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Adjuvant like effect of vaccinia virus 14K protein: A case study with malaria vaccine based on circumsporozoite protein.

Ph.D Thesis

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DECLARATION.

I, Aneesh Vijayan, declare that the thesis entitled **``Adjuvant like effect of vaccinia virus 14K protein: A case study with malaria vaccine based on circumsporozoite protein´´** and the work presented in it, carried out at CNB-CSIC under the guidance of Prof. Mariano Esteban, are my own. No part of this work has previously been submitted for a degree or any other qualification at this university or any other institution.

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Abbreviations.

Ab	Antibody.
Ag	Antigen.
APC	Antigen Presenting Cell.
CD	Cluster of differentiation.
CMI	Cell Mediated Immunity.
CSP	Circumsporozoite Protein.
CTL	Cytotoxic T Lymphocyte.
DMEM	Dulbecco´s Modified Eagle´s medium.
DNA	Deoxyribonucleic Acid.
dsRNA	Double stranded ribonucleic acid.
E.coli	*Escherichia coli*.
EDTA	Ethylene Diamine Tetraacetic Acid.
ELISA	Enzyme Linked Immunosorbent Assay.
ELISPOT	Enzyme Linked Immunosorbent Spot Assay.
GPI	Glycophosphatidylinositol.
HBsAg	Hepatitis B virus surface antigen.
HA Locus	Hemagglutinin Locus.
i.d	Intradermal.
IFN	Interferon.
Ig	Immunoglobulin.
IL	Interleukin.
iNOS	Inducible Nitric Oxide Synthases.
i.p	Intraperitoneal.

IPTG	Isopropyl beta-D-thiogalactopyranoside.
iRBC	Infected Red Blood Cell.
IRF	Interferon Regulatory Factor.
kDa	Kilodalton.
LPS	Lipopolysaccharide.
mAb	Monoclonal Antibody.
MFI	Mean Fluorescence Intensity.
MHC	Major Histocompatibility Complex.
MSP-1	Merozoite Surface Protein 1.
MVA	Modified Virus Ankara.
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells.
NO	Nitric Oxide.
NYVAC	New York Vaccinia Virus.
OD	Optical Density.
PBS	Phosphate Buffer Saline.
PFU	Plaque Forming Units.
rRNA	Ribosomal RNA.
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.
STAT-1	Signal Transducer and Activator of Transcription 1.
TCA	Trichloroacetic Acid.
T_{CM}	Central Memory T-cells.
T_{EM}	Effector Memory T-cells.
T_{EMRA}	Terminally Differentiated Effector Memory T-cells.
TLR	Toll Like Receptor.
TNF	Tumor Necrosis Factor.

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ABSTRACT

Development of subunit vaccines for malaria that elicit a strong, long-term memory response is an intensive area of research, with the focus on improving the immunogenicity of a circumsporozoite (CS) protein-based vaccine. In this study, we found that a chimeric protein, formed by fusing vaccinia virus protein 14K (A27) to the CS of Plasmodium yoelii, induces strong effector memory CD8+ T cell responses in addition to high-affinity Abs when used as a priming agent in the absence of any adjuvant, followed by an attenuated vaccinia virus boost expressing CS in murine models. Moreover, priming with the chimeric protein improved the magnitude and polyfunctionality of cytokine-secreting $CD8^+$ T cells. This fusion protein formed oligomers/aggregates that led to activation of STAT-1 and IFN regulatory factor-3 in human macrophages, indicating a type I IFN response, resulting in NO, IL-12, and IL-6 induction. Furthermore, this vaccination regimen inhibited the liver stage development of the parasite, resulting in sterile protection. In summary, we propose a novel approach in designing CS based pre-erythrocytic vaccines against Plasmodium using the adjuvant-like effect of the immunogenic vaccinia virus protein 14K.

INTRODUCTION

Malaria, meaning ‘bad air’ in Italian due to its prevalence in marshy areas, continues to present a major public health challenge and burden on economic development in many countries. *Plasmodium falciparum*, the main causative agent of malaria in humans, is known to cause approximately 225 million cases and about 781,000 deaths annually. Malaria continues to be a key factor involved in the mortality and morbidity among young children and mothers in African and Sub-Saharan areas (World Malaria Report, 2010). Areas which were previously declared malaria free are also under constant threat of resurgence due to changes in global weather and globalization. The situation has further worsened due to emergence of drug resistance parasites and ineffective vaccines. With several drugs in pipeline an effective vaccine is need of the hour in the fight against malaria.

To date vaccines have played an important role in the elimination of many diseases. However development of a malaria vaccine is curtailed by the ability of the parasite to deceive the immune system. Natural immunity to malaria is observed in endemic areas which also requires repeated exposure and takes considerable time to develop. However this kind of immunity ranges from partial to complete protection and is usually observed in older population. Therefore the development of an effective malaria vaccine is essential. Even though a large repertoire of antigens from various stages of malaria is available selecting a successful candidate is still an ongoing task. A successful vaccine should overcome several constraints such as it should provide long lasting protection irrespective of genetic variations that exists between human as well as parasite populations. Additionally it should be easy to produce and transport with minimal costs. In addition the vaccine may incorporate antigens from different developmental stages of the parasite. With the advent of new technologies in vaccine development an effective malaria vaccine might not be a distant dream now.

1.1 PLASMODIUM.

Plasmodium, the causative agent of malaria, belongs to the order *coccidia* a member of the *Protista* animal kingdom and *Apicomplexa* phylum . The family of *Plasmodium* is large with 172 different members infecting various eukaryotic species ranging from mammals, birds and reptiles. Only four members are known to infect humans viz; *P.falciparum*, *P.vivax*, *P.malariae* and *P.ovale*. Of these *P.falciparum* is the most prevalent with high mortality rates. Studies involving mouse malaria models, caused by *P.yoelii* and *P.bergeii*, helped us to understand malaria biology and are essential in development of various vaccines.

1.2 LIFE CYCLE OF MALARIA.

The vicious cycle of malaria requires two different species, one involving the host and other a vector.

1.2.1 DEVELOPMENT INSIDE HOST.

The host cycle is initiated with the blood meal by the infectious female *Anopheles* mosquito. During the meal the mosquito injects the sporozoites, contained in its salivary gland, into the subcutaneous tissue of the host. Recent studies has shown that not all sporozoites inoculated enters the bloodstream, some of them migrate into the draining lymph nodes (Chakravarty et al., 2007). The sporozoites are then transiently circulated in the blood before it home to the liver. The parasites pass through a number of hepatocytes before it establishes an infection, aided by CD81 marker on hepatocytes, and then replicates (Silvie et al., 2003). The whole process takes approximately 30 minutes and is known as the pre-erythrocytic stage. Inside hepatocytes the parasite undergoes several cycles of asexual replication to produce exoerythrocytic schizonts, each of which contains several thousands of merozoites; this typically takes about 2 to 15 days. These exoerythrocytic schizonts ruptures releasing the infectious merozoites which then infects the circulating RBC’s initiating the erythrocytic stage. During this stage the ring shaped parasite develops in a parasitophorus vacuole into trophozoites. This stage lasts for 48 to 72 hours during which the infected RBC’s harboring the merozoites ruptures releasing them which then infect more RBC’s. This stage is responsible for the clinical manifestation of the disease. Some

merozoites escape and undergo sexual replication forming the male and female gametes. Several factors are known to promote gametogenesis. These gametes are taken up by the mosquito during the blood meal.

1.2.2 Development inside vector.

Among the different species of mosquitoes only the female sex of the *Anopheles* genus transmits malaria in humans. The vector is not just a repertoire for harboring the male and female gametes taken up from the host but its biological variations effects the development and transmission of the parasites (Beier, 1998; Chugh et al., 2011). During a blood meal the female mosquito ingests the sexual stage of the parasites which then undergoes fertilization to produce a zygote which differentiates into motile ookinete. Fertilization occurs in the midgut of the mosquito following which the ookinete penetrates through the epithelial cell wall of the midgut to form oocyst. The oocyst undergoes meiosis to produce sporozoites which then migrates into the salivary gland. These sporozoites are then inoculated into the host during the blood meal.

1.3 Pathology and clinical manifestation of malaria.

Pathological symptoms of malaria are associated with the asexual replication of the parasite during the erythrocytic stages. The clinical manifestation of the disease is linked to the frequent bouts of fever linked to the rupture of erythrocytes and the subsequent release of merozoites. Other symptoms includes myalgia, nausea etc and in severe cases leads to seizures, coma, renal failure, jaundice etc. Studies have elucidated the role of toxins and other factors that when released during the rupture of erythrocytes results in the activation of pro-inflammatory cytokines such as IL-12, IL-1, TNF-α etc. The main culprit is GPI (Glycosyl Phosphatidyl Inositol), a glycolipid (Arrighi and Faye, 2010). Evidences suggest that purified GPI from parasite is able to stimulate a pro-inflammatory response (Kamena et al., 2008).

In patients with severe malaria comparatively high levels of parasitemia has been reported (Anstey and Price, 2007). This usually leads to an increase in the expression of adhesion molecules such as CD36, ICAM-1 etc by vascular endothelial cells which aids the sequestration

of infected RBC's into post capillary venules effecting the oxygen supply (Cserti-Gazdewich et al., 2012; Serghides et al., 2003). This leads to organ failure and when the organ involved is the brain (cerebral malaria) it leads to coma and eventually death. The main parasitic protein involved in this process is called PfEMP-1 (*P.falciparum* Erythrocyte Membrane protein) (Miller et al., 2002). Another complication associated with high parasitemia is the binding of sequestered iRBC's with iRBC's or uninfected ones to, this is known as rosetting, which often hampers the development of immunity against malaria (Vigan-Womas et al., 2008).

1.4 Malaria Immunology.

Immunity to malaria is a slow process and is influenced by factors such as age, area of transmission and the frequency of exposure to the parasite. So naturally acquired immunity against malaria occurs in only malaria endemic areas and in older generation through continuous exposure. However such immunity is known to wane faster and in most cases is unable to prevent disease severity (Doolan et al., 2009; Greenwood, 1999). There is a fine line between immunity and immunopathology in malaria. The presentation of large repertoire of antigens further confuses the immune system in mounting an appropriate response (Fig 1). An important question which continues to baffle malaria immunologists is the requirement of a chronic infection in order to maintain natural immunity. Even though studies have shown the importance of both innate and adaptive responses, we are yet to define the correlates of protection in controlling malaria.

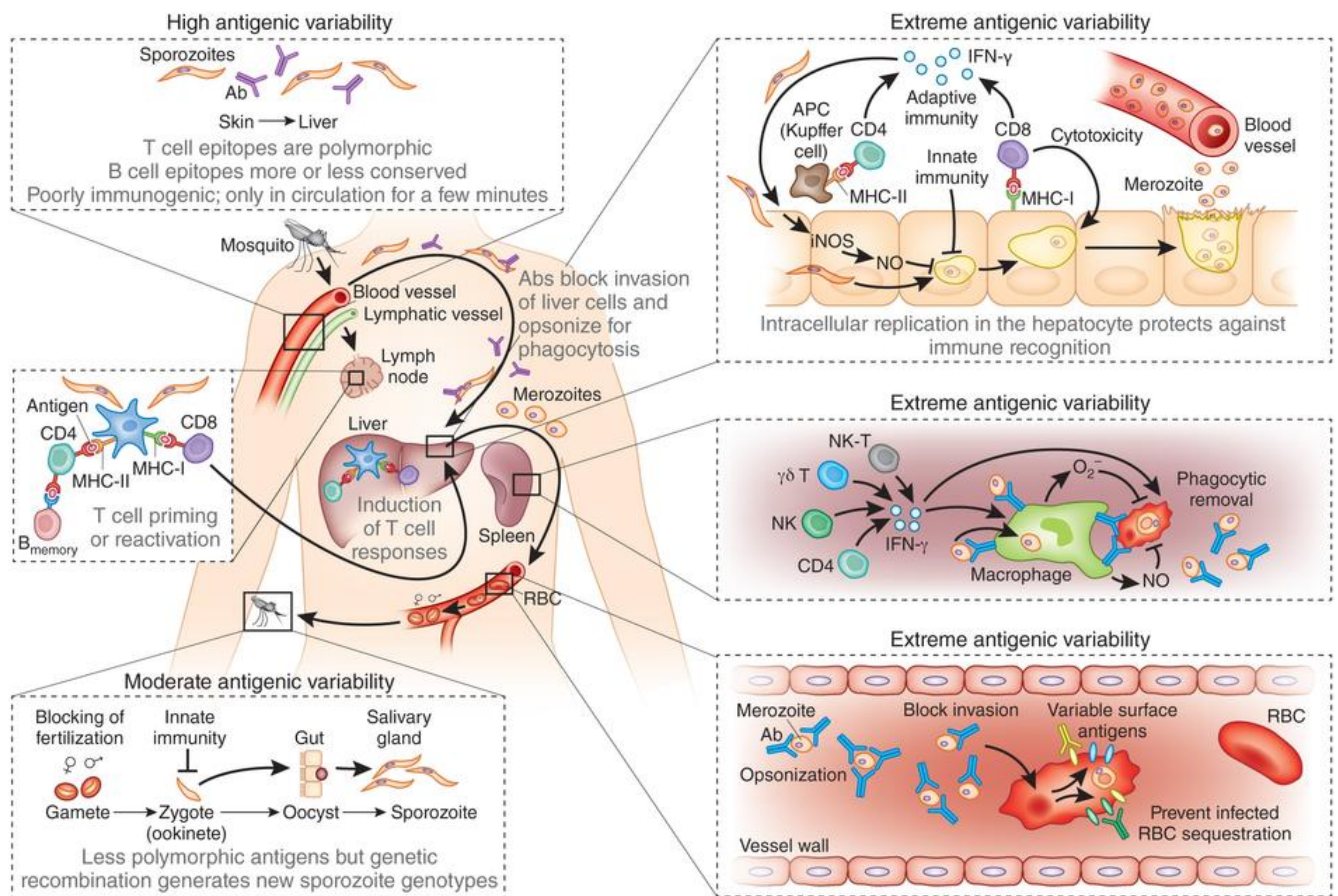


Figure 1: Immunology of malaria. A schematic representation of immune responses directed during various stages of parasite invasion (Riley and Stewart, 2013).

