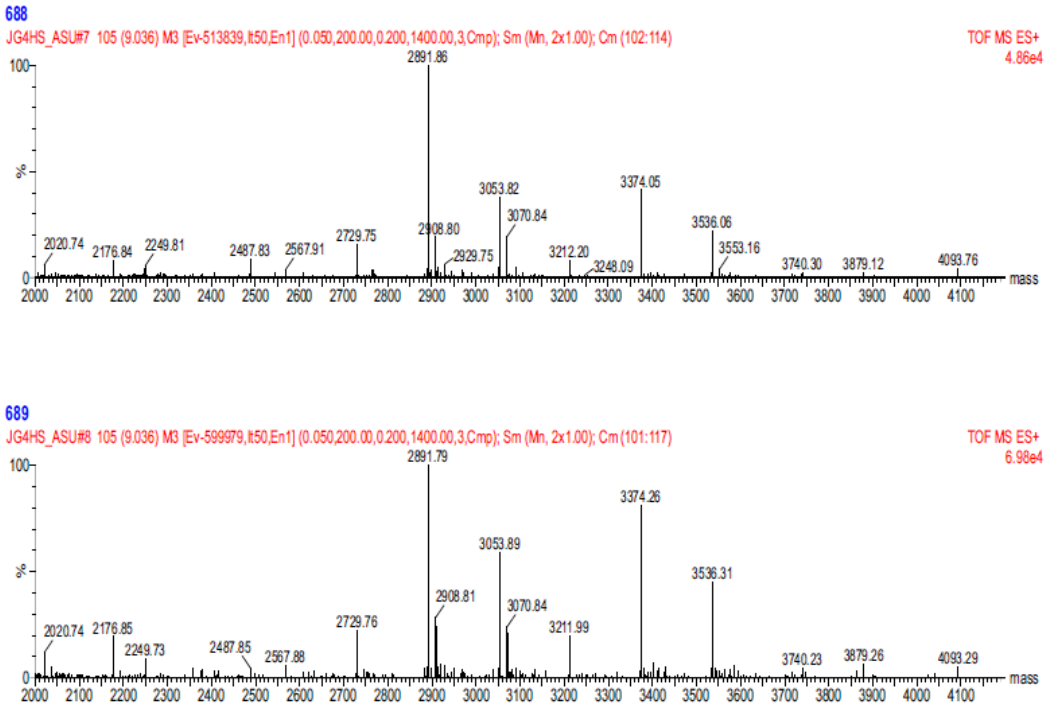

Fig. 2. MALDI-TOF/TOF MS of human mAb 6D8 extracted and purified from *N. benthamiana* leaves.

N-glycan compositions of human IgG produced in wild type (upper panel) and the humanized N-glycan *N. benthamiana* (lower panel) are similar.

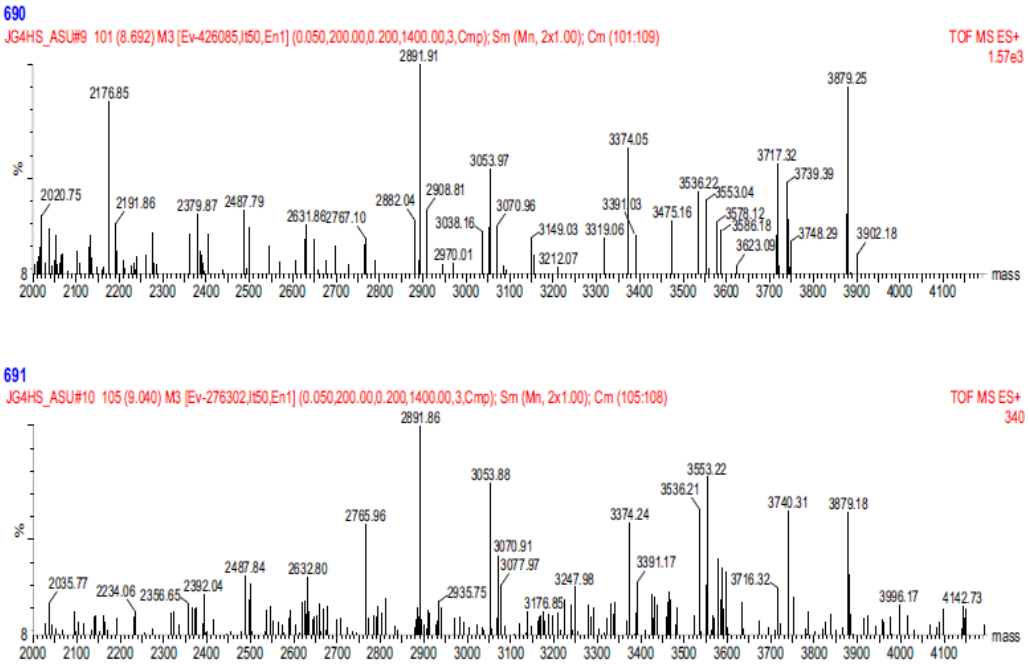

Fig. 3. MALDI-TOF/TOF MS of mouse mAb 6D8 extracted and purified from *N. benthamiana* leaves.

N-Glycan compositions of the mouse IgG produced in the wild type and the humanized N-glycan *N. benthamiana* are similar.

either the wild type or the humanized N-glycan plants contained N-glycan species that carried high mannose structures with eight mannose units (mass 2891) and nine mannose units (mass 3053).

PIC enhances anti-Ebola IgG production when it is co-administered with EIC whereas CL097 does not.

Both the hEIC and the mEIC were expressed in *N. benthamiana* leaves using a geminiviral replicon system (25, 26). After expression and purification, the hEIC and mEIC were used to immunize mice. We subcutaneously immunized mice with four doses of hEIC with PIC, CL097, or the combination of PIC and CL097. Anti-Ebola IgG in the sera (Fig. 4) significantly increased in mice immunized with hEIC compared to the PBS control group ($p=0.0248$). When hEIC with CL097 was used to immunize the mice, anti-Ebola IgG also significantly increased, as compared to the PBS control group, but it was not significantly different from that of the mice immunized with hEIC alone ($p=0.6198$). However, using hEIC with PIC to immunize the mice significantly increased the anti-Ebola response compared to immunization with hEIC alone ($p=0.0016$). Immunization using the combination of PIC and CL097 with hEIC produced antibody titers in mice that were not significantly different from those of mice that were immunized with hEIC and PIC alone ($p=0.5030$), but were significantly higher than those in mice immunized with CL097 and hEIC ($p=0.0019$).

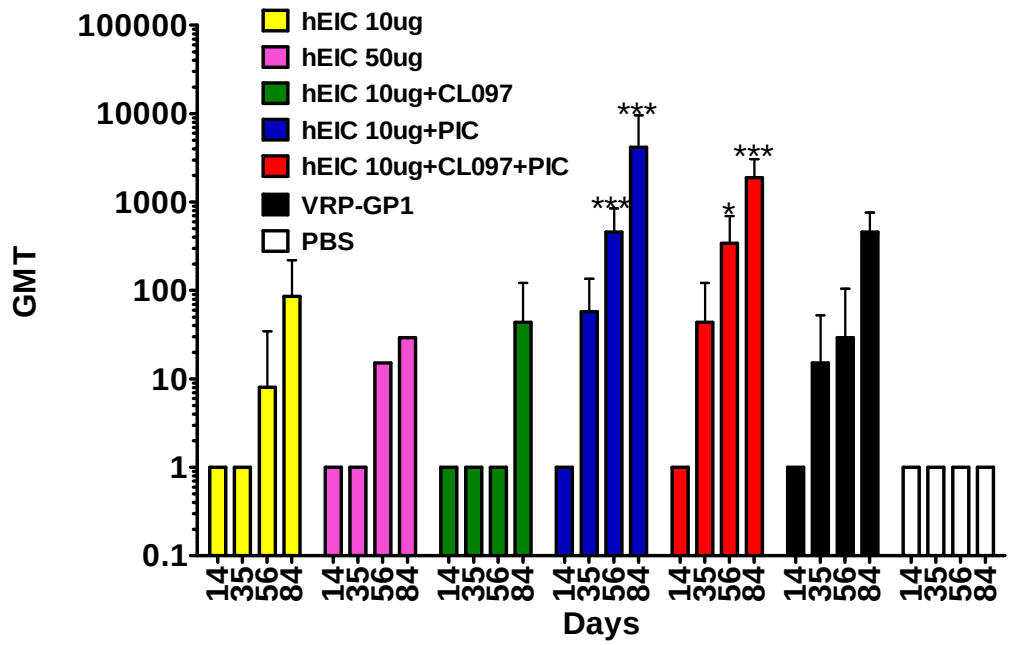

Fig. 4. Co-delivery of PIC with hEIC in mice induced the highest anti-Ebola IgG antibody response relative to other TLR agonist combinations and the PBS negative control.

Groups were subcutaneously immunized with 10µg hEIC alone, hEIC with CL097, hEIC with PIC, or hEIC with CL097+PIC on days 0, 21, 42, and 63. VRP-GP1 was used as the positive control. Anti-Ebola IgG titers were measured by ELISA on days 0, 14, 35, 56, and 84. GMT was defined as the highest serum dilution giving a positive reaction. The GMT for the PBS negative control group and preimmune (day 0) serum in all groups were ≤ 1 (not shown). The error bars indicate the standard deviation (SD). The data presented are representative of three independent studies. Statistically significant differences ($p<0.05$) in the levels of IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made

between immunization groups relative to PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Immunization with the EIC and PIC protected mice from a lethal Ebola virus challenge.

To assess the efficacy of the EIC against a lethal Ebola virus challenge, vaccinated mice were given 30,000 LD50 of Ebola virus i.p. 3 weeks after the last immunization. All studies with live Ebola virus were performed under BSL4 conditions in accordance with guidelines of the National Institutes of Health and the Centers for Disease Control. Four doses of hEIC co-delivered with PIC alone or co-administered with PIC and alum both induced 80% protection against the lethal virus challenge, as measured by survival (Fig. 5), which is not statistically different from the 90% protection afforded in the group that received four doses of VRP-GP1 ($p= 0.7630$ and 0.7835, respectively). The immunizations with four doses of hEIC alone or hEIC co-administered with alum did not protect the mice; all 10 mice died, which was not statistically different from the PBS negative control group ($p= 0.5577$ and 0.5772, respectively). When three doses of PIC and alum were co-administered with hEIC, the survival rate was 20%, which was not significantly different from PBS ($p= 0.0860$). The group that was immunized with three doses of 25µg of hEIC (compared to 10µg in other groups) with PIC and alum was the only group to show 50% protection, which was statistically different from PBS group ($p=0.0058$).

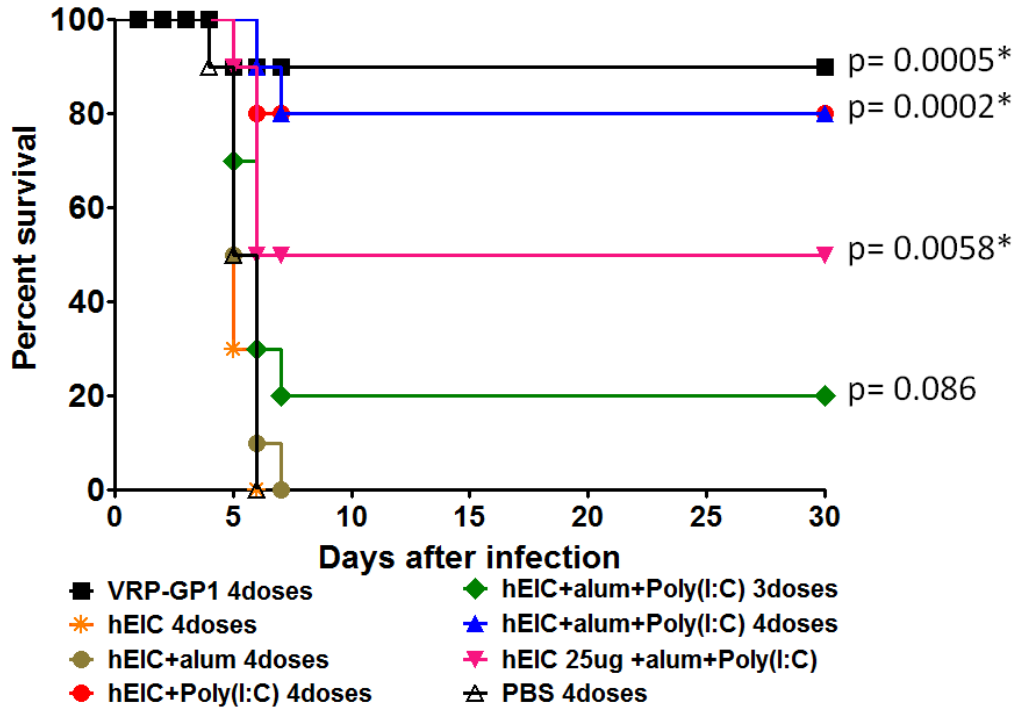

Fig. 5. The hEIC co-delivered with PIC significantly protected mice from the Ebola lethal challenge.

Immunization with 3 or 4 doses of 10µg of the hEIC, or 3 doses of 25µg of the hEIC, with or without candidate adjuvants was performed in mice (n=10 per group). Mice were immunized on days 0, 21, 42, and 63 for the 3 doses group or days 0, 21, 42, and 63 for the 4 dose groups and challenged i.p. with 1000 PFU (30,000 LD50) of the mouse-adapted Ebola virus on day 84. The survival was reported for 30 days. Statistically significant differences ($p<0.05$) in survival were determined by the log rank test, and comparisons were made between immunization groups relative to PBS control as indicated (*).

Table 1 displays the p values calculated from the log rank test to compare the survival rates between two paired immunization groups. The protection afforded by 10µg of the hEIC co-administered with PIC in four doses, 10µg of the hEIC co-delivered with PIC and alum in four doses, and 25µg of the hEIC co-administered with PIC and alum in three doses were not statistically different from the VRP-GP1 positive control group. Weight losses of the mice after they were challenged with Ebola virus are shown in Fig. 6. The mice immunized with VRP-GP1 experienced less weight loss than the mice in other groups. Mice in the groups immunized with hEIC + PIC or hEIC + PIC + alum showed disease symptoms and lost weight on day 4; the surviving mice recovered from the disease and gained more weight on day 7. Immunization with hEIC alone, thus delayed weight loss, which delayed the course of the disease, as compared to the PBS negative control group, in which the immunized mice showed weight loss on day 1 after the challenge.

The survival and weight loss of the mice immunized with the mEIC, with or without different adjuvants, are presented in Fig. 7 and Fig. 8, respectively. Fig. 7 shows that 70% of the mice immunized with the mEIC + PIC + alum were protected from Ebola infection, which is not statistically different from the mice immunized with VRP-GP1 ($p=0.5364$). The levels of anti-Ebola total IgG in mice immunized with the hEIC and the mEIC are shown in Fig. 9 and Fig. 10, respectively.

	VRP-GP1 4doses	hEIC 4 doses	hEIC+Alum 4doses	hEIC+PIC 4doses	hEIC+Alum+PIC 3doses	hEIC+Alum+PIC 4doses	25ughEIC +Alum+PIC 4doses	PBS 4doses
VRP-GP1 4doses	NA	0.0002***	0.0003***	0.763	0.0101*	0.7835	0.1488	0.0005***
hEIC 4doses	0.0002***	NA	0.2612	<0.0001***	0.0309*	<0.0001***	0.0018**	0.5577
hEIC+alum 4doses	0.0003***	0.2612	NA	0.0001***	0.1459	<0.0001***	0.006**	0.5772
hEIC+PIC 4 doses	0.763	<0.0001***	0.0001***	NA	0.0065**	0.948	506	0.0002***
hEIC+alum+PIC 3doses	0.0101*	0.0309*	0.1459	0.0065**	NA	0.0045**	0.1451	0.086
hEIC+alum+ PIC 4 doses	0.7835	<0.0001***	<0.0001***	0.948	0.0045**	NA	0.1405	<0.0001***
hEIC25ug +alum+PIC 3doses	0.1488	0.0018**	0.006**	0.1506	0.1451	0.1405	NA	0.0058**
PBS 4 doses	0.0005***	0.5577	0.5772	0.0002***	0.086	<0.0001***	0.0058**	NA

Table1. The P value calculated from the log rank test determines the statistical difference in survival from the Ebola virus challenge between two immunization groups when p value < 0.05.

* P<0.01-0.05, ** P <0.009-0.001, *** P<0.0009-0.0001, NA, not applicable

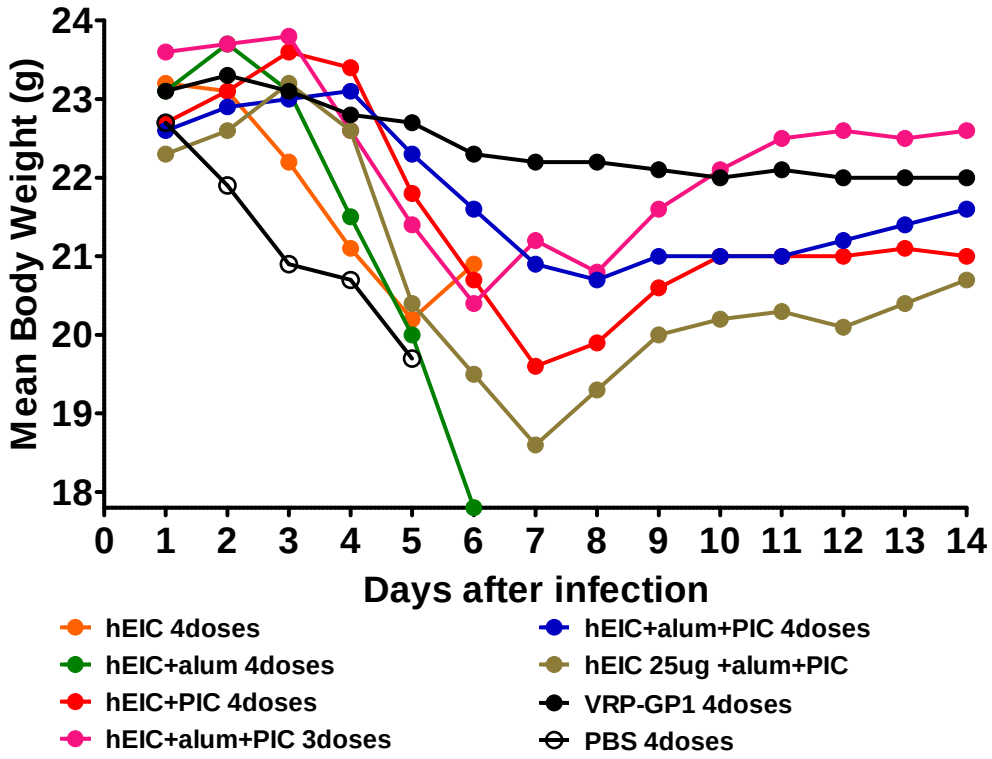

Fig. 6. Body weight change after Ebola virus infection in mice immunized with the hEIC alone or the hEIC with different adjuvants.

Immunization with 3 or 4 doses of the hEIC alone or the hEIC with candidate adjuvants was performed in mice (n=10 per group). The group immunized with hEIC 25μg + alum + PIC received 3 doses. All other hEIC-immunized mice received 10μg hEIC per dose. Mice were immunized on days 0, 21, 42, and 63 for the groups receiving 3 doses, or days 0, 21, 42, and 63 for the groups receiving 4 doses, and challenged i.p. with 1000 PFU (30,000 LD50) of the mouse-adapted Ebola virus on day 84. The body weight change was measured for 14 days.

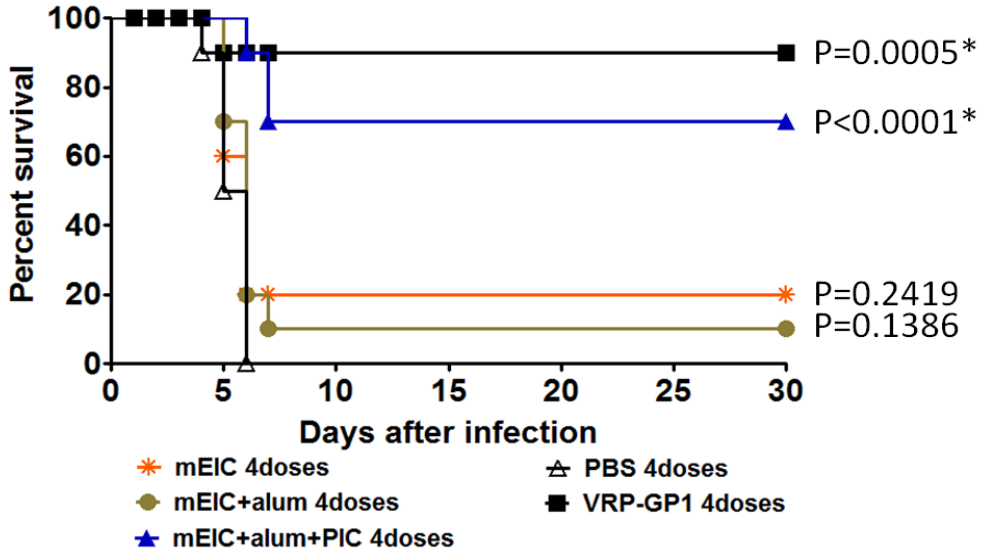

Fig. 7. The mEIC co-delivered with PIC significantly protected mice from lethal Ebola virus challenge.

Immunization with 4 doses of the mEIC alone or with the mEIC with candidate adjuvants was performed in mice (n=10 per group). Mice were immunized on days 0, 21, 42, and 63 and challenged i.p. with 1000 PFU (30,000 LD50) of the mouse-adapted Ebola virus on day 84. The survival was reported for 30 days. Statistically significant differences ($p<0.05$) in survival were determined by the log rank test, and comparisons were made between immunization groups relative to a PBS control as indicated (*).

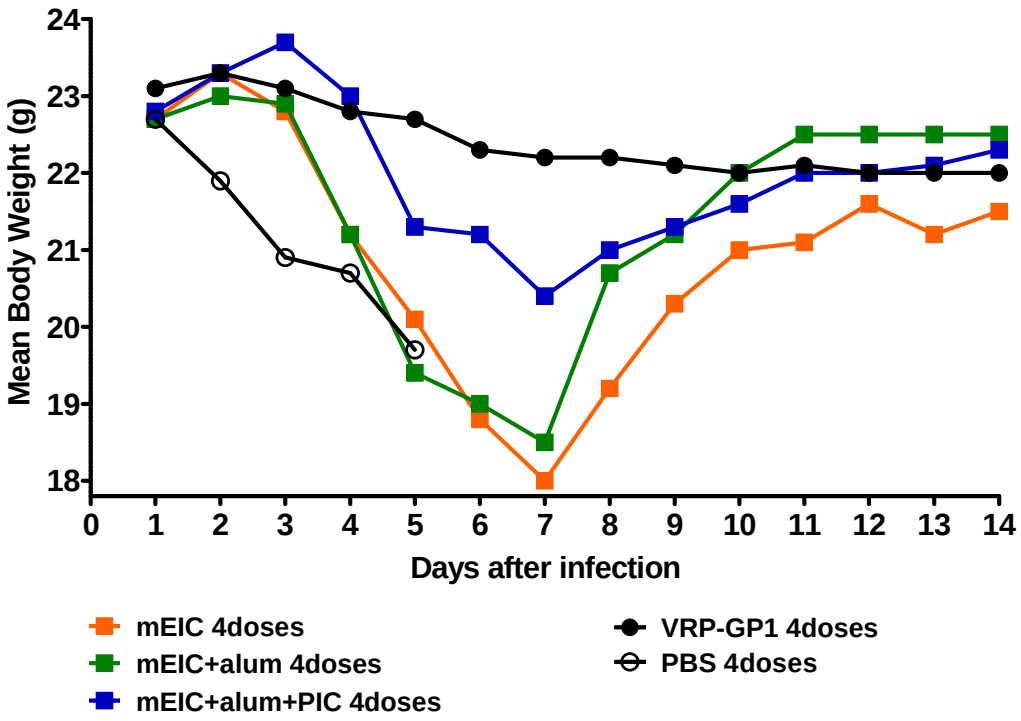

Fig. 8. Body weight change after Ebola virus infection in mice immunized with the mEIC alone or the mEIC with different adjuvants.