1.4.1 INNATE RESPONSES IN MALARIA.

Innate immune responses are carried out by a variety of cells including macrophages, monocytes, NK cells, granulocytes etc and generally acts as the first line of defense. They are also essential in shaping up the adaptive response via presentation of antigens and production of cytokines. An early innate response involving type I IFN response is essential in nipping the infection at the pre-erythrocytic stage (Arnold et al., 2010; Nussler et al., 1991; Shio et al., 2010). Like many parasitic diseases, TLR´s play a significant role in driving the immune response during malaria. Role of TLR´s are controversial due to its pros and cons during the infection (Coban et al., 2007; Franklin et al., 2011).

Macrophages, the sentinels of the innate immune response, have tremendous parasiticidal activity which involves direct and indirect actions. Several studies has emphasized the direct role of macrophages by phagocytosing iRBC´s which reduces the initial parasitemia by complement system (Silver et al., 2010) or ADCI (Langhorne et al., 2008). Additionally, recent studies have also shown macrophage dependent secretion of cytokines and reactive oxygen species aids not only in controlling the initial parasitemia but also helps to shape long term memory immune response. However recent studies have shown that like in *Leishmaniasis*, the sporozoite has also evolved mechanisms to impair the functions of macrophages. Excessive activation of macrophages results cytokine storm which does more harm than protection (Perkins et al., 2011). The abnormalities in iRBC´s results in rapid phagocytosis by the macrophages of both iRBC´s and the uninfected ones leading to severe anemia (SMA) (Kai and Roberts, 2008).

Thus the role played by macrophages in malaria is indispensable and a fine line exists between its role in protection or pathology.

1.4.2 Adaptive Response In Malaria.

Development of adaptive response against malaria via natural infection is a slow process and the major mediators involved are the antibodies and members of cell mediated immune system.

1.4.2.1 Humoral responses.

The role antibodies play in malaria is quite indispensable considering the fact that they are one of the early mediators of protection in malaria. Seminal studies based on transfer of purified antibodies from immune donors to naïve individuals have shown the role played by antibodies in building malaria immunity (Ak et al., 1993; Cohen et al., 1961). The humoral response to different antigens during each stage of parasite development whether within host or vector is known to provide protection. During the pre-erythrocytic stages antibody developed against circumsporozoite protein (CSP) (Tapchaisri et al., 1985) and sporozoite surface protein (SSP-2) (Rogers et al., 1992) prevents infection. In the next stage i.e. erythrocytic level, the iRBC´s express on its surface variety of highly polymorphic parasitic protein known as VSA (Variant Surface Antigens). Antibodies against VSA´s are known to protect individuals from severe

malaria (Chan et al., 2012). Additionally, antibodies against Pfemp-1 protein of malaria are also known to reduce the incidence of the disease especially in people residing in malaria endemic areas. Vaccines based on MSP-1 (Merozoite Surface Protein) capable of inducing antibodies are also gaining importance due to its effectiveness in reducing parasitemia. Another class of antibodies targeting the sexual stages of parasite, acting as a transmission blocking agents, are also of critical importance (Carter and Mendis, 1991). However not in all cases seropositivity against malaria antigens results in lifelong antibody titers. This could be explained by the defective development of B-cells (Dorfman et al., 2005).

Quality of the antibodies influences the outcome of the disease. Enhanced levels of cytophilic antibodies such as IgG1 and IgG3 have been reported in providing protection against malaria (Duah et al., 2010; Elliott et al., 2005). Antibodies produced against various surface antigens of merozoites were able to activate monocytes via FcRγII to release TNF and other mediators eliminating the infected erythrocytes in process known as antibody dependent cell mediated inhibition (ADCI) (Jafarshad et al., 2007). Antibodies are also known to neutralize the infection of RBC´s by merozoites (Williams et al., 2012). The role played by IgE antibodies in malaria is confounding due to reports which suggest its role in protection (Bereczky et al., 2004) as well as pathology (Perlmann et al., 1997).

An insight into the development of humoral response in malaria and the potential antigens could open up whole new aspects in our understanding of malaria and develop effective vaccines.

1.4.2.2 Cell mediated immune response.

In a seminal study the importance of cell mediated response in controlling malaria was first proven in animals that were thymectomized, making them more susceptible to infection (Brown et al., 1968). Protection in T-cells depleted animals by adoptive transfer of different antigen specific $CD4^+$ and $CD8^+$ T-cells further bolstered the importance of CMI in controlling malaria (Chakravarty et al., 2008; Stephens et al., 2005; Stephens and Langhorne, 2010).

$CD8^+$ T-cells:

Studies from animals vaccinated with multiple doses of γ-irradiated sporozoites, the only vaccine which induced sterile protection in animals, is mainly mediated by IFN-γ secreting $CD8^+$ T-cells (Malik et al., 1991). Evidence for the role of HLA Class I mediated $CD8^+$ protection against severe malaria was shown in children carrying the HLA-B53 MHC I allele in Gambia (Hill et al., 1992). Though most of our understanding about the role of $CD8^+$ cells in malaria was restricted to the pre-erythrocytic stage, recent advances have made it possible to evaluate the responses in the liver stage as well. Studies have illustrated that an effective $CD8^+$ response is influenced by IL-12 dependent production of IFN-γ, TNF-α and NO (Stevenson et al., 1995). $CD8^+$ responses known to provide protection during the pre-erythrocytic and liver stages, cannot mediate protection during the blood stages. Studies based on CD8 transfer and in $ß_2$ microglobulin deficient mice demonstrated that CD8 T-cells are not effective against the blood stages (van der Heyde et al., 1993). Initial studies considered liver to be the homing organ for anti-malaria $CD8^+$ T-cells, however in a pioneering work carried out by Zavala and colleagues showed that in fact the skin draining lymph nodes are the primary sites for the induction of $CD8^+$ T-cells against the liver stages (Chakravarty et al., 2007). Therefore in order to design effective CD8 T-cell based vaccine against malaria an important point to be considered is the skin immunity.

$CD4^+$ T-cells:

Accumulating evidence supports the role of $CD4^+$ T-cell response in regulating parasitemia or elimination of parasites (Meding and Langhorne, 1991; Shibui et al., 2009). Other than the direct effector function these cells are also important in the maintenance and survival of malaria specific $CD8^+$ T-cells (Overstreet et al., 2011). Even the most advanced malaria vaccine, RTS,S/AS01E a protein in adjuvant vaccine, mediates protection via memory $CD4^+$ T-cells secreting IFN-γ and TNF-α (Lumsden et al., 2011). In addition, effector $CD4^+$ T-cells producing IL-10 via IL-27 dependent path is known to protect from the severe immunopathology associated with malaria (Freitas do Rosario et al., 2012). Furthermore in malaria endemic areas $FOXP_3^-$ $CD_{45}RO^+CD4^+$ T-cells were linked to reduced pathological condition seen in severe malaria

(Walther et al., 2009). These cells were in fact independent of TCR stimulation but rather dependent on cytokines such as IL-10, TGF-ß and IL-2 (Scholzen et al., 2009).

Thus a strong balanced CMI response along with a quality humoral response is required to mount an effective strike against parasite. Therefore this concept should always pave the way while developing effective vaccines.

1.5 MALARIA VACCINES.

Vaccines are an essential tool in the armory for a fight against malaria. Considering the evolution of insecticide and drug resistance parasites, development of an effective vaccine is imperative. Development of a cost-effective vaccine is obscured because of the confounding ability of the parasite to manipulate the host immune system. In spite of the wide array of the antigens available, which are expressed by the parasite at various stages in its life cycle, pointing down a specific antigen has been quite intricate. Depending on the life cycle of the parasite, vaccines developed can be classified under three classes (Fig 2); viz (1). Pre-erythrocytic Vaccines (2). Erythrocytic Vaccines and (3). Transmission Blocking Vaccines. Since the most ideal vaccine should be the one that prevents the onset of clinical symptoms, we will be discussing more about the pre-erythrocytic vaccines.

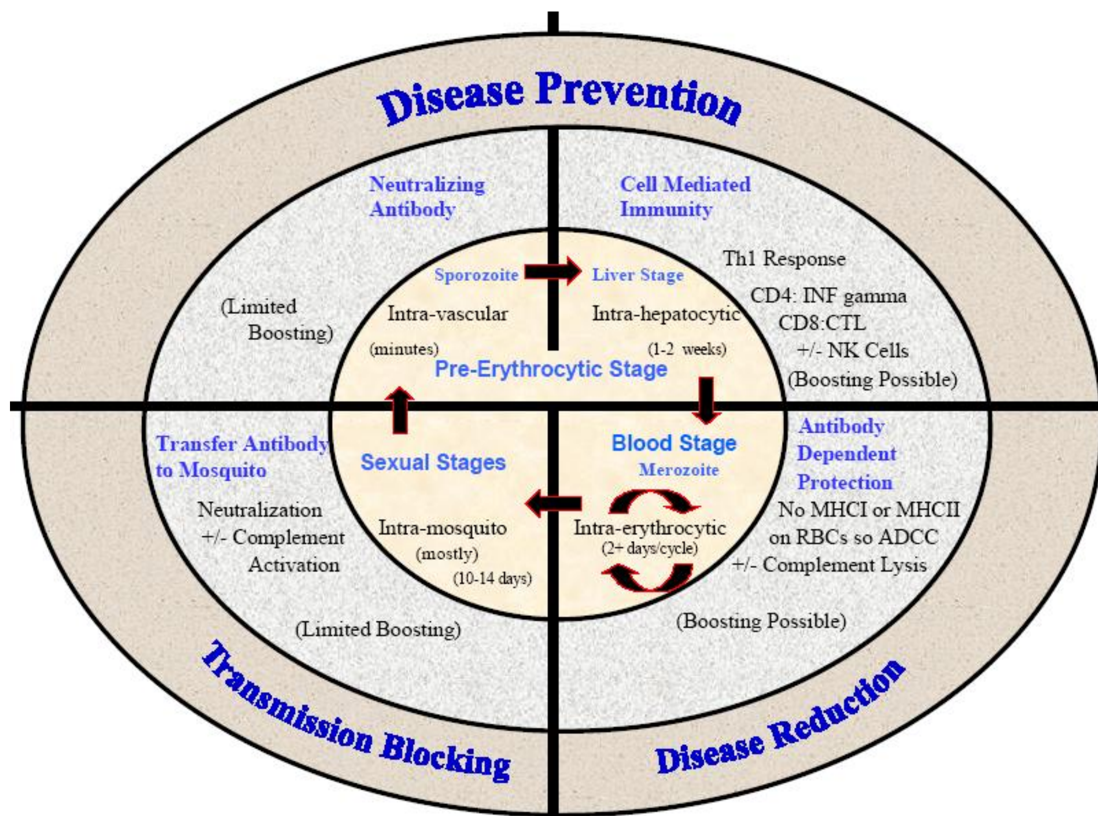


Figure 2: Types of malaria vaccines. Classification of malaria vaccines based on its action at different stages (Adapted from Malaria Path Initiative website).

1.5.1 PRE-ERYTHROCYTIC VACCINE.

Pre-erythrocytic vaccines involve antigens expressed right from sporozoite inoculation till the liver stage development. Therefore these vaccines prevent the occurrence of the disease. However an important drawback with this vaccine is that the window to mount an attack against the sporozoite is very short before it homes to the liver. In addition it has to overcome the problems associated with the polymorphic nature of T-cell epitopes. Even though the main protagonists during this stage are the antibodies, $CD8^+$ T-cells are also known to play a significant role during the liver stage. Liver being an immuno-tolerant organ, generating appropriate responses is a big hurdle. However there are overwhelmingly adequate data which supports the development of IFN-γ and TNF-α secreting $CD8^+$ T-cell in liver, especially in the γ-irradiated sporozoite vaccine model (Epstein et al., 2011). Activation of macrophages, by the

IFN-γ secreted by $CD8^+$ T-cell, helps in phagocytosis and also increases NO within hepatocytes (Seguin et al., 1994).

RTS,S vaccine, the current advanced vaccine against malaria, is a pre-erythrocytic vaccine. This vaccine comprises the CS protein fused with HbSAg in excess of HbSAg to form VLP´s (Stoute et al., 1997). Main mediators of protection in this model are $CD4^+$ T-cells in addition to antibodies (Lumsden et al., 2011). Even the golden model for malaria vaccines, γ-irradiated sporozoites, also promotes the development of intra-hepatic $CD8^+$ T-cells. The latest data from RTS,S phase III clinical trials shows an overall efficacy of 16.8% in children after 4 years (Olotu et al., 2013). The dismal performance of the vaccine can be attributed to the waning immunity especially the antibody titers of CS with time. Therefore better pre-erythrocytic vaccines could prove beneficial in controlling malaria.