Immunization with 4 doses of the mEIC with or without different adjuvants was performed in mice (n=10 per group). Mice were immunized on days 0, 21, 42, and 63 and challenged i.p. with 1000 PFU (30,000 LD50) of mouse-adapted Ebola virus on day 84. The body weight change was measured for 14 days.

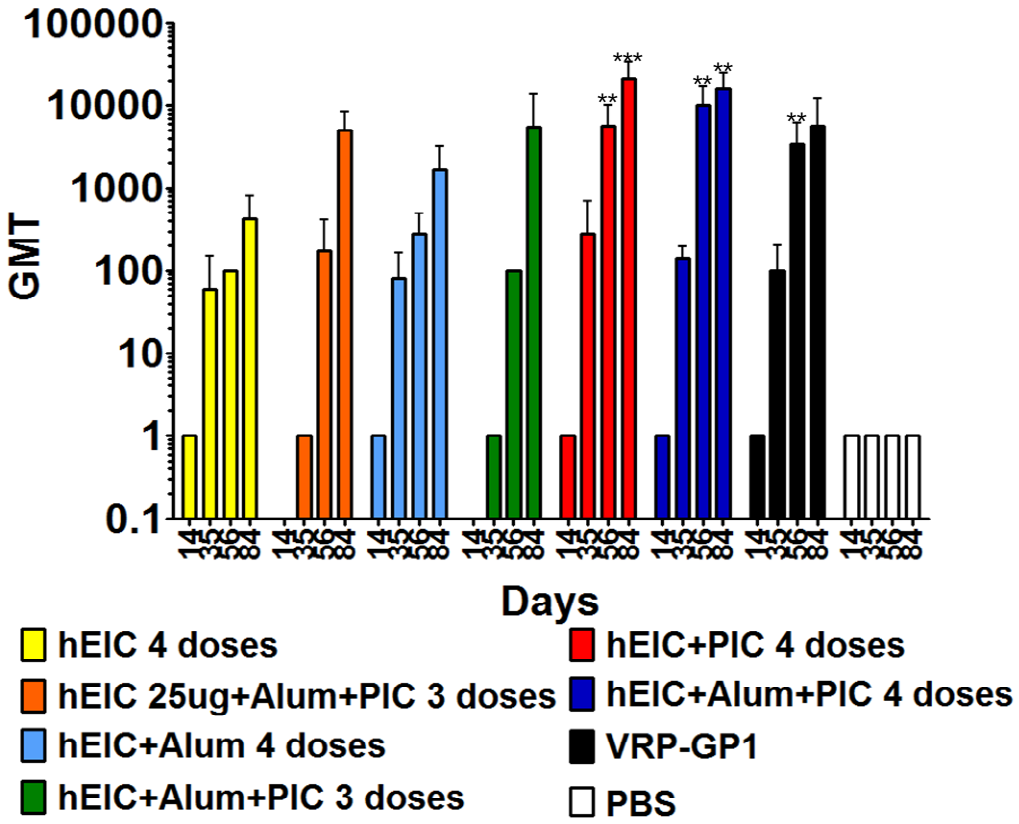

Fig. 9. Anti-Ebola IgG levels in mice immunized with the hEIC alone or with different adjuvants.

Mice (n=5 per group) immunized with the hEIC, as described for the challenge study (Figs. 5 and 6), were bled 2 weeks after each immunization and 3 weeks after the last immunization (on days 14, 35, 56, and 84 for the groups receiving 3 doses, or days 0, 14, 35, 56, and 84 for the groups receiving 4 doses). Individual serum samples for the mice in each immunization group were analyzed for anti-Ebola IgG by ELISA. The GMT for preimmune (day 0) serum in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the levels of anti-Ebola IgG were determined by the Kruskal-

Wallis test. Comparisons were made between immunization groups relative to PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

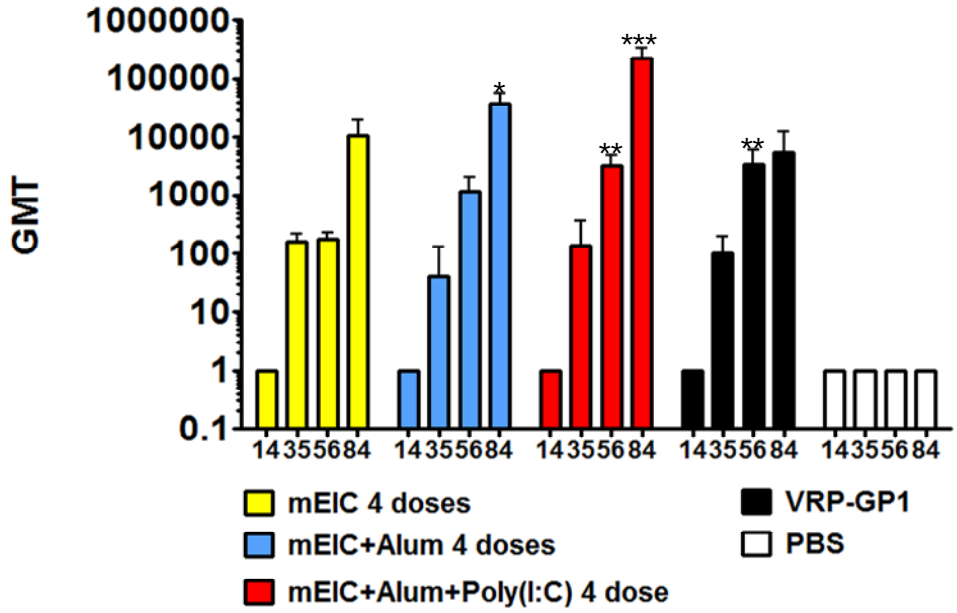

Fig. 10. Anti-Ebola IgG levels in mice immunized with the mEIC alone or with different adjuvants. Mice (n=5 per group) immunized as described for the challenge study (Figs. 7 and 8) were bled 2 weeks after each immunization and 3 weeks after the last immunization (on days 0, 14, 35, 56, and 84). Individual sera were analyzed for total IgG specific to the Ebola virus by ELISA. The GMT for preimmune (day 0) sera in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the level of IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made between the immunization groups relative to PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The levels of neutralizing antibody specific to Ebola virus in mice immunized with the hEIC and the mEIC are presented in Fig. 11 and Fig. 12, respectively. For mice immunized with the hEIC, the levels of anti-Ebola IgG were highest in the groups of mice immunized with 4 doses of the hEIC + PIC (red) and with the hEIC + PIC + alum (blue) (Fig. 9); the levels of virus-neutralizing antibody were highest in mice immunized with the hEIC + PIC + alum (blue) and hEIC + PIC (red), respectively (Fig. 11). The anti-Ebola IgG and virus-neutralizing antibody titers in these two groups were correlated with the level of protection against Ebola virus challenge, which was comparable to that in the mice immunized with VRP-GP1 (Fig. 5). Immunization with the mEIC + PIC + alum protected 70% of the mice from lethal Ebola virus challenge, which is not significantly different from the level of protection in the mice immunized with VRP-GP1 ($p=0.5364$) (Fig.7). The levels of anti-Ebola IgG in the mice immunized with the mEIC + PIC + alum was highest among the groups (Fig.10), and was directly correlated to the protection level (Fig.7). However, the levels of virus-neutralizing antibody (Fig. 12) were not correlated with the protection level, because mice immunized with the mEIC + PIC + alum produced the same levels of neutralizing antibody as mice immunized with the mEIC + alum.

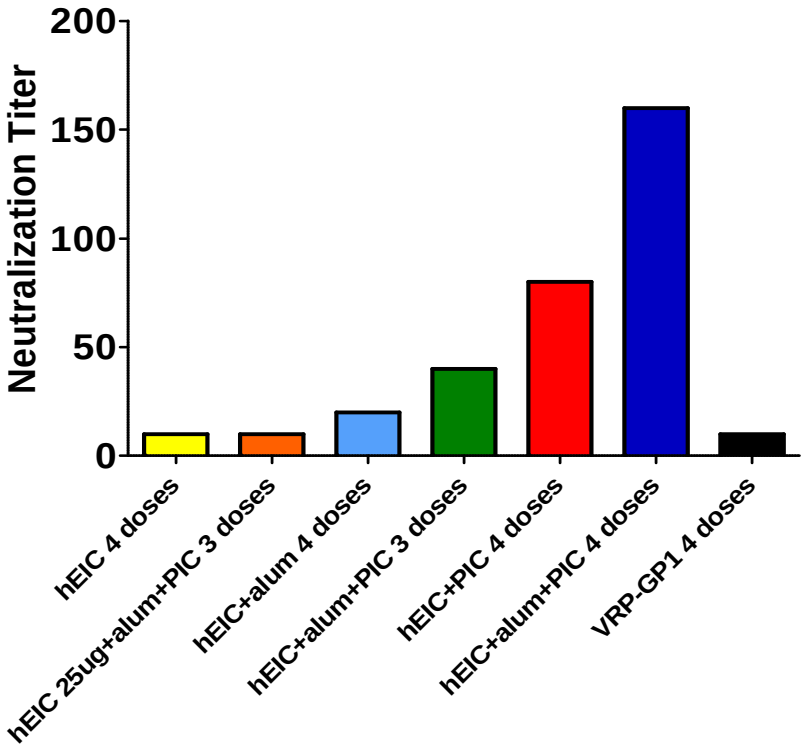

Fig. 11. Immunization with the hEIC co-delivered with PIC induced the production of Ebola virus-neutralizing antibodies in mice.

Mice (n=5 per group) immunized as described for the challenge study Figs. 5 and 6), were bled 2 weeks after each immunization and 3 weeks after the last immunization. Pooled sera collected on day 84 were analyzed for neutralizing antibody specific to the Ebola virus. Serial dilutions of serum were mixed with Ebola virus and applied to Vero cell monolayers. Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 80% compared with the control wells.

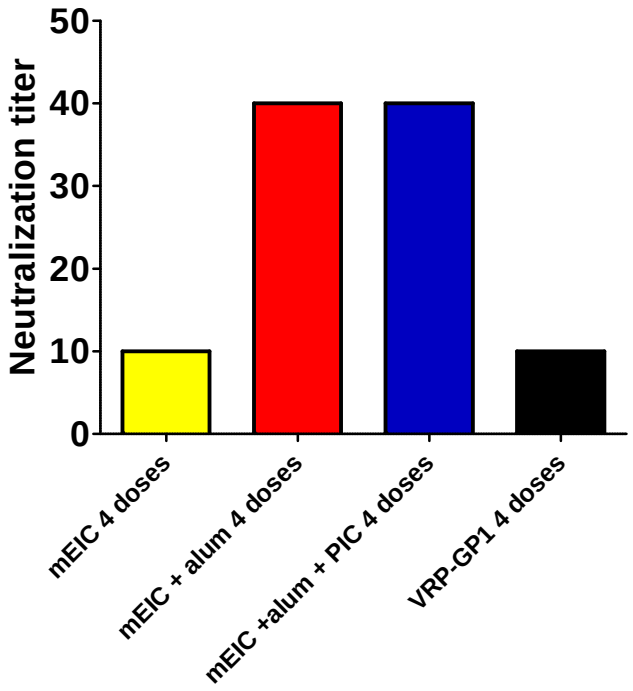

Fig. 12. Immunization with the mEIC + alum + PIC induced the production of Ebola virus-neutralizing antibodies in mice.