1.5.1.1 Circumsporozoite protein: - A potent pre-erythrocytic antigen.

Most of the pre-erythrocytic stage vaccines target the proteins that are expressed on the surface of the sporozoite. The most valued antigens that are targeted during this stage are the CS protein and TRAP protein. However a vaccine based on CS protein was found to be more promising and has successfully entered the phase III clinical trials in the form of RTS,S.

CS protein is a major monomeric protein found on the surface of the sporozoites. The importance of CS protein as a major vaccine candidate was first reported by Nussenzweig and colleagues (Nussenzweig and Nussenzweig, 1985). Most of the protection in γ-irradiated and genetically attenuated sporozoite vaccine model was mediated by CS specific humoral and CMI responses (Kumar et al., 2009). The protein maintains similar structural, biochemical and immunological properties across different species of *Plasmodium*. The central domain of all *Plasmodium* species contains a repeat region which contains immunodominant repeat regions of B and T-cell epitopes (Lal et al., 1987). This region is usually flanked by conserved region I at the N-end and region III and region II+ at the C-end. Binding of sporozoites to the GAG chains of HSPG of hepatocytes is mediated by the region II of CS protein (Pinzon-Ortiz et al., 2001). Native CS has rod like structure which in anchored to the surface of the sporozoites by a GPI motif found at the C

terminal region (Plassmeyer et al., 2009). In a seminal study it was shown that CS binds to the ribosomes disrupting protein synthesis in infected hepatocytes (Frevert et al., 1998). CS protein also aids in the survival of sporozoites in hepatocytes by blocking the translocation of p65 into the nucleus thereby blocking the NFkB pathway. In addition it also promotes the expression of various genes vital in the metabolic process to allow the parasite to thrive inside the infected cells (Singh et al., 2007). Inspite of certain disadvantages vaccines based on CS protein are continuingly making progress.

1.5.2 ERYTHROCYTIC VACCINE.

Vaccines designed against this stage targets the blood stage antigens. Immune response generated at his stage can only reduce the intensity of the disease by reducing the parasitemia. Antigens expressed at this stage undergo rapid mutations and are highly polymorphic to overcome the pressure from the immune responses. Due to the lack of MHC I molecules on RBC´s an ideal erythrocytic vaccine candidate must induce high titer, high avidity antibodies. These antibodies, mainly directed against merozoites, prevent merozoites from infecting new RBC. However reports of malaria specific T-cell responses during this stage are also reported. This is mainly due the cross presentation of the antigens by professional APC (Miyakoda et al., 2008). In fact IFN-γ secreting $CD8^+$ T-cells against blood stage antigens were reported in patients in malaria endemic areas (Sinigaglia et al., 1985).

MSP is the most widely studied blood stage protein. Abundance of this immunogenic protein on the surface of the merozoites makes it an ideal candidate for vaccine development. Clinical trials based on this antigen have progressed to phase II (Schwartz et al., 2012). MSP_{19}, a cleavage product of proteolysis, is highly conserved and is the main vaccine candidate. Rapid sero-conversion associated with this vaccine results in high titer antibodies capable of neutralizing merozoites (Hirunpetcharat et al., 1997). Sera transfer from vaccinated mice to immunocompromised mice did not result in protection even though a delay in parasitemia was observed suggesting that other factors also play an important role in this vaccine model. Some of the other blood stage vaccine candidates include AMA-1, PfEMP-1, RESA etc.

Thus a blood stage vaccine can ameliorate the symptoms associated with malaria the individual still remains susceptible to infection. Therefore sterile protection with these vaccines is not possible which is reflected by reduced number of candidates entering clinical trials.

1.5.3 Transmission Blocking Vaccines.

Vaccines belonging to this category mainly target the sexual stage antigens of the parasite. The main goal with this vaccine is to reduce the disease morbidity in an area by preventing the development of the parasite in the vector thereby preventing new infections. Mostly antibody mediates this kind of protection wherein during a blood meal the antibodies taken up by the vector hampers with the proper development of the gametocytes in the vector (Carter, 2001). Although various vaccine candidates have been tested such as PfS25, PfS28, PfS230 etc, only PfS25 has gone to clinical trials (Wu et al., 2008).

1.6 Vaccine Design Strategies.

Traditional vaccines were mostly live or attenuated organism which provided long term sterile immunity and therefore was effective in eliminating many diseases. However dependence of traditional means of preparing vaccines especially one involving live/attenuated organisms is not much popular today because of the ability of the pathogen to revert back to its virulent state. With mankind facing life threatening diseases, development of improved vaccines with minimal side effects has become a necessity.

1.6.1 DNA Vaccine.

A DNA vaccine is one of the new generation vaccines wherein a plasmid of bacterial DNA encoding the desired antigen under a strong mammalian promoter is inoculated into the individual. The presence of bacterial backbone containing appropriate selection gene makes it easy for the large scale production. The strong CMI response associated with DNA vaccines makes it a very viable technology for producing effective vaccines (Davis et al., 1995). The discovery of DNA vaccines was quite a surprise for the scientific community considering that for direct transfection of cells would be impossible. However studies show that the naked DNA is

rapidly taken up by the muscle tissues which is then expressed and presented in context of MHC I (Ulmer et al., 1993). Endogenously synthesized proteins can be then cross-presented by professional antigen presenting cells. This could explain why DNA based vaccines induce strong CTL responses. Overwhelmingly abundant data signifying the viability of DNA based approach in designing vaccines for influenza (Kim and Jacob, 2009), tuberculosis (Hanif et al., 2010), leishmaniasis (Masih et al., 2011), malaria (Hoffman et al., 1997b) etc has been reported.

Inspite of being a good vector for expression of foreign gene, there are potential risks and disadvantages associated with DNA vaccines. They have recently come under renewed scrutiny for a number safety factors such as integration of the DNA into host gene (Wang et al., 2004) and also by their induction of tolerance against the antigen (Mor et al., 1996). When compared with conventional vaccines, DNA vaccines are poor inducers of antibodies (Gramzinski et al., 1998; Polack et al., 2013). A potential risk that could be linked with DNA vaccines are the induction of inflammatory cytokines due to the bacterial DNA backbone of the plasmid construct. Even though such an induction of cytokines can have an adjuvant like effect, too much production can lead to chronic inflammation.

1.6.2 Subunit Vaccine.

Family of these vaccines is not limited to the native recombinant proteins but also incorporates short peptides. Modern technology has facilitated the production of recombinant proteins from various expression systems which closely resemble the native proteins (Hansson et al., 2000). These vaccines mostly drive the humoral immune responses against the antigen. However the inclusion of adjuvants aids in skew the Th2 response towards a Th1 response. An advantage with these vaccines is the maintenance of conformational stability of the immunodominant epitopes.

Subunit vaccines based on peptides are currently being evaluated for their efficacy against malaria (Mahajan et al., 2010), cancer (Naz and Dabir, 2007) and other diseases. Synthesis of peptides also rules out the potential contamination associated with purification from heterologus systems. Since the peptide vaccine basically involves inoculation with immunodominant B-cell and T-cell, it prevents unnecessary responses directed against other non-protective epitopes

(Crowe et al., 2006). For the peptides it becomes essential to couple them with a carrier protein. One such malaria vaccine was based on the coupling of the central repeat domain of *P.falciparum* CS protein to the tetanus toxoid (Herrington et al., 1987). However to overcome the dependence on tetanus toxoid Tam and colleagues designed a multiple antigen peptide, in which different branches of the repeat region were synthesized on a polylysine core to form a macromolecular structure (Nardin et al., 1995). In another study a MAP was synthesized by incorporating antigens from different stages of the parasite cycle which generated responses directed against different stages of parasite development (Mahajan et al., 2010).

So despite the ease with which subunit vaccines can be produced most of them require an adjuvant to obtain a strong response.

1.6.2.1 Adjuvants.

Subunit vaccines are poorly immunogenic and therefore require additional components to improve its immunogenicity. These components, known as adjuvants, help to create a favorable niche for the antigen processing and presentation. This can be by enhancing the innate responses which in turn primes an effective adaptive response (McKee et al., 2007; Yuki and Kiyono, 2003). Majority of the adjuvants target the pattern recognition receptors (PRR) for instigating an effective response. One of the earliest adjuvant developed was the Freunds adjuvant, which contained heat killed mycobacteria, signaling via NLR, inflammasome and TLR´s (Freund and Bonanto, 1946). Depending on the adjuvants used, it is possible to skew the response from Th2 to Th1 or the induction of $CD8^+$ T-cells instead of $CD4^+$ T-cells and vice-versa (Carson and Raz, 1997; Cribbs et al., 2003; Morrow et al., 2010). They can also influence humoral responses by effecting the isotype (Gomez et al., 1998), quantity (Gavin et al., 2006) and avidity (Khurana et al., 2010) of the antibodies produced against the antigen.

Though the number of adjuvants being developed has increased considerably over the last decade, the number of licensed ones for human uses is very limited. In fact the only licensed adjuvant for human purpose is Alum. Despite considerable advances made in immunology development of an effective adjuvant with minimal side effects is still an area of active research.

One of the main reasons for the slow incorporation of new adjuvants with licensed vaccines is the fear of aggravating the inflammatory responses leading to auto immune disorders (Zandman-Goddard and Shoenfeld, 2005). A recent study about ASO3, a proprietary adjuvant from Glaxo, was reported to increase the incidence of childhood narcolepsy (Nohynek et al., 2012). A variant of this adjuvant, ASO1, is currently used for the RTS,S malaria vaccine.

1.6.3 POXVIRAL VECTORS.

Since the elimination of smallpox by vaccinia virus, use of poxvirus as effective delivery vehicle for heterologus antigens has gained increased popularity. Ability of vector to accommodate large foreign gene combined with the low cost and ease of production makes it an ideal vaccine vector candidate (Pastoret and Vanderplasschen, 2003). Furthermore an effective long lasting humoral and CMI against the heterologus antigen could be developed with these vectors (Smith et al., 1983). Attempts to further enhance the immunogenicity of these vectors gave rise to highly attenuated strains of vaccinia such as Modified Virus Ankara (MVA) and NYVAC. Moreover deletion of immunomodulatory genes from these attenuated strains, such as C6L (Garcia-Arriaza et al., 2011), F1L (Perdiguero et al., 2012) resulted in the production of better vaccine candidates. Earlier studies from our lab also show the importance of the vectors to stimulate mucosal immune cells making them an ideal tool in the fight against diseases which infects via mucosal routes (Gherardi and Esteban, 2005; Gherardi et al., 2003).

An alternative approach to further enhance the immune responses is the prime-boost strategy (Dunachie and Hill, 2003; Ramshaw and Ramsay, 2000). Priming agents such as recombinant DNA, protein, VLP´s, adenoviruses etc when combined with poxvirus are known to induce long lasting immune response (Gomez et al., 2012; Rodriguez et al., 2012; Sanchez-Sampedro et al., 2012). The antigen specific memory $CD8^+$ T-cells induced by this approach exhibits an effector phenotype and are highly polyfunctional for IFN-γ, TNF-α and IL-2 (Sanchez-Sampedro et al., 2012). Since an effective malaria vaccine should produce high levels of $CD8^+$ T-cells, prime-boost approach is an effective measure for fighting malaria (Li et al., 1993; Schmidt et al., 2010). In fact many clinical trials based on poxvirus prime-boost in combination with different agents are reported (Table 1).

A27 (14K) Vaccinia Protein:

Poxviruses are known to contain many genes that encode for immunogenic proteins such as A27L, A4L, A33, H3L, B5, L1 and many more (Davies et al., 2005; Xiao et al., 2007). Poxvirus antigens have also being used as a strategy to enhance the immunogenic characteristics of other antigens when expressed from viral vectors (Rodriguez et al., 1991). In fact our laboratory has shown that HIV envelope protein fused at the C-terminus with 14K (A27L) or 39K (A4L) protein of vaccinia virus enhanced the immunogenicity of Env, when expressed from the virus vector and inoculated in mice by homologous prime/boost approach, as indicated by an increase in broadly reactive antibodies and $CD8^+$ T-cell responses to Env (Collado et al., 2000).

Previous study from our lab has shown the structural organization 14K protein (Vazquez et al., 1998). 14K protein comprises of a structure less region from amino acids 1 to 28 responsible for producing neutralizing antibodies, a helical region from residues 29 to 37, a triple coiled-coil helical region from residues 44 to 72, and a Leu zipper motif at the C terminus. Recently it was reported that amino acid region between 21-32 is responsible for the binding of 14K protein to the heparin sulfates on the cell surfaces, which in turn is aided by the coiled-coil region (Ho et al., 2005).

Table 1: Prime-boost regimen for malaria vaccine in clinical trials.

Priming Agent	Boosting Agent	Immune responses		
		CD4⁺	*CD8⁺*	*IgG*
AdCh63_ME-TRAP	MVA_ME-TRAP(Reyes-Sandoval et al., 2010)	-	+++	-
FP9-CS	MVA-CS(Imoukhuede et al., 2006)	+++	++	-
DNA-CS	MVA-CS(Schneider et al., 1998)	-	++	-
RTS,S/ASO2	MVA-CS(Dunachie et al., 2006)	-	+	+

OBJECTIVES

Development of an efficient vaccine against malaria is a long sought goal of vaccinologist. Decades of research has finally yielded a vaccine, RTS,S in combination with a powerful adjuvant ASO1E. However, with 50% protection and that too limited for 6 months in children, and with 16.8% after 4 years, this vaccine is far away from being a successful one. With reports suggesting the side effects of adjuvant, the negative factors outweigh its benefits.

The molecular complexity of an antigen is known to influence its immunogenicity. Large oligomeric antigens are more efficient in mounting an effective immune response compared to its monomeric counter parts (Kovacs et al., 2012; Qian et al., 2012). Hence, based on this principle the main objective of this work was to improve the immunogenicity of CS protein based on this principle.

AIMS OF CURRENT STUDY.

- Develop a novel way to enhance the immunogenicity of CS protein of *Plasmodium* by fusing it with the oligomeric A27 (14KDa) vaccinia virus protein (referred to as CS-14K).
- Expression and purification of the fusion protein CS-14K from *E.coli*.
- Physical and biochemical properties of CS-14K.
- Characterization of innate properties of CS-14K protein in cell-cultures.
- Evaluate the immunological properties (adaptive and memory responses) of CS-14K in mice based on a prime-boost strategy with poxvirus vector MVA expressing CS.
- Analyze the efficacy of CS-14K in protecting mice from malaria.
- Define immune correlates of protection.

MATERIALS & METHODS

3.1 CELL LINES.

In this study the following cell lines were utilized:

DF-1 (ATCC: CRL-12203)

DF-1 is an immortalized chicken embryo fibroblast cell line. This adherent cell line is mainly used in the propagation of virus. They are grown in Dulbecco´s Modified Eagle Medium supplemented with 2mM of L-Glutamine, 100 µg/ml of streptomycin, 100 IU/ml of penicillin and 10% of heat inactivated fetal calf serum. The cells are maintained at 39ºC with 5% CO_2 and 95% humidity. The medium is changed two times a week and are passaged with trypsin EDTA following which they are subcultivated at 1:5 ratio.

J774 (ATCC: TIB 67)

Mouse macrophage cell line (Balb/C). These adherent cell line exhibit antibody dependent phagocytosis and synthesize large amounts of lysozyme. The cells were maintained in RPMI1640 medium contained L-Glutamine supplemented with 100 µg/ml of streptomycin, 100 IU/ml of penicillin, 50µM β-mercaptoethanol and 10% of heat inactivated fetal calf serum. The cells are maintained at 37ºC with 5% CO_2 and 95% humidity. The cells are dislodged using cell scrapper during passages and are subcultivated at a ratio of 1:6.

THP-1 (ATCC: TIB 202)

It is a human monocyte cell line isolated from the peripheral blood from a patient suffering from acute monocytic leukemia. They are grown in suspension and maintained in RPMI 1640 medium containing L-Glutamine supplemented with 100 µg/ml of streptomycin, 100 IU/ml of penicillin, 50µM β-mercaptoethanol and 10% of heat inactivated fetal calf serum. To differentiate into macrophages, cells were treated with 0.5mM PMA (Sigma Aldrich, Spain) overnight.

3.2 Generation of Recombinant Virus.

3.2.1 Construction of Virus.

Construction of recombinant MVA virus expressing circumsporozoite protein of *P.yoelii* 17XNL strain (MVA-CS) has been describe previously (Gonzalez-Aseguinolaza et al., 2003). Briefly the gene encoding the entire CS protein of *P.yoelii* was isolated from the plasmid, pBS-PY1993, and cloned into the vaccinia insertion vector pJR101, which contains the p7.5 promoter for CS expression and the flanking regions for the HA locus of MVA. The recombinant virus was generated by homologous recombination in DF-1 cells. After several rounds selection based on the expression of B-galactosidase expression the stable recombinant virus was selected.

3.2.2 Purification of Virus.

Twenty confluent P150 plates of chicken embryo fibroblast, from 11 day old embryonated SPF eggs (Intervet, Spain), was infected with 0.01 PFU/cell of recombinant virus in 5ml of serum free DMEM medium. Following incubation for 1 hour at 37ºC with 5% CO_2 the inoculum was removed by aspiration and fresh DMEM medium with 2% FCS was added for 72 hours. After incubation the cells were centrifuged down and washed once with PBS and resuspended in 20 ml of 10 mM Tris-HCl pH9.0. Cells were lysed using sonication (Misonix Sonicator 3000). The sonicated lysate was layered onto 45% sucrose solution and centrifuged in a Beckman SW-28 rotor. The viral pellet was resuspended in 10 mM Tris-HCl pH 9.0 in desired volume.

3.3 Generation of Recombinant Plasmids.

pCI-Neo-CS. The PyCSP gene was amplified MVA-CS using the primers CS-XhoI-F (5´-ACTTA*CTCGAG*ATGTGTTACAATGAAGAAAATG-3´) and CS-NotI-R (5´-ATT*GCGGCCGC*TTTAAAATATACTTGAAC-3´) to yield a 972 bp fragment lacking the N-terminal signal sequence and C-terminal GPI sequence. The gene was inserted into a mammalian expression vector, pCI-Neo, that had been previously digested with XhoI and NotI followed by SAP treatment (Shrimp Alkaline Phosphatase). The CS gene in both the virus and plasmid were

sequenced (Secugen; Spain). The plasmid was purified using Qiagen Mega Prep Kit according to manufacturer's protocol. Expression of CS from pCI-Neo-CS was confirmed by transfecting DF-1 cells followed by western blot analysis with CS specific antibodies.

pGEX-CS / pGEX-CS-14K. The CS gene from pCI-Neo-CS plasmid was amplified using the primers CS-EcorI-F (5´-ACTTA*GAATTC*ATGTGTTACAATGAAGAAAATG-3´), CS-NotI-R (5´-ATT*GCGGCCGC*TTTAAAATATACTTGAAC-3´) for pGEX-CS and with primers CS-EcorI-F and CS-14K-NotI-R (5´-ATT*GCGGCCGC*TATTAAATATACTTGAAC-3´) for pGEX-CS-14K. The A27L ORF from Vaccinia strain WR (Accession number- YP_233032, www.ncbi.nlm.nih.gov/genbank/) was amplified with the primers A27L-NotI-F (5´-GCTGCTA*GCGGCCGC*GAGGCTAAACGCGAAG-3´) and A27L-XhoI-R (5´-CC*CTCGAG*TGGGTTACTCATATGGACG-3´) to generate a 276 bp fragment which lacks the first 28 amino acids from the original sequence. The chimeric gene fragment was generated by digesting with Not I followed by ligation. The fusion gene fragment was then inserted into pGEX-6p-1 plasmid to produce pGEX-CS-14K plasmid.

The recombinant plasmid was transformed into DH5α *E.coli*. The positive clone was selected and purified using Megaprep Kit (Qiagen).

3.4 Recombinant Protein Purification.

The recombinant proteins were purified from *E.coli* strain DH5-α. The starter culture was diluted 1:100 in fresh LB media and allowed to grow at 37ºC till the OD_{600} reached 0.7, following which IPTG was added to a final concentration of 1 mM. The culture was then incubated in an orbital shaker at 18ºC and 200 rpm for 24 h. After the incubation the cells were harvested and the pellet was suspended in extraction buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA and protease inhibitor tablets ROCHE). The cells were then lysed with lysozyme, added to a final concentration of 1mg/ml and incubated on ice for 20 min. Following lysozyme treatment 1% sarkosyl detergent and 1% triton X-100 was added and incubated at 37ºC for 10 min. Clarified supernatant from the lysis was incubated with Glutathione Sepharose 4B beads at 4ºC overnight.

The beads were then washed using three washes with wash buffer I (Extraction buffer with 0.5% Triton X-114) and three with wash buffer II (Extraction buffer with 0.1% Triton X-114) followed by two washes with extraction buffer. The purified protein was then eluted with 20mM reduced glutathione. The protein was desalted using Amicon centrifugal concentrator and the protein concentration was determined using Bradford reagent. The GST tag from the protein was cleaved using preScission protease according to manufacturer's protocol (GE Healthcare). The proteins were tested for LPS contamination using chromogenic Limulus Amebocyte Lysate kit (QCL-1000, Lonza) which was maintained below 2 EU per microgram of protein.

3.5 Neutralization Assay.

Sera from immunized rabbit were inactivated at 56ºC for 30 min, following which two fold serial dilutions of the serum were made and incubated with 200 PFU of MVA at 37ºC for 1 h. Afterwards, confluent DF-1 cells were infected in triplicate and were visualized by immunostaining with anti-WR serum after 48 h. As a control, non-immune serum from non immunized animal was used. The number of plaques obtained from each serum dilution was normalized to this control value.

3.6 Nitrite Measurement.

NO synthesis was measured by estimating the levels of nitrite present in the supernatant. Briefly, J774 cells were stimulated with proteins. For *in vivo* nitrite analysis, 10^6 splenocytes from vaccinated animals, sacrificed after 53 days post boost was treated with 5 µg/ml of CS protein. Nitrite accumulation was measured by treating 50 µl of supernatant with 50 µl of Griess reagent I (1% sulfanilamide solution in 2.4 N HCl) for 10 min in dark followed by the addition of 50 µl of Griess reagent II (0.1% naphthylethlyenediamine in 2.4 N HCl) for 10 min. The assay was read by a spectrophotometer at 540 nm.

3.7 RNA EXTRACTION AND RT-PCR.

Total RNA was extracted from cells treated with respective proteins for 24 h, using RNeasy mini Kit according to manufacturer´s instructions. Analysis of RNA was carried out using RT-PCR as described in the kit for reverse transcriptase (Invitrogen). Briefly, 1 µg of RNA was reverse transcribed into cDNA using oligo dT primers (Invitrogen). For relative quantitative PCR, 2 µl of cDNA was used as a template with primers specific for iNOS, IL-12p40 and GAPDH (Maffei et al., 2004). All the experiments were done in triplicates and the bands from gel electrophoresis were quantified using Adobe Photoshop CS4.

3.8 CONFOCAL MICROSCOPY.

Immunostaining was carried out as described previously (Guerra et al., 2003). Briefly, after fixation (30 min; 4% formaldehyde in PBS; 37ºC), permeabilisation in 0.1% Triton X-100 (Sigma) and blocking with 10% FCS in PBS, cells were incubated with primary antibody (anti CS antibody 1:500; C3-anti14K 1:400; NF-κBp65 1:500) along with DNA staining dye, DAPI (1:200) for 1hour at room temperature. Following extensive washing with PBS secondary antibodies (Alexa 546 goat antimouse and Alexa 488 goat antirabbit; 1:500) were applied for 1 hour at room temperature. The slides were washed three times with PBS and mounted in Prolong Antifade medium and analyzed with Bio-Rad Radiance 2100 confocal laser microscope.

3.9 ANIMALS AND IMMUNIZATION.

All animal procedures were approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of Centro Nacional de Biotecnologia (CNB-CSIC). Female Balb/C mice (H-2^d), 6-8 weeks old, were obtained from Harlan U.K. A standard immunization protocol based on a heterologus prime-boost approach designed in the laboratory was followed (Garcia-Arriaza et al., 2010). In short, animals were primed with DNA (100 µg; DNA-CS or DNA-ϕ) or protein (20 µg; CS or CS-14K) via intradermal (i.d) route and were boosted after two weeks with 2 x 10^7 PFU of the respective sucrose-purified viruses (MVA or MVA-CS) through intraperitoneal (i.p) injection. All the preparations were made in endotoxin free PBS.

3.10 *P.YOELII* SPOROZOITE CHALLENGE.

Challenge experiments were performed as previously described (Bruna-Romero et al., 2001). Briefly, sporozoites were obtained from the salivary glands of *Anopheles stephensi* mosquitoes. Two weeks post-boost, mice were challenged with 2×10^4 sporozoites via intravenous route through tail vein. After 42 hours, animals were sacrificed and levels of *P.yoelii* 18s rRNA levels were assessed by qRT-PCR. To determine sterile protection two weeks post immunization, mice were challenged intravenously with 300 *P. yoelii* sporozoites. Parasitemia was monitored by performing daily blood smears from days 3 to 21.

3.11 ELISA AND ANTIBODY AVIDITY MEASUREMENT.

Antibodies present in the serum of immunized animal were determined using ELISA as previously described (Garcia-Arriaza et al., 2010). Purified CS protein was coated to the 96 well Nunc Maxisorp plates at concentration of 2 µg/ml in coating buffer ($NaHCO_3$ Na_2CO_3) at 4ºC overnight. Bound antibodies were detected using 1:2000 dilution of alkaline phosphate conjugated goat anti mouse antibody total IgG or IgG1 or IgG2a (Southern Biotechnology Associated, Birmingham). Plates were developed by adding TMB substrate (3,3',5,5' Tetramethylbenzidine; Sigma) and stopping the reaction with 1M H_2SO_4. OD was read at 450 nm. Endpoint titer values were determined as the last positive dilution of serum giving an absorbance value three times higher than naïve serum. For analyzing the avidity of antibodies, an initial serum dilution which gave an absorbance of 2.7 ELISA units was selected. Following incubation, with serum, the antigen-antibody interaction was disrupted using a range of dilutions from 0 to 5 M Urea (Invitrogen) in Tris-HCl pH 8.0 for 15 min before the addition of secondary antibody. Effective concentration of urea which reduced the initial value of absorbance by 50% (EC_{50}) was then calculated by linear regression (R^2=0.99) between 1-5 M Urea concentration (Log of 50% reduction = 1.699).