Mice (n=5 per group) immunized as described for the challenge study (Fig. 7 and 8) were bled 2 weeks after each immunization and 3 weeks after the last immunization. Pooled sera collected on day 84 were analyzed for Ebola virus-neutralizing antibodies. Serial dilutions of the sera were mixed with Ebola virus and applied to Vero cell monolayers. Neutralization titers were determined to be the last dilution of the serum that reduced the number of plaques by 80% as compared with control wells.

Immunization with EIC + PIC induced a mixed Th1/Th2 immune response that correlated with protection against virus challenge.

The IgG isotope induced by immmunization can vary with the type of immunogen and the adjuvant used. In general, IgG2a is associated with a Th1-polarized response, and IgG1 is associated with a Th2 response. We compared the IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) induced by the hEIC with different adjuvants after each immunization; results are shown in Figs. 13, 14, 15, and 16. Immunization with the hEIC alone induced IgG1, IgG2b, and IgG3, but not IgG2a (Fig. 17A). When PIC or the combination of PIC + alum were co-administered with the hEIC, the IgG2a and an increase in the levels of IgG1, IgG2b, and IgG3 were induced (Fig. 17B and Fig. 17C, respectively), which showed a pattern similar to VRP-GP1 immunization (Fig. 17D). IgG1 was produced in the mice in all groups (Fig. 13), whereas IgG2a (Fig. 14) was produced only in mice immunized with VRP-GP1 or with the hEIC + PIC. Alum co-administered with the hEIC induced low levels of IgG2a that were not significantly different from the levels of IgG2a induced by the hEIC administered alone or the PBS negative control groups (data not shown) ($p=0.4533$). In Fig. 15, IgG2b was produced in all groups, but the levels were higher in groups immunized with three doses of 25µg hEIC + PIC + alum, four doses of 10µg hEIC + PIC, and four doses of 10µg hEIC ≠ PIC + alum, compared to groups receiving four doses of 10µg hEIC alone. The levels of IgG3 were very low in all groups (Fig. 16).

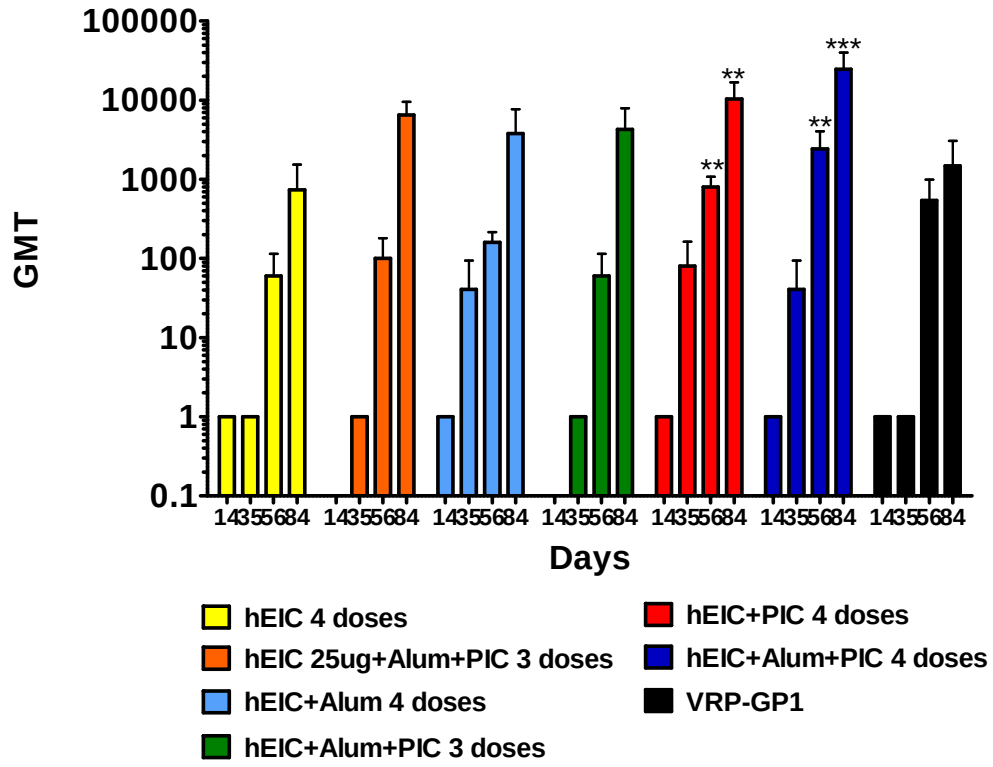

Fig. 13. The levels of IgG1 in the sera of mice immunized with the hEIC alone or with different adjuvants.

Mice (n=5 per group) immunized with the hEIC as described for the challenge study (Figs. 5 and 6) were bled 2 weeks after each immunization and 3 weeks after the last immunization. Individual sera from days 0, 14, 35, 56, and 84 were analyzed for IgG1 specific to Ebola virus. The GMT for preimmune (day 0) serum in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the level of the IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made between immunization groups relative to the PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

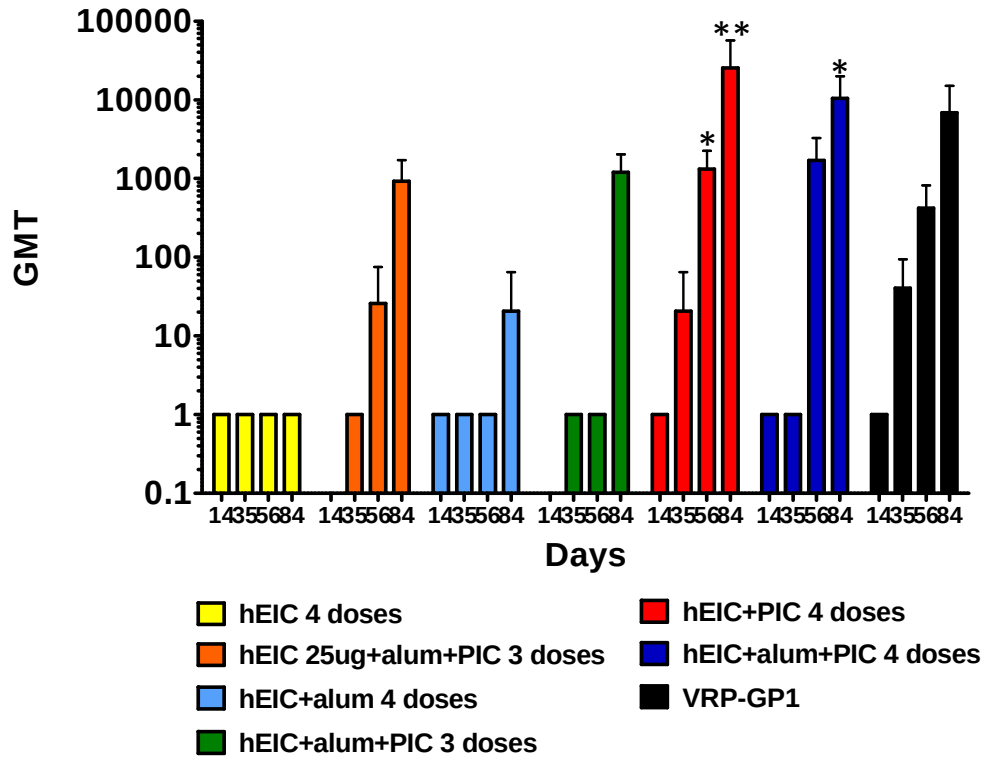

Fig. 14. The levels of IgG2a in the sera of mice immunized with the hEIC alone or with different adjuvants.

Mice (n=5 per group) immunized with the hEIC as described for the challenge study (Figs. 5 and 6), were bled 2 weeks after each immunization and 3 weeks after the last immunization. Individual sera from days 0, 14, 35, 56, and 84 were analyzed for IgG2a specific to Ebola virus. The GMT for preimmune (day 0) serum in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the level of the IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made between immunization groups relative to the PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

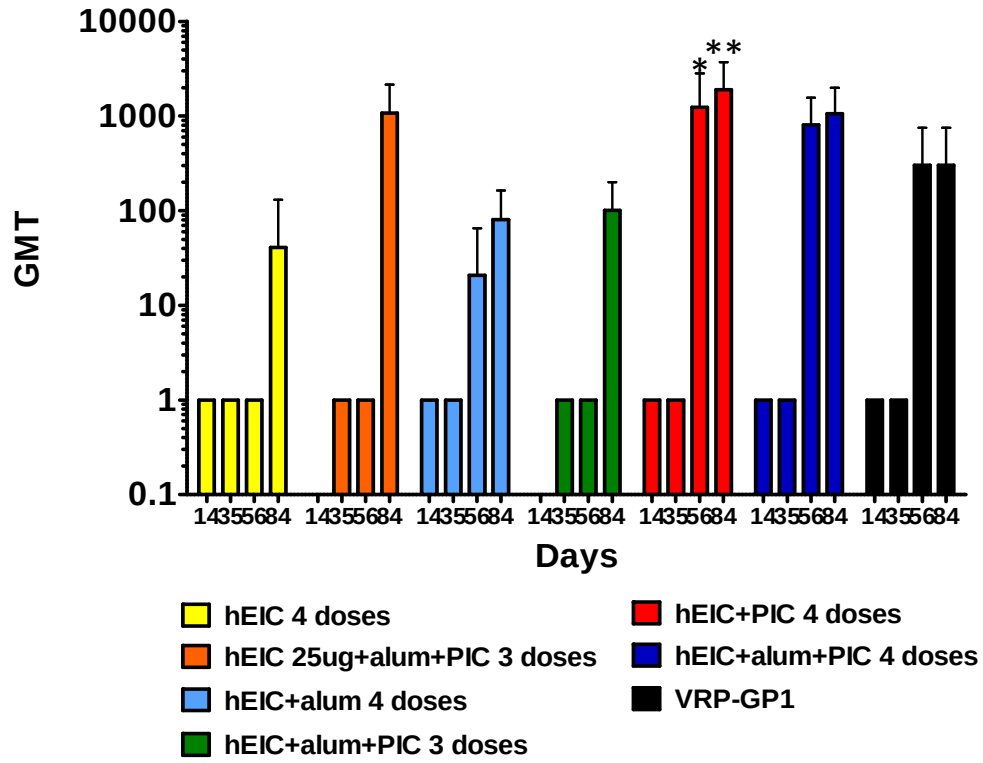

Fig. 15. The levels of IgG2b in the sera of mice immunized with the hEIC alone or with different adjuvants.

Mice (n=5 per group) immunized with the hEIC as described for the challenge study (Figs. 5 and 6) were bled 2 weeks after each immunization and 3 weeks after the last immunization. Individual sera from days 0, 14, 35, 56, and 84 were analyzed for IgG2b specific to the Ebola virus. The GMT for preimmune (day 0) serum in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the level of IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made between immunization groups relative to the PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

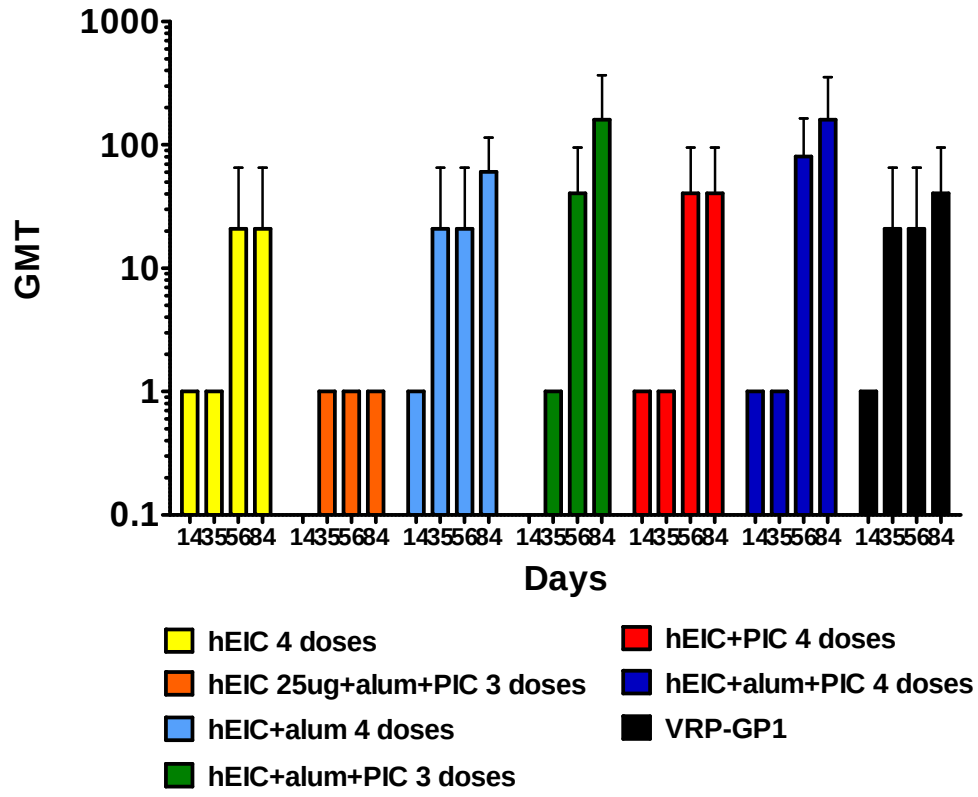

Fig. 16. The levels of IgG3 in the sera of mice immunized with the hEIC alone or with different adjuvants.

Mice (n=5 per group) immunized with the hEIC as described for the challenge study (Figs. 5 and 6) were bled 2 weeks after each immunization and 3 weeks after the last immunization. Individual sera from days 0, 14, 35, 56, and 84 were analyzed for the IgG3 specific to the Ebola virus. The GMT for preimmune (day 0) serum in all groups were ≤ 1 (data not shown). The error bars indicate the SD. Statistically significant differences ($p<0.05$) in the level of the IgG anti-Ebola were determined by the Kruskal-Wallis test. Comparisons were made between immunization groups relative to the PBS control. * $P < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

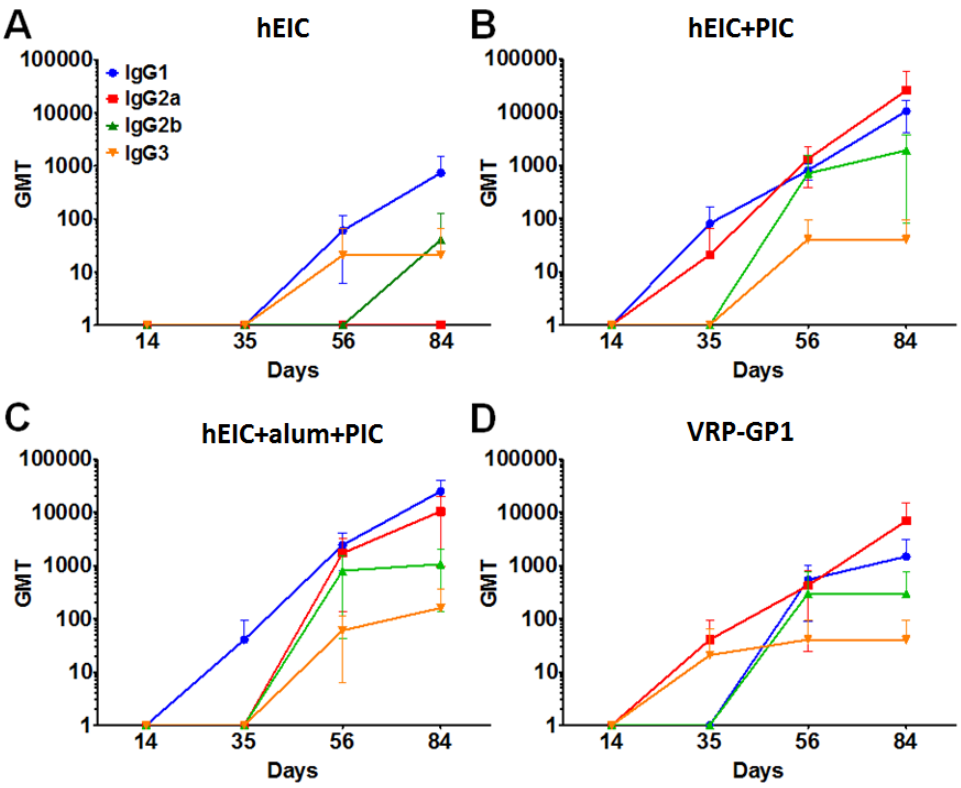

Fig. 17. IgG isotypes induced by immunization of mice with the hEIC + PIC or hEIC + PIC +alum induced a mixed Th1/Th2 immune response.

Mice (n=5 per group) immunized as described for the challenge study (Figs. 5 and 6), were bled 2 weeks after each immunization (days 14, 35, and 56) and 3 weeks after the last immunization (day 84). The anti-Ebola antibody isotypes were measured by ELISA. IgG1 (blue), IgG2a (red), IgG2b (green), and IgG3 (orange) levels are presented for mice immunized with (A) the hEIC, (B) the hEIC + PIC, (C) the hEIC + PIC + alum, and (D) VRP-GP1.

The hEIC induced the production of anti-6D8 antibody in mice.

To evaluate whether mice immunized with the hEIC produced IgG specific for the 6D8 epitope, a competition binding assay was performed. When the humanized mAb 6D8 was mixed with serum from the mice immunized with the hEIC + PIC or with VRP-GP1, the binding of the resulting mouse serum to Ebola virus was decreased (Fig. 18). The result suggests that plant-produced humanized mAb 6D8 competed with a component of the mouse serum for the binding sites on the Ebola virus particles, and implies that antibody specific to the 6D8 epitope exists in the serum of mice immunized with the hEIC and PIC.

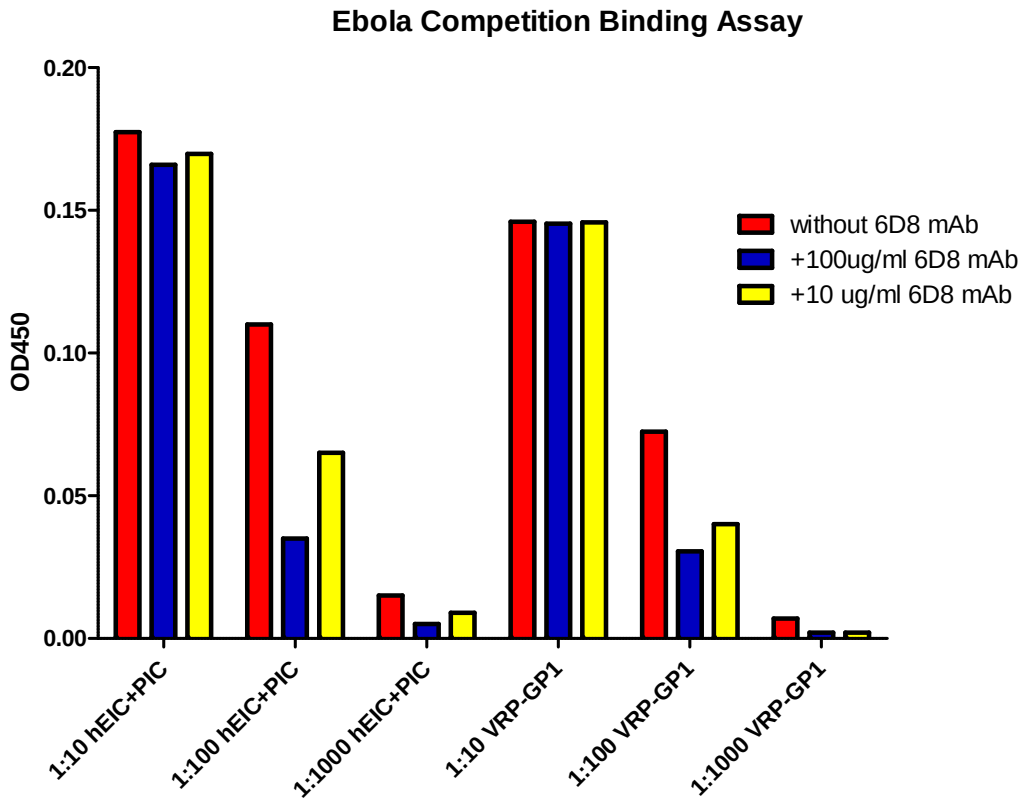

Fig. 18. Competition ELISA to examine serum antibody specific to the 6D8 epitope in mice immunized with the hEIC + PIC or with VRP-GP1.

Sera were pooled from mice immunized with hEIC + PIC or with the VRP-GP1, diluted 1:10, 1:100, or 1:1000, and mixed with plant-produced humanized mAb 6D8. The mixtures were used to probe irradiated Ebola virus that was bound to an ELISA plate. Goat anti-mouse IgG conjugated with HRP was used to detect the bound mouse IgG.

DISCUSSION

Since the first Ebola virus outbreak approximately 30 years ago, no vaccine or drug against the Ebola infection in humans has been approved. There are different types of Ebola vaccines in development. Previous studies have demonstrated that both humoral and cell-mediated immune responses are critical to mediate the protection against a lethal Ebola virus challenge (32, 44, 49, 65, 68, 69). In this study, we determined that the EIC produced in *N. benthamiana* leaves induced humoral immune responses in mice. We anticipate that the cell-mediated immune response induced by EIC will be examined with future research.

In this study, purified hEIC and mEIC were produced in either normal wild type or humanized N-glycan *N. benthamiana* (57), which synthesize complex N-glycans without xylose and core α-1,3-fucose. Because the constructs that we used to produce both immune complexes have a SEKDEL sequence at the C-terminus, the proteins were expected to be retained in the ER and thus obtain similar N-glycan compositions in both the wild type and the humanized N-glycan plants. Both enzymes that were suppressed in the humanized N-glycan plants, β-1,2-xylosyltransferase (XylT) and α-1,3-fucosyltransferase, are localized in the Golgi apparatus, and thus had no effect on the N-glycans on the EIC produced in the wild-type plants, if they were indeed retained in the ER.

Many studies showed that an IC, the combination between antigen and monoclonal antibody against that antigen, can induce both arms of

the adaptive immune responses (1, 9, 40, 41). Most of these studies used a mixture of antigen and the antibody specific to that antigen. The only fusion IC that induced an immune response was the tetanus toxin fragment C (TTFC) IC, with the fusion of TTFC at the C-terminus of an anti-TTFC antibody (9). These studies indicated that the IC preferentially binds to antigen-presenting cells via the Fc receptor and induces the adaptive immune response. For the plant-produced EIC, the structure of the Ebola GP1 is unknown. It is possible that the GP1 might block the Fc region on the mAb 6D8 to which it is fused. This could explain why the EIC cannot bind to the Fc receptor on the antigen-presenting cell and thus cannot induce the adaptive immune response. Another possibility is that the humanized mAb 6D8 used in the hEIC might affect the immunogenicity of the hEIC in mice.

Published studies demonstrated that the administration of the IC formed with xenogeneic antibody were not able to induce humoral and cell-mediated immune responses, whereas using a syngeneic antibody in the IC did induce both immune responses (40, 41). The researchers showed that mice immunized with human serum albumin (HSA) bound tp rabbit anti-HSA produced lower antibody responses to HSA compared with mice immunized with HSA bound to mouse anti-HSA. However, our comparison between the immunogenicity of the hEIC and the mEIC in mice showed no statistical difference in either the antibody levels or the protection against lethal Ebola virus challenge. The P value from the Mann

Whitney test of the antibody levels between the groups of mice immunized with four doses of the hEIC or four doses of the mEIC was 1; and the p value from the log rank test of the protection levels between the groups of mice immunized with four doses of the hEIC + alum + PIC or four doses of the mEIC + alum + PIC was 0.6389. This result indicates that using a syngeneic antibody in the plant-produced EIC could not increase its immunogenicity in mice. However, different adjuvants co-delivered with the EIC induced strong immune responses, leading to effective protection from lethal Ebola virus challenge.