3.12 IFN-γ ELISPOT ASSAY.

The vaccine- specific cellular immune response in mice was determined using ELISPOT assay measuring the secretion of IFN-γ by splenocytes after stimulation with different peptides pools. Briefly, 10^6 splenocytes were plated in triplicate in 96-well nitrocellulose-bottomed plates previously coated with 6 µg/ml of anti-mouse IFN-γ mAb R4-6A2 (Pharmingen, San Diego, CA). CS peptide was resuspended in RPMI 1640 supplemented with 10% FCS and added to the cells at a final concentration of 1 µg/ml. Plates were incubated at 37°C, 5% CO_2 for 48 h, washed extensively with PBS containing 0.05% of Tween20 (PBS-T) and incubated 2 h at room temperature (RT) with a solution of 2 µg/ml of biotinylated anti-mouse IFN-γ mAb XMG1.2 (Pharmingen, San Diego, CA) in PBS-T. Afterwards, plates were washed with PBS-T and 100 µl of peroxidase-labeled avidin (Sigma, St Louis, Mo) at 1:800 dilution in PBS-T was added to each well. After 1 h of incubation at RT, wells were washed with PBS-T and PBS. The spots were developed by adding 1 µg/ml of the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St Louis, Mo) in 50 mM Tris-HCl, pH 7.5 containing 0.015% hydrogen peroxide. The spots were counted with the aid of a stereomicroscope. The responses were given as the number of spot-forming cells per million of splenocytes. In all the cases the background levels were subtracted to each specific peptide pool.

3.13 MULTIPARAMETER FLOW CYTOMETRY.

Flow cytometry and intra cellular cytokine staining (ICS) analysis were performed to detect the different phenotype of lymphocytes secreting cytokines, as reported previously (Garcia-Arriaza et al., 2010). Briefly, 4×10^6 splenocytes were stimulated with 1µg/ml of CD^{8+} peptide, $PyCS_{280-288}$ (SYVPSAEQI), with Golgi Plug (BD Bioscience) for 6 h in a 96 well plate. The cell were then washed and Fc receptors were blocked using anti CD-16/CD-32 following which the cells were stained with surface specific mouse antibodies namely, CD4 Alexa700, CD3 FITC, CD8 Percp; for adaptive response studies or CD8 FITC, CD44 PeCy5 and CD62L PE for memory studies. Cells were permeabilized using the BD Cytofix/Cytoperm™ Kit (Becton Dickinson) and were stained for intracellular cytokines, IFN-γ APC, IL2 PE and TNF-α PeCy7 for adaptive or

with IFN-γ PeCy7, IL2 APC and TNF-α Pe for memory. A million cells were then passed through a LSRII flow cytometer (Becton Dickinson) and the data was analyzed with FlowJo (Tree Star. Inc) and Spice (ver 5.0). Appropriate controls were used and the values from unstimulated samples were subtracted.

3.14 STATISTICAL ANALYSIS.

Statistical analysis was performed using Minitab for Windows. For ELISA in order to determine the differences between groups we performed a linear regression of the logarithmic absorbance versus the logarithmic dilution, removing those samples clearly not following a linear trend ($R^2 >$ 0.98). Then we computed the logarithmic dilution at which this regression line was crossing the threshold given by twice the absorbance of the control values for that mouse. Doing this for the 4 mice we have 4 estimates of the logarithmic dilution beyond which the absorbance is smaller than twice the absorbance given by the control. We will refer to this value as the critical logarithmic dilution. Finally, we compared with a Student- T Test the hypothesis that the critical logarithmic dilution between the groups. For ICS and ELISPOT, statistical analysis was done based on previously described method (Garcia-Arriaza et al., 2010). Briefly, Given the total number of cells, N_T, and the number of cells responding to a given antigen, N_{Ag}, an estimate of the proportion of cells responding to this antigen is given by $\hat{p}_{Ag} = \frac{N_{Ag}}{N_T}$. The Bayesian *a posteriori* distribution of $\hat{p}_{Ag}$without any *a priori* assumption (i.e., assuming that the true proportion is uniformly distributed between 0 and 1) is the Beta distribution with parameters $(N_{Ag}+1, N_T - N_{Ag}+1)$. Let us call $f_{\hat{p}_{Ag}}(x)$the corresponding probability density function *a posteriori*. Analogously, we can derive the distribution of the proportion of cells responding to RPMI, obtaining the distribution $f_{\hat{p}_{RPMI}}(x)$. To test whether the antigen response is significantly larger than the RPMI response, we computed the probability density function of the variable $\hat{p}_{AgCorrected} = \hat{p}_{Ag} - \hat{p}_{RPMI}$as $f_{\hat{p}_{AgCorrected}}(x) = \int_{-\infty}^{\infty} f_{\hat{p}_{Ag}}(\chi) f_{\hat{p}_{RPMI}}(x+\chi) d\chi$. The cumulative density function of this variable is defined in the usual way $F_{\hat{p}_{AgCorrected}}(x) = \int_{-\infty}^{x} f_{\hat{p}_{AgCorrected}}(\chi) d\chi$.

The α percentile of this variable is defined as x_α such that $F_{\hat{p}_{AgCorrected}}(x_\alpha)=\alpha$. We computed the symmetric 95% confidence interval for the RPMI corrected proportion as $[x_{0.025}, x_{0.975}]$. Finally, we consider $\hat{p}_{Ag}$ to be significantly larger than $\hat{p}_{RPMI}$ if $x_{0.025}>0$. In such a case, $[x_{0.025}, x_{0.975}]$ gives the 95% symmetric confidence interval for $\hat{p}_{AgCorrected}$. The average $\hat{p}_{AgCorrected}$ is computed as the expected value of $f_{\hat{p}_{AgCorrected}}(x)$ (note that this expected value needs not be in the middle of the confidence interval, $\frac{x_{0.025}+x_{0.975}}{2}$).Whenever two corrected proportions need to be summed, $\hat{p}_{AgCorrected_{1+2}}=\hat{p}_{AgCorrected_1}+\hat{p}_{AgCorrected_2}$, we convolved their probability density functions to obtain the probability density function of the summed proportion, $f_{\hat{p}_{AgCorrected_{1+2}}}(x)=\int_{-\infty}^{\infty} f_{\hat{p}_{AgCorrected_1}}(\chi) f_{\hat{p}_{AgCorrected_2}}(x-\chi)d\chi$, in this way confidence intervals for any sum of corrected proportions can be obtained. Antigen responses were not added unless each component was significantly larger than the corresponding RPMI.

In the ELISPOT experiment, three replicates were obtained for each kind of antigen. The average response to that antigen was computed using only the corrected proportions significantly larger than the corresponding RPMI.

RESULTS

4.1 Characterization of Recombinant Proteins.

Circumsporozoite (CS) is a monomeric surface protein of Plasmodium sporozoite, the causative agent of malaria (Nussenzweig and Nussenzweig, 1985). However, the native CS from sporozoites is also known to form some aggregates/oligomers which may facilitate the binding of CS to hepatocytes (Pinzon-Ortiz et al., 2001). Considering that CS is largely a monomer, we hypothesized that higher levels of CS aggregates/oligomers would be required to attain improved immunogenicity and protection.

Based on this hypothesis we decided to fuse CS with an immunogenic protein of vaccinia virus, A27 (14K), which facilitates the formation of higher oligomers via its coiled-coil domain.

4.1.1 Expression and purification of recombinant proteins.

The CS of plasmodium is a GPI anchored protein thereby making its purification difficult. Elimination of GPI motif is beneficial, with some studies supporting its role in improving the immunogenicity (Bruna-Romero et al., 2004). Therefore in this study we removed the signal peptide and GPI motif of CS in both DNA and protein vaccines with the exception of MVA vector which expresses the full length CS protein.

A schematic representation depicting the construction of various plasmids used for the production of recombinant protein is shown in ***Figure 3***. Both CS and CS-14K gene was then inserted into pGEX-6P-1 vector for expressing the proteins in *E.coli.* The proteins were then purified according the protocol as mentioned in *Materials and Methods*.

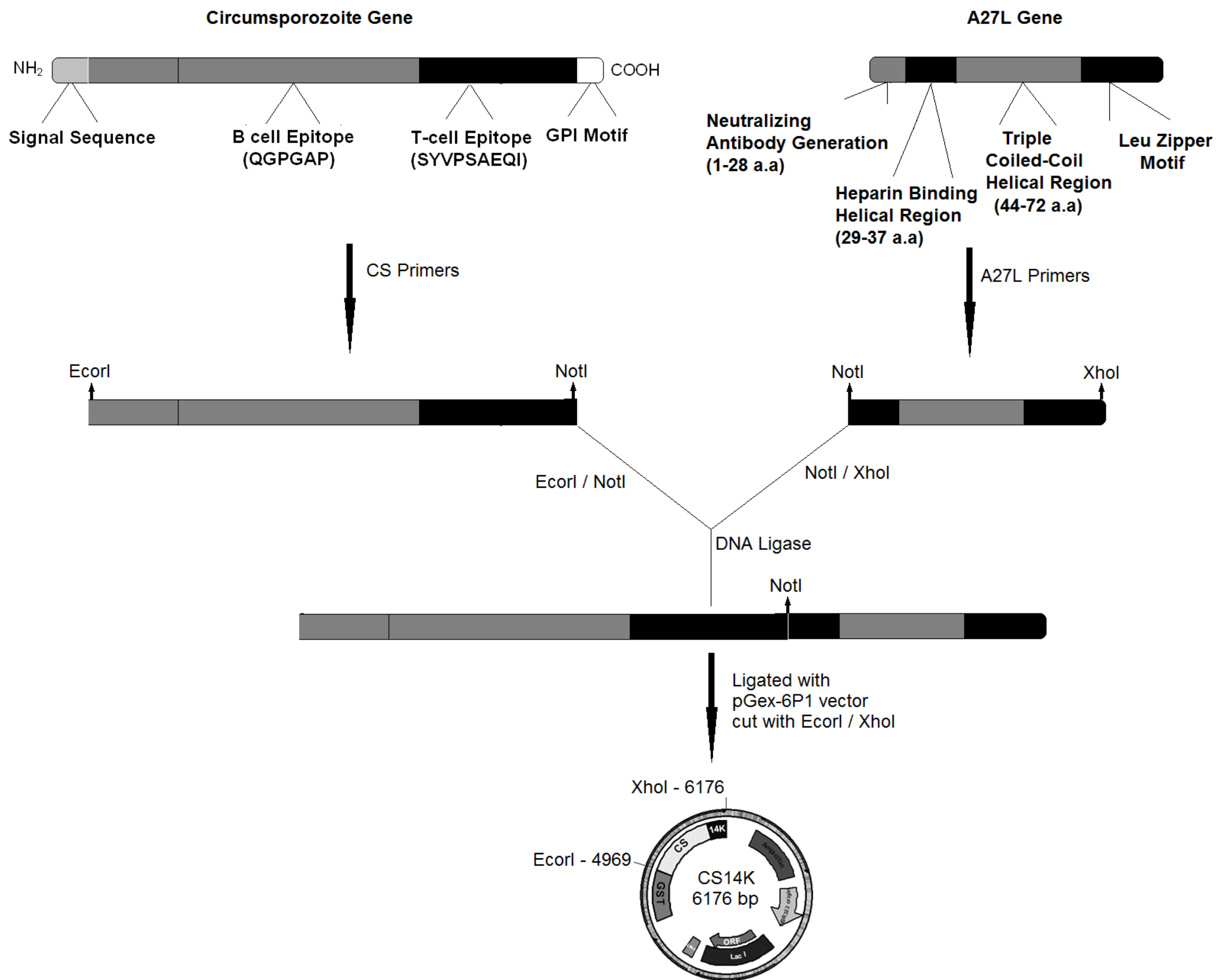


Figure 3: Design and construction of plasmid encoding CS-14K. A schematic representation of gene encoding the CS protein of *P.yoelii* and that of 14K of vaccinia virus is shown. For generating the CS fragment of chimeric protein, the signal sequence represented by 37 amino acids of amino terminal and GPI motif involving the 19 amino acids at the carboxy terminal of CS gene was removed by PCR amplification. The C terminal of amplified CS fragment was then fused in frame to the N terminal of gene, A27L of vaccinia, lacking the first 28 amino acids. All PCR amplifications were carried out using appropriate primers carrying restriction enzyme sites.

This fusion construct was then fused in frame with the GST tag present within the pGEX-6P-1 vector.

Briefly, DH5-α *E.coli* strain expressing the recombinant protein was grown at 18ºC for 24 hours induced with 1mM IPTG. The cells harvested were then suspended in extraction buffer (50mM Tris-HCl; pH 7.5, 250mM NaCl, 1mM EDTA and protease inhibitor cocktail tablets). Lysis was carried out with lysozyme (1mg/ml) followed by treatment with 1% Sarkosyl and 1% Triton X-100. Clarified supernatants were allowed to bind to Glutathione Sepharose 4B beads at 4ºC overnight. The beads were then washed and proteins were eluted with 20mM reduced Glutathione. Proteins were desalted and concentrated using Amicon centrifugal concentrators. GST tag from the proteins were cleaved using PreScission protease. Purified proteins dialyzed against PBS were then irradiated and aliquots of 1mg/ml were made and frozen at -20ºC.

The proteins were analyzed on SDS-PAGE under both reducing and non reducing conditions to study the extent of oligomerization. Also the reactivity of the proteins against NYS1, a well defined antibody against *P.yoelii* CS protein was also done. Under reducing conditions CS-14K has a molecular mass of ~60 kDa, compared with ~50 kDa for CS, whereas under non-reducing conditions CS-14K has a size apparently >250 kDa, in contrast to 50 kDa for CS (***Figure 4***).

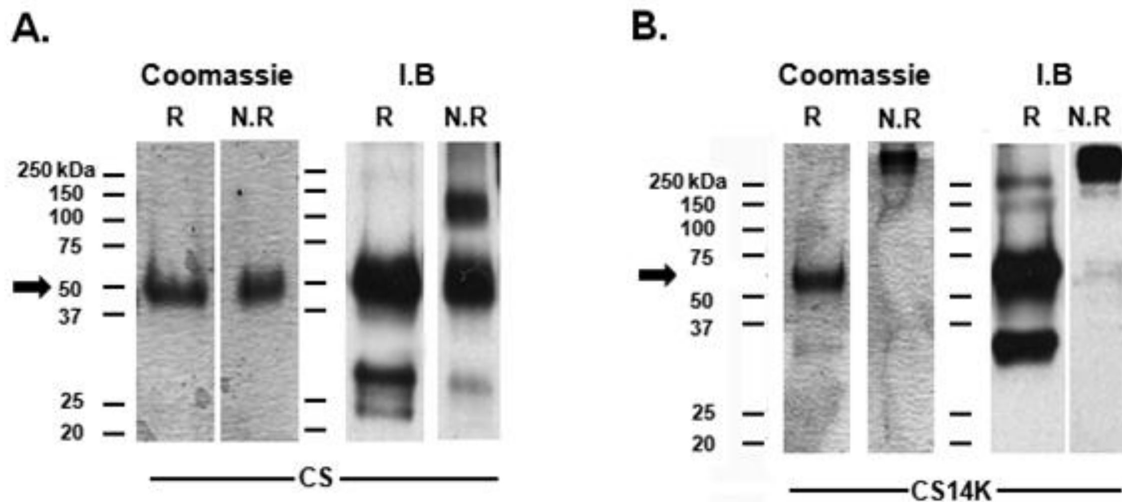


Figure 4: Biophysical and biochemical properties of recombinant proteins: Coomassie-Blue stained SDS page and immunoblot with monoclonal antibody against CS (NYS1; 1:1000 dilution) of 1 µg of recombinant ***(A)*** CS protein or ***(B)*** CS-14K protein, under reduced (R) and non reduced (NR) conditions.

Formation of oligomers/aggregates is not antigen dependent since the fusion of 14K to *P.falciparum* CS protein as well as GP120 of HIV-1 also formed oligomers/aggregates (***Figure 5 A & B***). Even the formation of the oligomers is not dependent on the system used for producing the chimeric protein. The GP120 fused to the 14K protein was produced in mammalian cells, CHO-K1 (***Figure 5 B***).

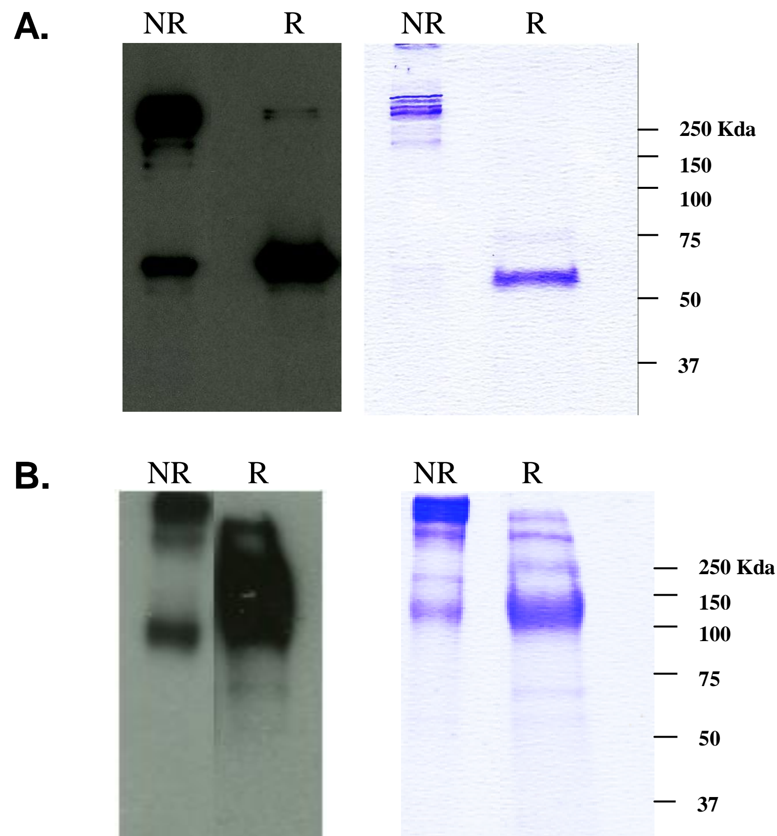


Figure 5: 14K fusion protein forms oligomers/aggregates irrespective of antigen used. ***(A).*** 14K fused with CS protein of *P.falciparum*. Protein is recognized by a monoclonal antibody against CS ***(B).*** 14K fused with GP120 protein of HIV-1, clade IIIB, purified from mammalian cells. Immunoblot analysis carried out with GP120 antibody. NR and R indicates under non-reducing and reducing conditions.

Additional experiments by analytical ultracentrifugation revealed that CS-14K protein has a main sedimenting species with a standard s-value of 4.3 S ± 0.1S. The CS-14K has high tendency to form oligomers/aggregates which is also evident when the protein were run on 5-40% sucrose gradient. The presence of higher oligomers was detected even in the last fraction (40%). However majority of the protein was found between 3rd and 7th fractions (***Figure 6***).

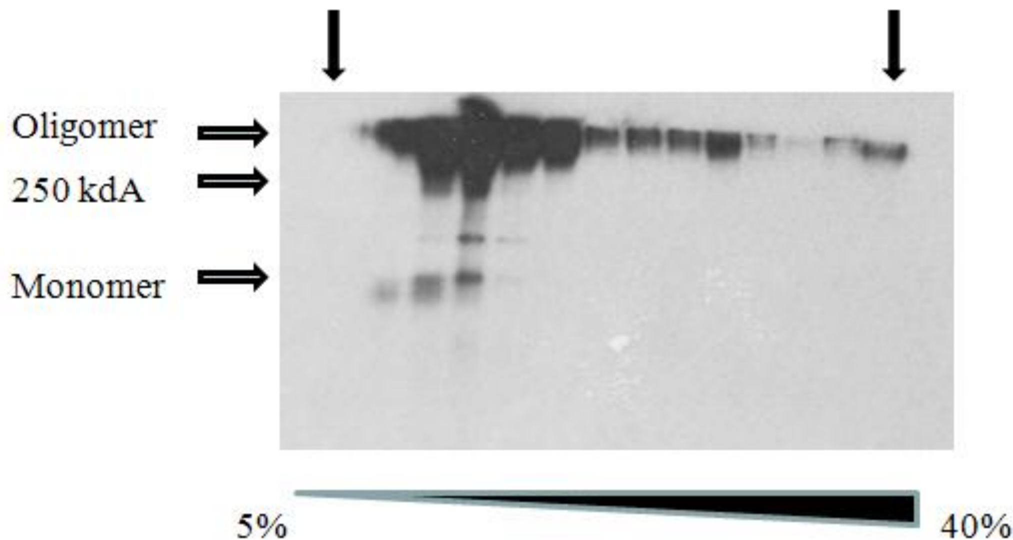


Figure 6: Sucrose gradient analysis of CS-14K protein: Ultracentrifugation of 100µl of CS-14K protein was carried out on 5-50% w/v sucrose gradient at 50,000 rpm for 4 hrs. Fractions of 300 µl were collected from top to bottom of the tube. Proteins in each fraction were then precipitated using TCA/Acetone mixture. Protein was then run in a 7% SDS-PAGE gel under non reducing condition. The protein was transferred into a nitro-cellulose membrane and probed with NYS1 antibody.

4.1.2 Characterization of CS protein expressed by MVA.

Attenuated poxvirus provides a good platform for developing effective vaccines because of its ability to accommodate and express large proteins in addition to their enhanced immunogenic nature. MVA expressing full length CS protein of *P.yoelii* was previously generated in our lab.

New plaques of the recombinant virus were picked and grown in DF-1 cells to prepare new stocks for animal studies. A single plaque was selected for the analysis. Insertion of CS gene does not cause any change in the viral replication as seen from the viral growth curve. The stocks were routinely checked to ensure it is free of mycoplasma and bacteria. To determine the kinetics of CS expression by the vector MVA-CS, we performed time-course analysis of cells infected

with the MVA recombinant expressing CS (***Figure 7 A***). We observed that after infection with MVA-CS, CS was detectable 2 h p.i., and these expression levels remained elevated up to 6 h p.i. Next, to study if CS expressed by MVA undergoes post translational modifications, we incubated infected cells in the presence of tunicamycin, an inhibitor of glycosylation, and observed that most of the CS expressed in the virus infected cells was glycosylated, as indicated by the reduced intensity and size reduction of the protein bands (***Figure 7 B***).

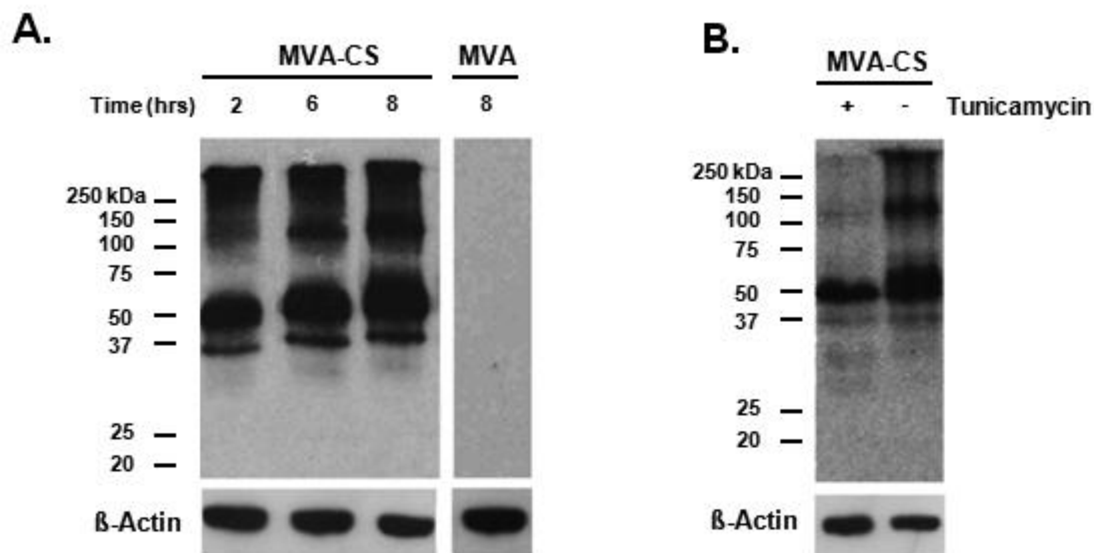


Figure 7: Kinetics of CS expression by MVA-CS: Western blots of DF-1 cells infected with MVA-CS virus at 1 MOI for ***(A).*** 2, 6 and 8 hours or for ***(B).*** 16 hours in the presence and absence of 10 μg/ml of tunicamycin, and probed with NYS1 antibody.

We also investigated if there was any difference in the localization of proteins in macrophages expressed by virus or upon transient transfection. To study the subcellular localization of full length CS during infection with MVA-CS, J774 cells were infected at an MOI of 5 PFU/cell for 18 hours. We observed MVA-CS expresses CS as punctuated spots with complete cytoplasmic spread (***Figure 8***). In contrast, when macrophages were transfected with DNA encoding CS and CS-14K, we observed that CS and CS-14K were strongly localized around the nuclear membrane of the cell.