Several TLR agonists are currently being evaluated as adjuvants for vaccine development. Among all TLR agonists, TLR7/8 agonists, such as Resiquimod, Gardiquimod, CL075, and CL097, have been shown to enhance vaccine efficacy by inducing both specific humoral and cell-mediated immune responses (8, 17, 36, 48). Moreover, many studies have demonstrated that PIC, a synthetic analog of double-stranded RNA (dsRNA), is an effective adjuvant of vaccines (11, 27, 37, 47, 55). The combination of PIC with other adjuvants also showed synergistic effects (2, 10, 22, 42, 72). Most studies showed that the combination of different immunostimulators could induce the immune response more effectively and DC could cross-prime CD8 T cells in vivo, which is essential for enhanced vaccine immunogenicity (15). PIC induced efficient cross-presentation from mature DC (14). TLR3 engagement induced cross-priming in virus-infected cells (53). Thus, conjugating the appropriate TLR

ligand to the vaccine might promote cross-presentation and help to elicit improved immunity.

In this study, we focused on testing the adjuvant effect of TLR7/8 and TLR3 agonists because TLR7/8 and TLR3 recognize single-stranded and double-stranded RNA, respectively. The Ebola virus has a negative sense RNA genome, and it uses double-stranded RNA as the intermediate in RNA replication. Thus, both TLRs should be activated when the cells are infected by the Ebola virus. From our data, use of CL097 (a highly water soluble derivate of R848) did not enhance antibody immune responses specific to the Ebola virus, but co-administration with PIC did (Fig.2). A previous study showed that a combination of MyD88-dependent and MyD88-independent pathway agonists created synergy, but the combination of CL097 and PIC in our study, which are MyD88-dependent and MyD88-independent pathways agonists, respectively, did not yield a synergistic effect. This was borne out by the observation that the antibody levels were the same for the use of PIC alone as for the combination of PIC + CL097 (Fig. 4). From this result, PIC shows potential for use as an adjuvant for the Ebola vaccine, because it enhanced the antibody response specific to Ebola virus when co-delivered with the EIC. Thus, we chose to add PIC to the hEIC for immunization of mice in the challenge study.

Alum was also added to the EIC to immunize another group of mice in the experiment, because it is the classical adjuvant for vaccines and

until recently, the only FDA-approved adjuvant for human use (7, 51). If the addition of alum could enhance the immune response produced with the EIC, it could prove practical for clinical trials in humans. We also tested the combination of PIC + alum, which is the combination between the immunostimulator and the classical adjuvant, respectively. Our data, however, showed that the levels of antibody specific to Ebola virus in the group of mice immunized with the hEIC alone and the hEIC + alum were not statistically different ($p=0.7715$) (Fig. 6a). The mice in both groups were not protected from the lethal Ebola virus challenge, which was similar to the mice in the PBS negative control group (Fig. 5a). This result implies that alum failed to induce specific anti-Ebola immunity in a murine system. In contrast, PIC co-administered with the hEIC induced high levels of total IgG and neutralizing antibody specific to the Ebola virus (Fig. 6) and also provided protection from virus challenge (Fig. 5a). The antibody responses and the protection from Ebola virus challenge in the group immunized with the hEIC + PIC or in the group immunized with the hEIC + PIC + alum were not significantly different ($p= 1$ and 0.9480, respectively). This result confirms that hEIC + alum cannot induce a strong immune response and the combination of alum + PIC did not create any synergistic enhancement. However, co-administering PIC with the the hEIC did show enhancement of the anti-Ebola antibody responses and protection against the virus challenge. Our research demonstrated that PIC is a promising and effective adjuvant for an Ebola virus vaccine.

PIC was determined to be a good adjuvant for vaccine development in previous studies, because it can induce inflammation and long-lasting T cell immunity (52, 55, 60). PIC showed the induction of IL12 (62) and type I interferon (59) via TLR3 and the adaptor protein, TRIF (12). Moreover, it was able to induce a Th1 response to human papillomavirus in rhesus macaques (55). PIC directly enhanced the survival of activated CD4 T cells both in vitro and in vivo (21). It was observed that fully functional CD8 memory cells were generated without CD4 help in the presence of PIC (23). PIC was used in a phase I-II trial in patients with leukemia, but the trial was stopped due to the toxicity of the treatment (50). In animals vaccinated with the peptide and PIC, the response was optimal only when PIC was immunized at the same time as the peptide, and the response was partially dependent on cytokines produced by NK cells (52).

In our study, PIC showed the potential to adjuvant an adaptive Th1 immunity, because there was an increase observed in IgG2a in mice immunized with the hEIC co-delivered with PIC, whereas there was no IgG2a found in the serum of mice immunized with the hEIC alone (Fig. 5). The presence of IgG2a indicates a Th1 response. VRP-GP1 also showed the induction of all IgG subtypes, which is similar to the hEIC co-administered with PIC, and these groups were not statistically different in the protection against the lethal Ebola challenge. Immunization with the hEIC alone induced Th2, but not Th1, immune responses, because no

IgG2a was induced. Moreover, alum co-administered with the hEIC did not increase the level of IgG2a. This supports previous studies that alum predominantly induced the differentiation of the Th2 cells and mediated an antibody immune response (54). The hEIC alone and the hEIC + alum groups both showed the induction of Th2, but not Th1, immune responses, and all mice in these two groups died from the virus challenge. In contrast, the mice immunized with the hEIC + PIC, the hEIC + alum + PIC, and VRP-GP1 showed the induction of both Th1 and Th2 immune responses, and 80-90% of the mice in these groups survived the Ebola virus challenge. This implies that protection from the virus challenge requires a mixed Th1/Th2 immune response. In another study, the anti-GP mAb that was efficient in protecting mice from the virus challenge was predominantly IgG2a (69). This evidence supports the use of PIC with the EIC in ours study, because these mice produced more IgG2a than those that did not receive PIC.

Although we did not test the protective efficacy against a lethal Ebola virus challenge in mice that were treated with PIC alone (without the antigen), the protection that we observed likely resulted from a combination of the EIC and PIC. PIC was shown to induce type I interferon (59), and the interferon system is one of the earliest host defenses, becoming operative within hours of infection, and is recognized as a potent antiviral agent. However, the expression of interferon is transient, decreasing 4 days after induction (4). Therefore, it is unlikely

that PIC treatment without the antigen could protect the mice challenged 3 weeks after their last immunization, because the level of antiviral interferon would not be high enough 3 weeks after treatment. This suggests that the protection against a lethal Ebola challenge occurred from the effect of a combination of the EIC and PIC, and not from PIC alone.

As shown in Fig 11, the hEIC co-administered with PIC or a combination of PIC and CL097 induced the production of neutralizing antibody specific to Ebola virus. However, VRP-GP1, which protected the mice from a lethal challenge, did not induce the neutralizing antibody. This indicates that the neutralizing antibody is not the only factor that can protect against an Ebola infection. Although VRP-GP1 did not induce neutralizing antibody specific to the Ebola virus, it induced a cytotoxic T cell response, which mediates protection (44). The cytotoxic T cell induced by the EIC will be evaluated in the future. However, we believe that a prime-boost strategy that utilizes immunization with both VRP-GP1 and hEIC + PIC could enhance protective efficacy by providing both cellular and humoral immunity.

We found that immunization with the hEIC + PIC combination or with VRP-GP1 induced the production of IgG specific for the 6D8 epitope in Ebola GP1, because the plant-produced humanized mAb 6D8 inhibited the binding to Ebola virus (Fig.18). This is an important finding because it

suggests that the formation of an immune complex did not prevent the presentation of the 6D8 epitope.

In conclusion, this study presented the first evidence that protection against the lethal Ebola virus challenge can be afforded by plant-produced EIC in mice. This study also demonstrated that PIC is a good adjuvant for the EIC because it can induce antibody responses against Ebola virus, a mixed Th1/Th2 response, and protection from Ebola virus infection. In future research, the EIC will be tested for its ability to confer protection against an Ebola lethal challenge in non-human primates.

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Chapter 5

CONCLUSION

Plants have been used as bioreactors to produce therapeutic proteins for more than 20 years. Besides the low cost and large-scale production of safe and biologically active mammalian proteins, plants also are able to perform most post-translational modifications required for biological activity and suitable pharmacokinetics of recombinant therapeutic proteins. Among the many plant species, such as cereals, legumes, vegetables, and fruit plants, investigated for their ability to produce biopharmaceuticals, outstanding features are possessed by two tobacco species—*Nicotiana tabacum* and *Nicotiana benthamiana*. Foreign proteins can be expressed in these plants through either transient expression or stable transformation. *N. benthamiana* was shown to be useful in producing various therapeutic proteins. In this study, we used *N. benthamiana* as a bioreactor to produce the EIC.

The antigen-antibody immune complexes are able to activate both humoral and cell-mediated immune responses. The administration of the antigen in the form of an IC enhances the capacity of DC to prime CD4+ and CD8+ cytotoxic T cells. Thus, the uptake of Ag in the IC is improved, compared with uptake of soluble Ag. Due to these capacities of the IC, it has potential to be applied to vaccine development.

The goal of this dissertation was to develop an EIC from *N. benthamiana* for use as an Ebola vaccine. To produce the EIC, Ebola

glycoprotein (GP1) was fused at the C-terminus of the heavy chain of humanized IgG monoclonal antibody (mAb) 6D8, which specifically binds to a linear epitope on GP1. Co-expression of the GP1-heavy chain fusion and the 6D8 light chain using a geminiviral vector in *N. benthamiana* leaves produced an assembled immunoglobulin, which was purified by ammonium sulfate precipitation and protein A/G affinity chromatography. The IC formation was confirmed by a C1q binding assay, which showed that the recombinant protein bound to the complement factor C1q more effectively than antibody alone. Size measurements of the purified recombinant protein suggested that the EIC contained four to six molecules. Mice were subcutaneously immunized with the purified EIC and showed production of anti-Ebola antibodies.

Although the immunized mice produced antibodies against Ebola virus, the level of the antibody was not high enough to protect the mice from Ebola infection. The humanized antibody was replaced with mouse mAb 6D8 to produce the mEIC since previous studies had demonstrated that using syngeneic antibody in antigen-antibody immune complexes could induce both humoral and cell-mediated immune responses. However, the levels of anti-Ebola antibody produced with immunization with either the mEIC or the hEIC were observed to be similar. This indicates that using syngeneic antibody in our EIC did not increase the efficacy of the IC. To improve the immunogenicity of the EIC, different adjuvants were tested with the EIC .

In this study we evaluated the adjuvant effect of two TLR agonists co-delivered with the EIC. Mice were immunized subcutaneously in four doses with the EIC alone, or with the EIC co-administered with a TLR3 agonist (PIC) or with a TLR7/8 agonist (CL097), alone or in combination with PIC. The use of PIC showed good results as an adjuvant for the plant-produced EIC, because the mice immunized with the EIC + PIC produced high antibody response against Ebola virus. In contrast, CL097 was not a good adjuvant for the EIC, because it did not enhance the immune responses when immunized with the EIC. Furthermore, the combination of PIC and CL097 did not result in a synergistic enhancement.

To determine the efficacy of the EIC as an Ebola vaccine, the immunized mice were intraperitoneally injected with 30,000 LD50 live Ebola virus. The immunization with the EIC + PIC protected 80% of the mice from a lethal Ebola virus challenge, comparable to the VRP-GP1 positive control. Alum was also tested as an adjuvant by immunizing mice with the EIC + alum, but only 20% of the mice were protected. These data support PIC as an effective adjuvant for the EIC.

The profile of IgG subtypes showed that the EIC alone induced a predominant Th2 immune response, as indicated by the production of IgG1, but no IgG2a. However, a mixed Th1/Th2 response was produced in mice immunized with EIC + PIC, and the mice in this group were protected from the Ebola infection. This suggests that a mixed Th1/Th2

immune response may be necessary for the protection against lethal Ebola virus challenge.

In the immunization study, we used VRP-GP1 as the positive control because it has been shown to protect mice from Ebola infection in previous studies. Since VRP-GP1 is an alphavirus vector, it undergoes limited replication in antigen-presenting cells of the immunized host, thus creating a viral replicon RNA that can act as a powerful adjuvant through Toll-like receptor signaling. Although VRP-GP1 could not stimulate the production of neutralizing antibody against the Ebola virus, it enhanced the antibody response specific to Ebola virus, induced a mixed Th1/Th2 response, and conferred protection against the lethal Ebola virus challenge. Thus, the comparable response with the use of the plant-derived EIC is a significant observation that indicates strong potential for the efficacy of a plant-produced EIC vaccine.

The production cost of our EIC is likely to be substantially lower than for VRP-GP1 production, which requires mammalian cell culture. The vaccine efficacy of the EIC might be improved by several different strategies: for instance, testing different linkers used between the mAb 6D8 heavy chain and the GP1, using genetically engineered *N. benthamiana* to produce the EIC with human glycosylation, using a truncated GP1 to produce the EIC, increasing the dose of the EIC, trying a different prime-boost strategy, or testing the stability of the EIC after freeze-drying.

In conclusion, the EIC was produced in *N. benthamiana* leaves using a geminiviral replicon system. The protein was purified with ammonium sulfate precipitation and protein A/G affinity chromatography to remove most plant proteins. The EIC co-delivered with PIC induced high levels of total IgG antibody and neutralizing antibody specific to the Ebola virus, activated mixed Th1/Th2 responses, and protected mice from a lethal Ebola challenge. These results support the plant-produced EIC as a good vaccine candidate against Ebola virus infection.

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CHAPTER 1

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Appendix A

COMPARISON OF THE EIC EXPRESSION BETWEEN THE GEMINIVIRAL REPLICON AND THE ICON SYSTEM

Objective

To compare the expression level of the humanized Ebola immune complex (hEIC) expressed from different constructs of the Geminiviral replicon and the ICON systems.

Materials and Methods

The geminivector constructs were described in Chapter 2. For magnICON constructs, Hgp(TMV) and K(PVX) were infiltrated with pICH17388, pICH21380, and pICH14011 (1) at final OD600 0.2. Plants were maintained in a growth chamber, and the leaves were harvested on day 4 after infiltration. Soluble proteins were extracted by grinding the leaves with a fastprep machine in 1ml of extraction buffer (phosphate-buffered saline (PBS), pH 7.5, leupeptin, and 0.1% Tween-20) per 0.1mg of leaves. After centrifugation at 13,000 rpm for 5 min, the supernatant was retained for subsequent analysis by ELISA (Materials and Methods in Chapter 2).

Result and Discussion

The IgG expression was analyzed by ELISA and shown in Fig. 1. The expression level of the geminivector using pBYRH2GP1kdel is the highest. Thus we continued to use this construct for all the hEIC production. As shown in Chapter 2, Fig. 6, purified hEIC presented

different protein species when detected with the anti-human heavy chain, due to protein degradation. The ELISA result represented the IgG expression. Because of the degradation, the level of the EIC is less than the IgG level.

Reference

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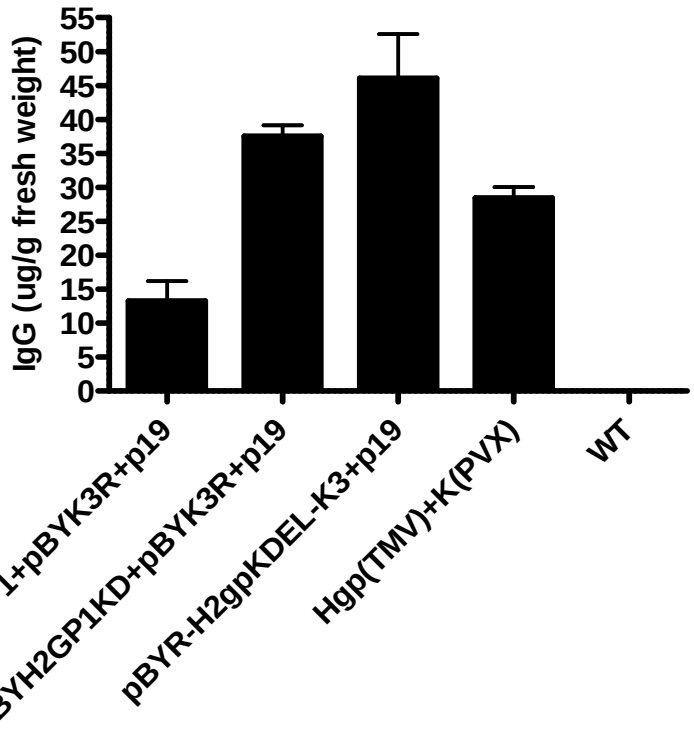

Fig. A1. IgG expression in *N. benthamiana* leaves

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Appendix B

COMPARISON OF THE EIC EXPRESSION AMONG DIFFERENT GP1 TRUNCATIONS

Different truncations of the GP1 were designed and constructed as shown in Fig.1—H2GP312 and H2GP355. The expression levels of the EIC from each of these constructs were measured by ELISA (Fig. 2). There is no significant difference among the EIC from full length GP1 and the EIC from H2GP312 and H2GP315. Moreover, the C1q binding assay confirmed that all three different versions of EIC bind to C1q similarly.

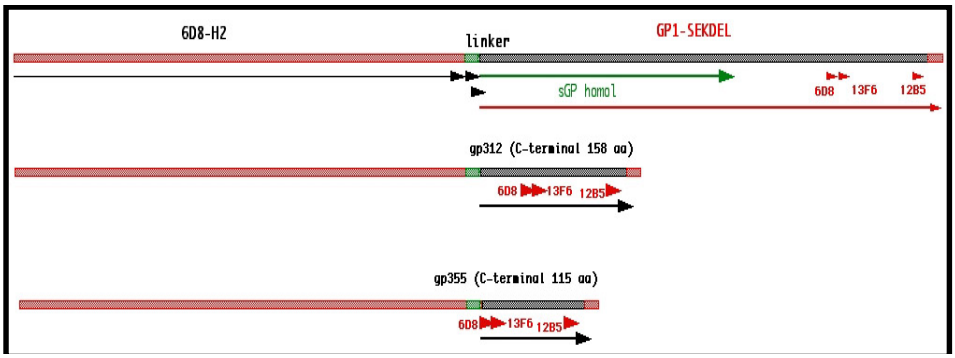

Fig. B1. Schematic representation of the full length and truncated Ebola GP1 fused to the C-terminus of the heavy chain of the mAb 6D8. H2GP312 has 158 amino acids (aa) of the GP1 from the C-terminus, whereas H2GP355 has 115 aa of GP1 from the C-terminus fused to the heavy chain.

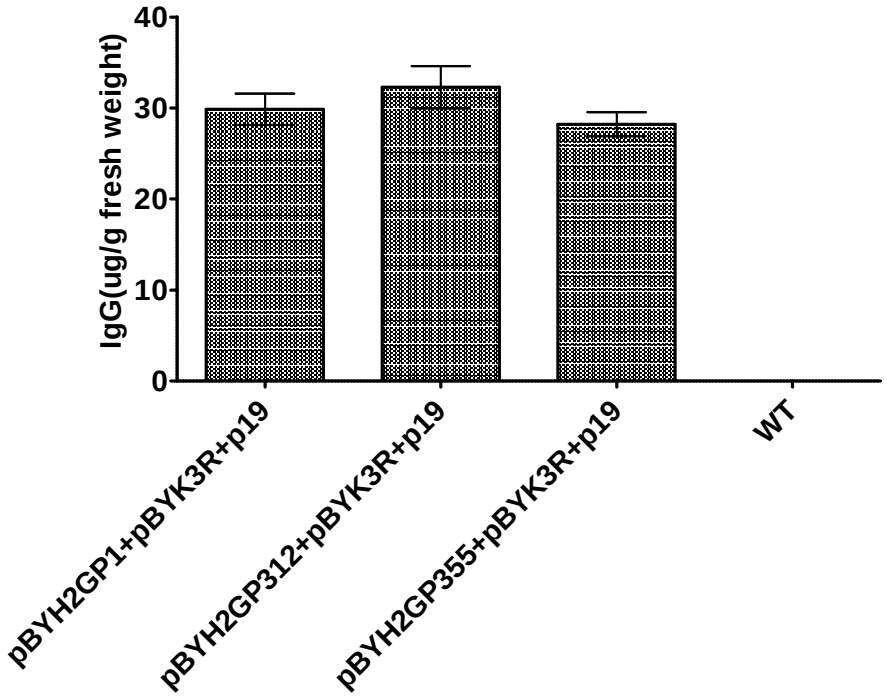

Fig. B2. The expression levels of EIC containing full length GP1 or different truncations of GP1.

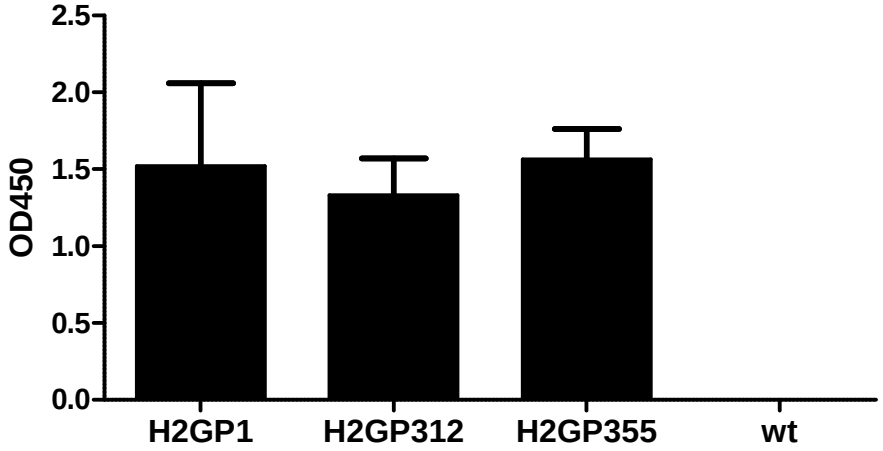

Fig. B3. C1q binding of EIC containing full length GP1 compared to different truncations of GP1.

Appendix C

A NOVEL SYSTEM for PRODUCING PHARMACEUTICAL PROTEINS IN HYBRID MAIZE

The objective was to test the development of a bipartite Gemini virus gene expression system in corn cell cultures. Cell lines containing the LIR and SIR sites and a reporter gene (GUS) were produced and retransformed with either a construct containing the Rep or a control construct. Levels of protein expression can be used to confirm high levels of replication upon “activation” with the Rep.

A gene expression construct pSCS20 using components of the Wheat Dwarf Virus (WDV) for GUS expression was designed and produced, and is shown in Fig. 1. The construct would then be used to genetically engineer the BMS cells. Transgenic BMS cells would be re-transformed with a construct containing the Rep gene from WDV, using pSCS24 or pSCS25 (Fig.2 and Fig.3). pSCS24 contained the Rep under the BLT4.9 promoter which is a weak promoter, whereas pSCS25 contained the Rep under the 35S promoter which is a strong promoter. The Rep gene would initiate excision on replication of the construct, including the scorable marker gene. The amount of expression could be tested and compared to non-induced transgenic lines.