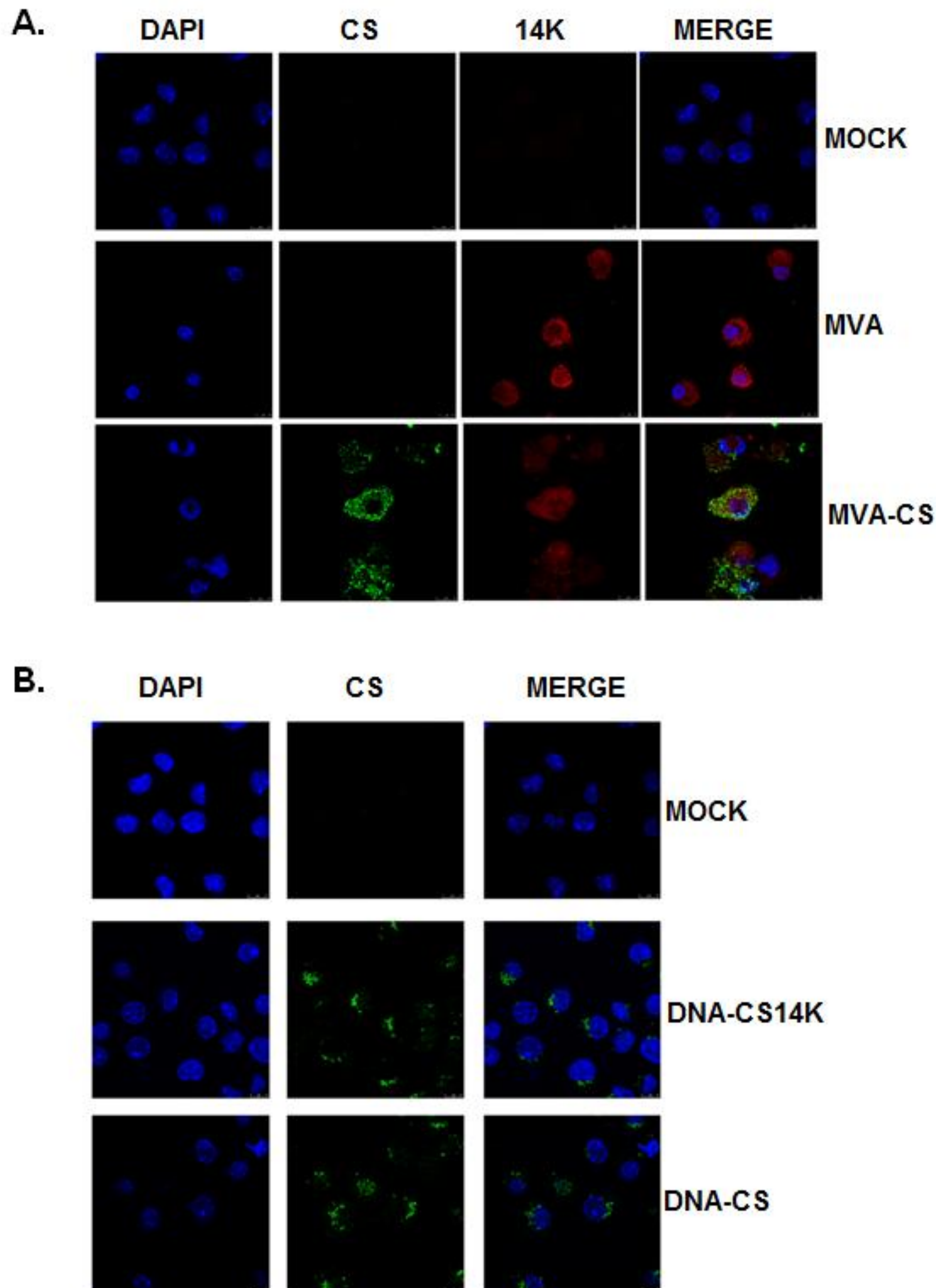


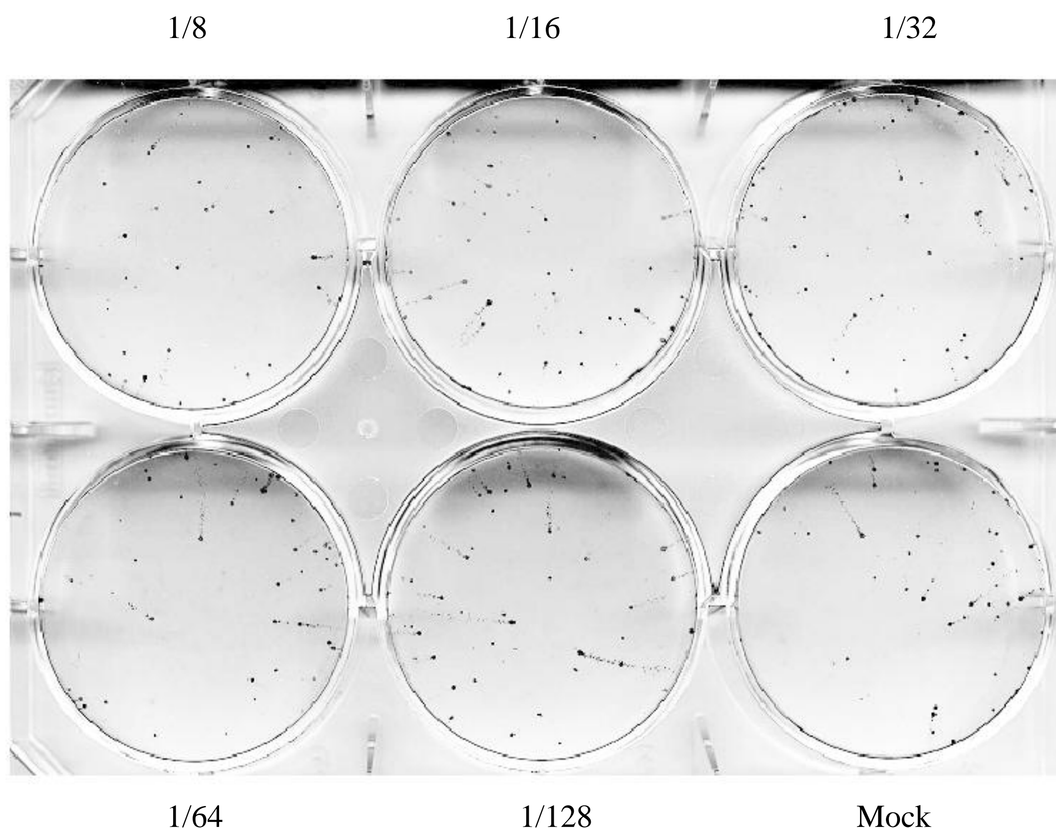
Figure 8: Localization of proteins in macrophages. Confocal microscopy analysis of CS and CS-14K localization in macrophages using NYS1 antibody at a dilution of 1:500. ***(A).*** Pattern of

CS expression by MVA-CS. Confluent J774 cells were infected with MVA-CS at an MOI of 5 PFU/cell for 18 hours. ***(B).*** Protein localization in transfected macrophages. CS and CS-14K encoding plasmids, pCINeo-CS (10 μg) and pCDNA-CS-14K (10 μg), both lacking the signal and GPI motifs were transfected into confluent monolayers of J774 cells for 24 hours.

4.1.3 ANTIBODIES AGAINST CS-14K DO NOT NEUTRALIZE VACCINIA VIRUS.

Since the fusion protein containing 14K protein is used in prime-boost strategy with MVA, can vaccination with this protein induce antibodies against MVA? In order to answer this question we produced antibodies against CS-14K protein in rabbit. The sera were heat inactivated and different serum dilutions were incubated with a known titer of MVA virus. We observed that even low serum dilution could not inhibit the infection of MVA virus (***Figure 9***).

1/8 1/16 1/32



1/64 1/128 Mock

Figure 9: CS-14K fusion protein does not induce neutralizing antibodies against MVA. Serum from rabbits injected with CS-14K protein was not able to neutralize the infectivity of MVA virus *in-vitro*. 200 PFU of MVA virus were incubated with two fold dilutions of serum from rabbit injected with CS-14K protein. Following infection of DF-1 cells with the serum treated virus, immunostaining was carried out using anti-vaccinia antibody used at a dilution of 1:1000. Virus incubated with rabbit serum was used as mock control. The numbers of plaques formed by the viruses were counted to determine the extent of infection.

4.2 CS-14K PROTEIN PRIMING CONFERS STERILE PROTECTION AGAINST MURINE MALARIA.

Sterile protection is the ultimate goal of any vaccine. In case of malaria achieving complete protection was demonstrated only via vaccinating with irradiated sporozoites (Mellouk et al., 1990). Although vaccine combinations based on prime / boost regimens involving DNA, protein or viral vectors have been studied, sterile protection still remains an elusive goal.

Here we demonstrate how a vaccine regimen based on CS-14K protein prime and MVA-CS boost, without any adjuvant, confers sterile protection. The protection was analyzed both at liver stage and the appearance of blood stage parasites

4.2.1 CS-14K ABROGATES THE LIVER STAGE DEVELOPMENT OF SPOROZOITES.

In order to analyze if the change in physical properties had an impact on the immunogenicity, we decided to vaccinate the animals based on a prime/boost strategy as depicted in ***Figure 10.*** Following vaccination the animals were challenged with 2×10^4 sporozoites via intravenous route. After 42 hours, animals were sacrificed and levels of *P.yoelii* 18s rRNA levels were assessed by qRT-PCR.

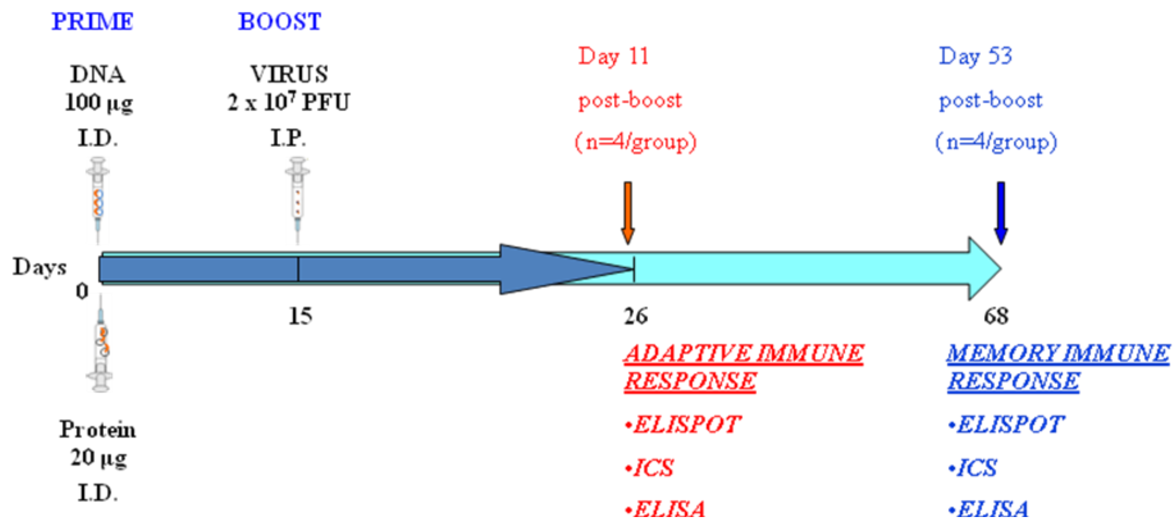


Figure 10: Vaccination strategy. Schematic representation of the heterologus prime-boost vaccination strategy. Animals were primed with either 100µg of plasmid (DNA-CS; DNA-Ø) or 20µg of protein (CS; CS-14K) via intradermal route. After two weeks they received a boost of $2{\times}10^7$ PFU of MVA or MVA-CS virus administered intraperitoneal. Mice were sacrificed on day 26 and 68 for studying adaptive and memory immune responses. For studying protection, animals were challenged 2 weeks post-boost with sporozoites.

We show that a heterologus protein prime / vaccinia virus boost regime was found to be more effective than an homologous protein prime / protein boost regime, a 32% reduction in the liver stage burden of parasites was observed in mice receiving CS-14K (CS-14K/CS-14K) compared to CS (CS/CS) protein. Furthermore, a MVA-CS boost significantly lowered the parasite levels in the liver. Moreover, the CS-14K protein priming followed by a MVA-CS resulted in a near complete inhibition (~99.9%; $p < 0.005$) of parasite development in liver compared to CS protein and DNA-CS (***Figure 11***).

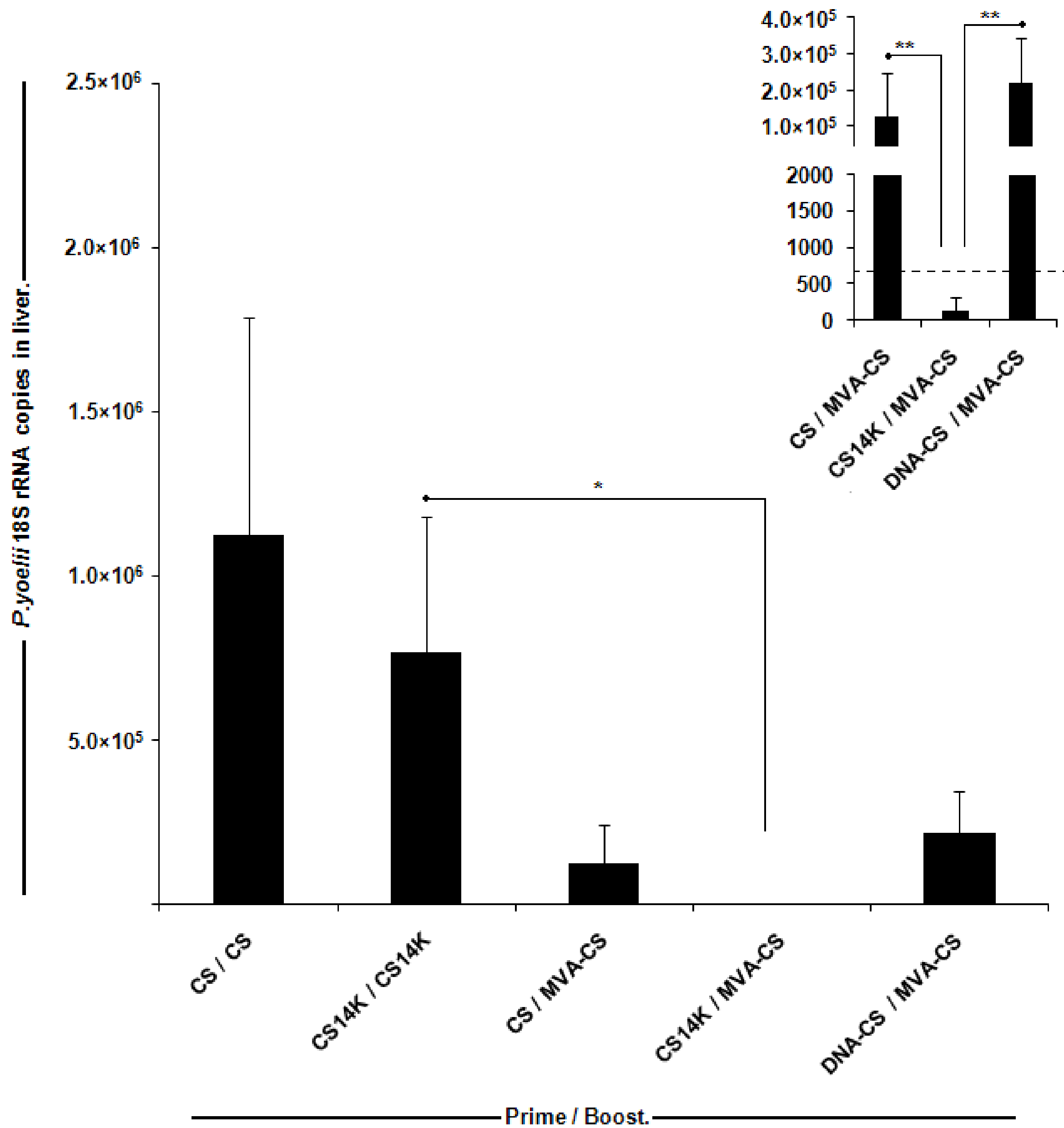


Figure 11: Strong inhibition of liver stage parasite development by CS-14K. Mice were immunized as described in *Materials and Methods.* After two weeks post boost animals were challenged with 2×10^4 *P.yoelii* sporozoites through intra-venous route via tail vein. After 42 hours the amount of parasites in the liver were estimated by measuring the number of copies of

18S rRNA by RT-PCR. The dotted line represents the minimum detectable levels by the highly sensitive qRT-PCR (Bruna-Romero et al., 2001). Data are expressed as mean ± s.e. (n = 6 mice per group). $*p < 0.05$; $**p < 0.005$.