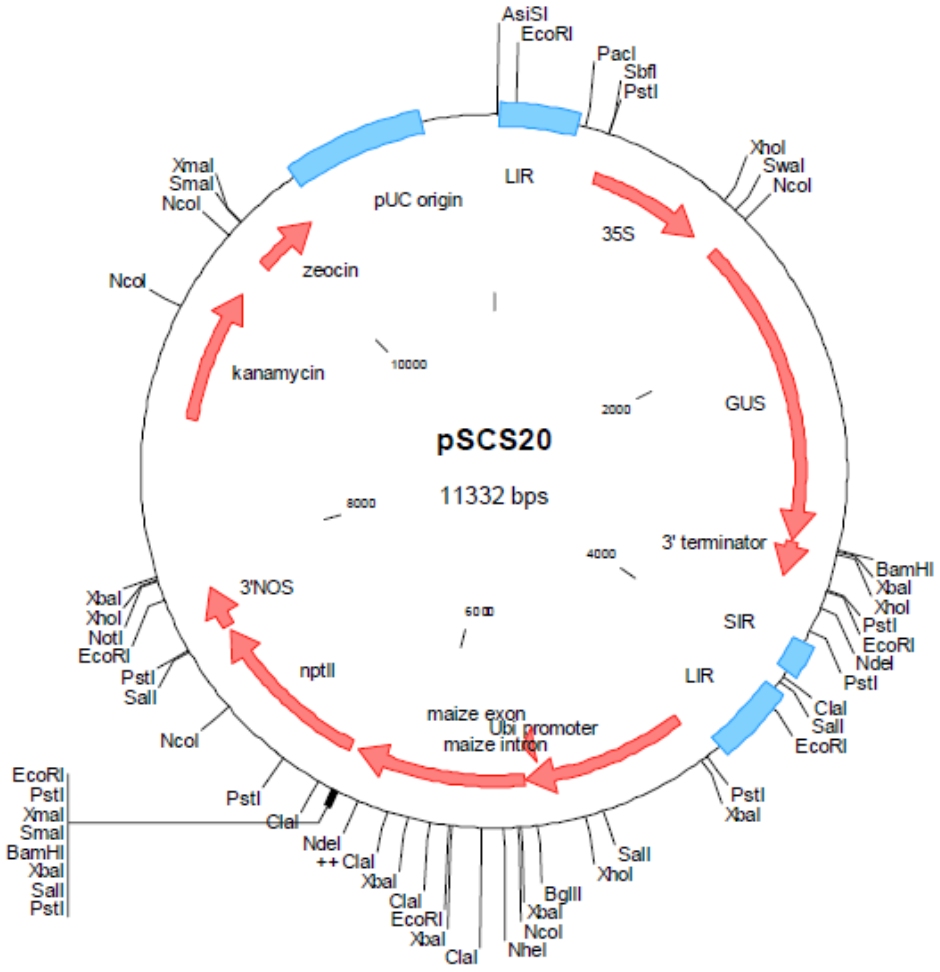

Fig. C1. Map of pSCS20.

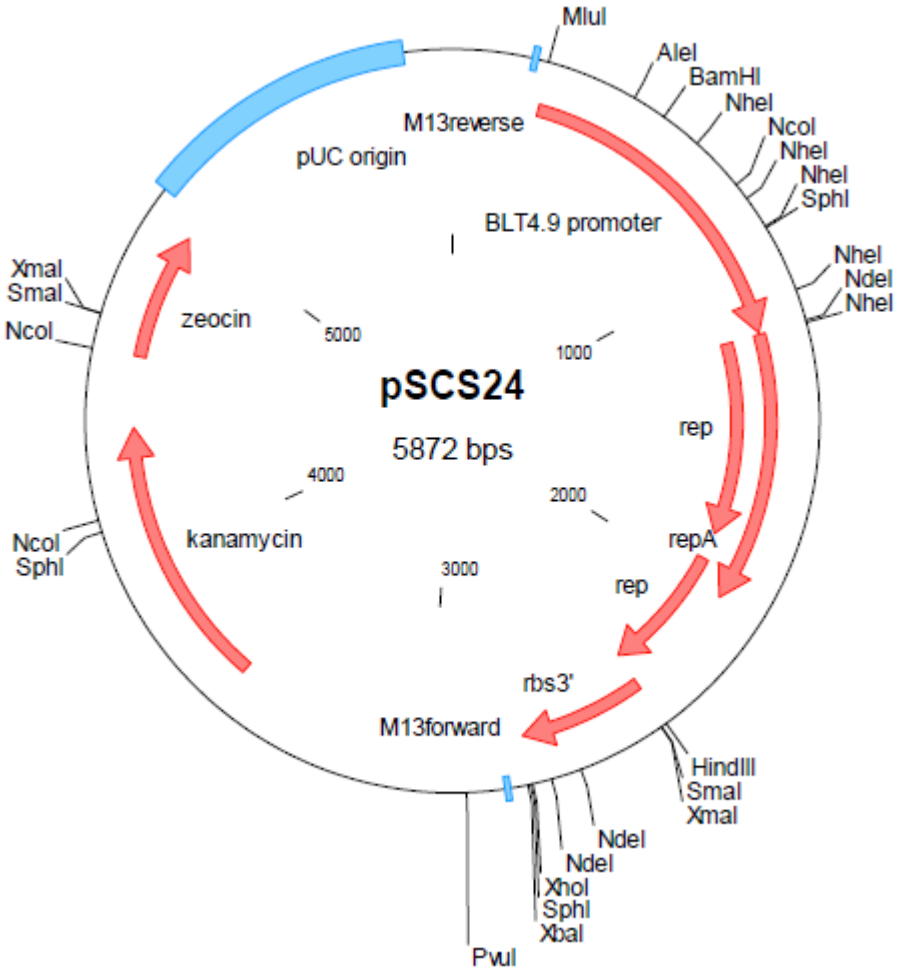

Fig. C2. Map of pSCS24.

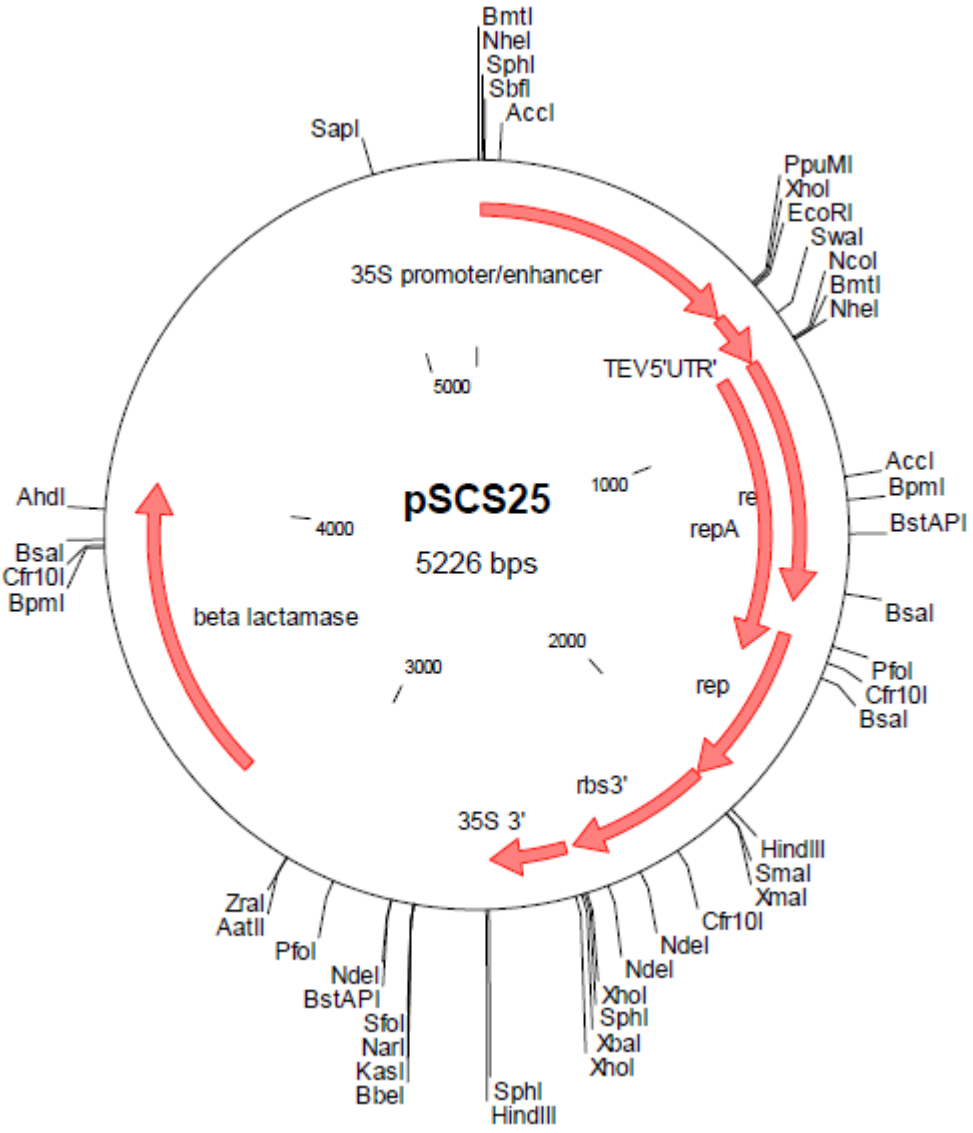

Fig. C3. Map of pSCS25

The transformation was done via particle-mediated gene insertion using a BioRad PDS 1000 He. The coating of the gold particle with DNA was performed using BioRad recommended protocol. Gold was 1.0 micron at 60mg/ml concentration. Gun parameters were chosen from those recommended by BioRad and as described in the protocol from the University of Minnesota. The rupture pressure was 1100 PSI, vacuum of 27.5 in of Hg, 1/4-in gap distance and 9cm from the stopping screen to the target tissue.

After we obtained the stable BMS cell line containing pSCS20 and re-transformed either pSCS24 or pSCS25, PCR to detect Rubisco as an internal control, GUS gene, and circular form were performed, and results are shown in Fig. 4. From the PCR screen, the GUS gene was detected in some clones, but the circular form, which is the intermediate Geminivector replication, was not detected. To summarize, the WDV Rep, transiently expressed in stable BMS cell lines containing the GUS gene in the WDV replicon, could not cut and amplify GUS in the WDV replicon. Thus, the expression of GUS did not increase.

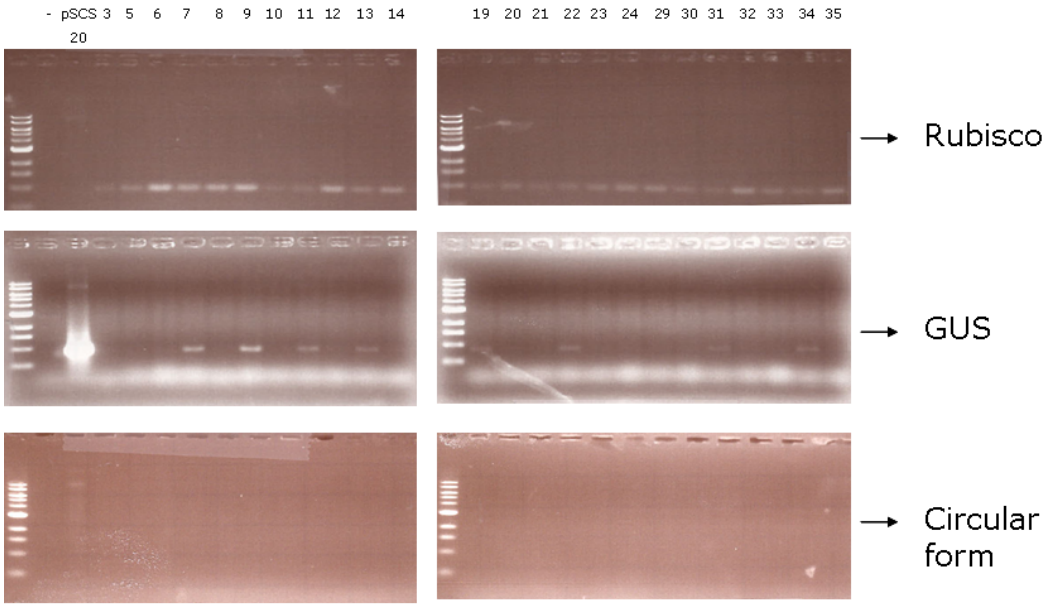

Fig. C4. PCR screen for Rubisco, GUS, and the circular form.