4.2.2 CS-14K PROVIDES STERILE PROTECTION AGAINST A LETHAL CHALLENGE.

Achievement of sterile protection against malaria by vaccination still remains unattainable. Since priming with CS-14K reduced the liver stage development of the parasite, we wanted to see if this was translated into sterile protection. In other words, we evaluated if the heterologus prime/boost strategy based on CS-14K/MVA-CS prevented the blood stage development of malaria in mice. For this two weeks post boost, animals were challenged with 300 sporozoites via intravenous. The presence of infected RBCs in the blood was detected from the blood smears from day 3 to 21. Animals that received CS-14K as a priming agent were completely protected in contrast to control groups which received PBS (***Table 2***).

Table 2: CS-14K induces sterile protection.

Priming[a]	**Boosting[b]**	**Challenge[c]**	**Protected/Challenged (% Protected)**	**Pre-patent period**
CS-14K	MVA-CS	300 sporozoites 14 days post-boost.	10/10 (100%)	----
PBS	PBS		0/10 (0%)	4 days

[a] 20µg of protein administered intra-dermal.

[b] 2×10^7PFU of virus administered intra-peritoneal.

[c] Challenge via intra-venous route.

4.3 Immune correlates of protection.

An important part of any vaccines is to evaluate the correlates of immune responses in the progression of disease. Most of the immune correlates can be classified into two, the **innate immune system** which comprises of various cellular receptors, cytokines and second line of defense i.e. the **adaptive response** consisting of humoral or Cell Mediated Immunity (CMI), based on which arm of the immune system is activated, antibody or T-cell response.

For the development of an efficient vaccine it is important to know not just its protective capability but also the mechanism behind. Here we analyzed the difference in the pathways and immune parameters activated by CS-14K compared with CS or DNA-CS priming in mice.

4.3.1 CS-14K protein modulates innate immune responses in macrophages.

Fusion of antigens to immunomodulatory fragments such as TLR´s (Kastenmuller et al., 2011) or to complement proteins (Ogun et al., 2008), aimed at improving immunogenicity by aggregation of proteins or by exploiting the innate immune signaling of macrophages is an intense area of research. Since fusion of 14K protein to CS facilitates its aggregation and protects the mice against malaria, we sought to investigate the modulation of innate immune responses in macrophages.

4.3.1.1 Nitric Oxide.

Ability of macrophages to induce NO to inhibit the growth of parasite has been well documented (Seguin et al., 1994); therefore we sought to evaluate the ability of the fusion protein to induce NO production. Comparing the NO levels in supernatant of J774 cells treated with fusion protein or normal protein, it was apparent that only CS-14K resulted in NO production (***Figure 12 A***). However, when the cells were stimulated with proteins along with recombinant IFN-γ, elevated levels of NO were found in all samples. Further analysis revealed a significant increase in NO ($p = 0.044$) at mRNA levels in CS-14K treated macrophages compared to CS protein treatment (***Figure 12 B***).

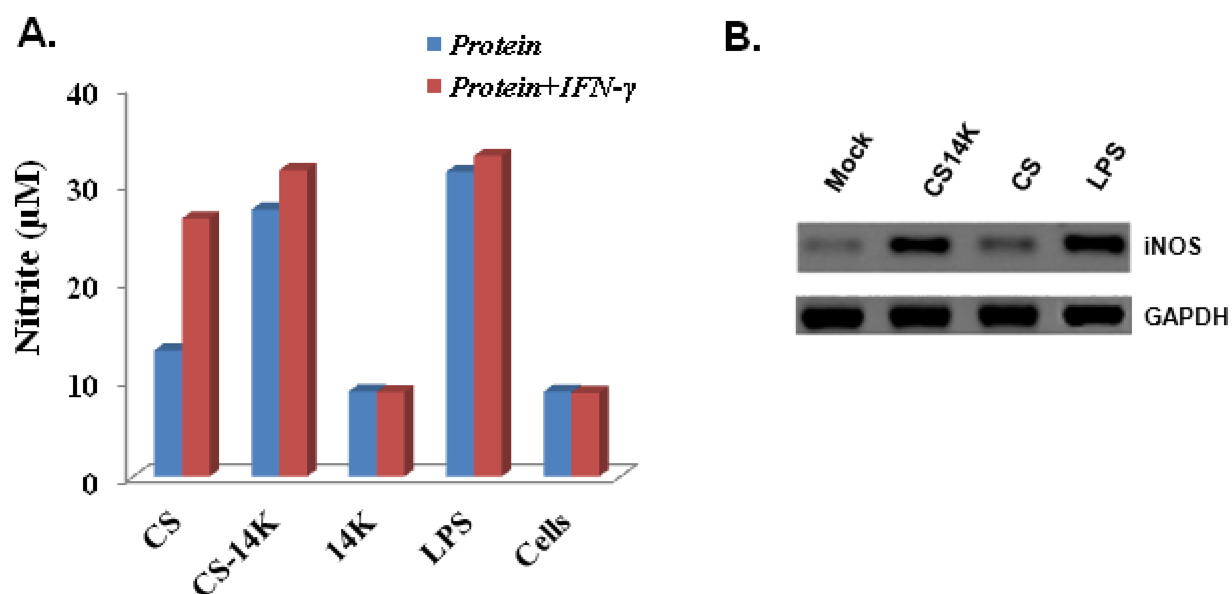


Figure 12: CS-14K improves NO production in macrophages. J774 macrophage cells were stimulated with 5 µg/ml of proteins or as positive control with 1 µg/ml of LPS. IFN-γ was used at a final concentration of 10 ng/ml. ***(A).*** Nitrite accumulation in the supernatant was determined, an estimate of NO production, after 48 hours by using Griess reagent. ***(B).*** Semi-quantitative RT-PCR analysis of NO expression. RNA was extracted and the corresponding cDNA was obtained as mentioned in *Materials and Methods*. Proportionate volume of amplified DNA was run on 8% agarose gel.

Having established that CS-14K can mediate the induction of NO in cultured macrophages, we sought to assess the ability of splenocytes from vaccinated mice to respond to purified CS protein by evaluating NO production. Consequently, splenocytes harvested from animals receiving different prime-boost vaccine regime 53 days post-boost were stimulated with 5 µg/ml of purified CS protein for 48 hours. Mice that were primed with DNA-CS showed no detectable NO. On the other hand, mice that received proteins as priming agents were able to induce NO production (***Figure 13***). While CS protein was able to increase NO by low levels, CS-14K increased NO production by four fold (p = 0.001). These data indicates a reduced capability of

CS-based vaccination to induce NO compared to CS-14K. The unique nature of CS based malaria vaccines to induce NO has not been reported before.

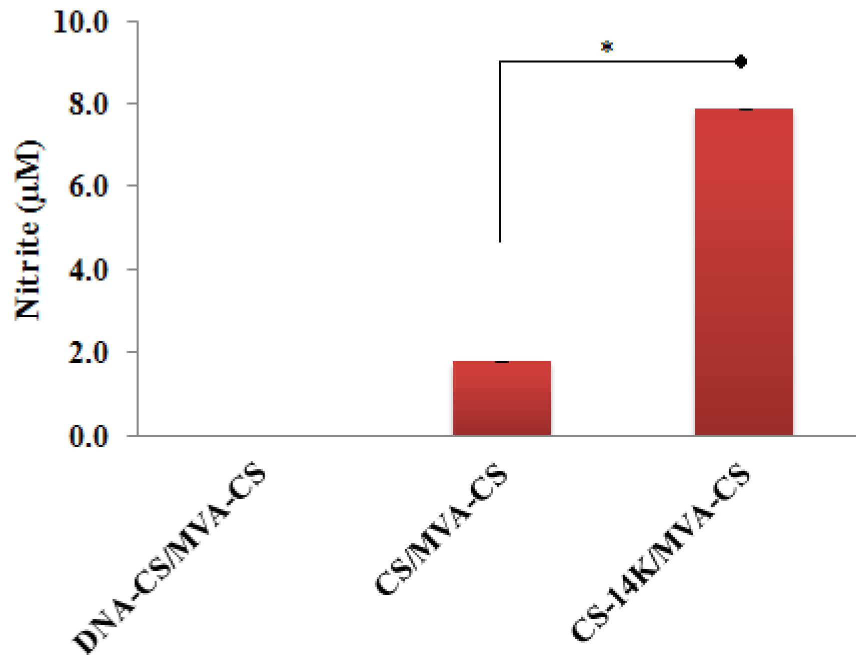


Figure 13: CS-14K priming elevates NO production in mice. Splenocytes from vaccinated groups harvested after 53 days post-boost were incubated with 5 *µg/ml* of recombinant CS protein for a period of 48 hours. Amount of NO in control groups, was deducted from DNA-CS and protein primed groups. Data are expressed as mean ± s.e. of triplicate observation (n = 4 mice per group) and are representative of two independent experiments. *$p < 0.05$.

4.3.1.2 Macrophage Signaling and Cytokine Secretion.

Cytokines secreted by activated macrophages forms an important part of innate immune responses. Among the wide array of cytokines secreted, prominent ones such as IL-12, IL-6 and TNF-α (Type I Interferon response) has significant role against the pre-erythrocytic stages of malaria (Arnold et al., 2010; Shio et al., 2010). Some of these cytokines helps to prime long lasting $CD8^+$ T-cells. Given the significance of TLR activation and the induction of type I

interferon responses, experiments focused on the analysis of the downstream molecules involved in IFN signaling such as IRF-3 and STAT-1 were performed in addition to cytokine analysis.

To study cytokines secreted, J774 cells were stimulated with 5 µg/ml of respective protein or 1µg/ml of LPS as positive control. Given the role played by the above mentioned cytokines in controlling malaria we analyzed the amount of these cytokines secreted by the macrophages using Luminex. Chimeric protein was able to induce a significant increase in IL-6 in macrophages (***Figure 14***). While not statistically significant, we also observed increased TNFα production at 24 h with CS-14K treatment. To detect IL-12 activation we measured the mRNA levels following protein treatment. Again CS-14K significantly effected IL-12p40 activation (p = 0.05).

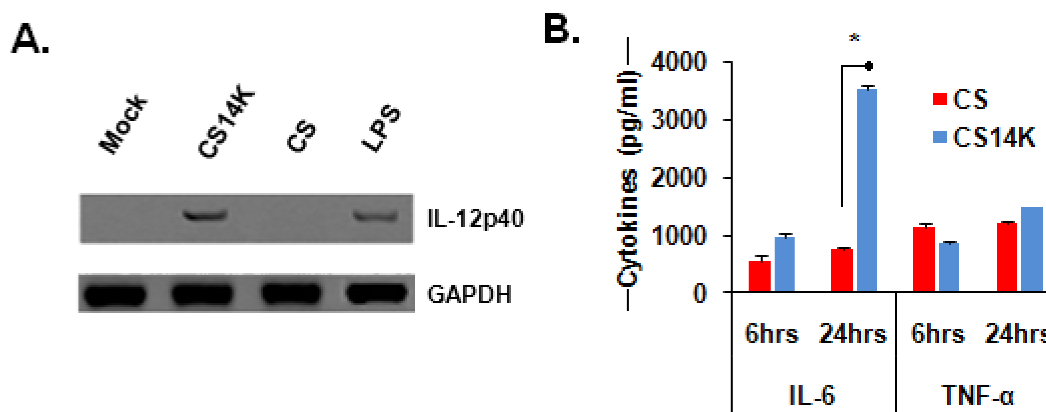


Figure 14: CS-14K protein induced cytokine production in macrophages. Fusion protein can activate innate sensing in macrophages. J774 macrophage cells were stimulated with 5 µg/ml of protein. As positive control, cells were stimulated with 1 µg/ml of LPS. ***(A).*** Semi-quantitative RT-PCR analysis of IL-12p40 expression. RNA was extracted and the corresponding cDNA was obtained as mentioned in *Materials and Methods*. Proportionate volume of amplified DNA was run on 8% agarose gel. ***(B).*** Cytokine analysis by Luminex assay. Supernatant were collected after 6 and 24 hours of protein treatment. Cytokine levels in the supernatant were measured by Luminex.

From the data we inferred that the chimeric protein was able to induce a type I IFN response based on cytokine secretion. To corroborate the results at the cellular level we decided to study the signaling molecules involved in type I IFN response namely IRF-3 and STAT-1. The analysis was carried out using THP-1 cells (***Figure 15 A***). It is notable that CS-14K was particularly effective in activating STAT-1 and IRF-3. To ensure the activation of STAT-1 and IRF-3 is not influenced by any dsRNA contamination, the cells were stimulated with proteins previously digested with RNAse III (***Figure 15 B***).

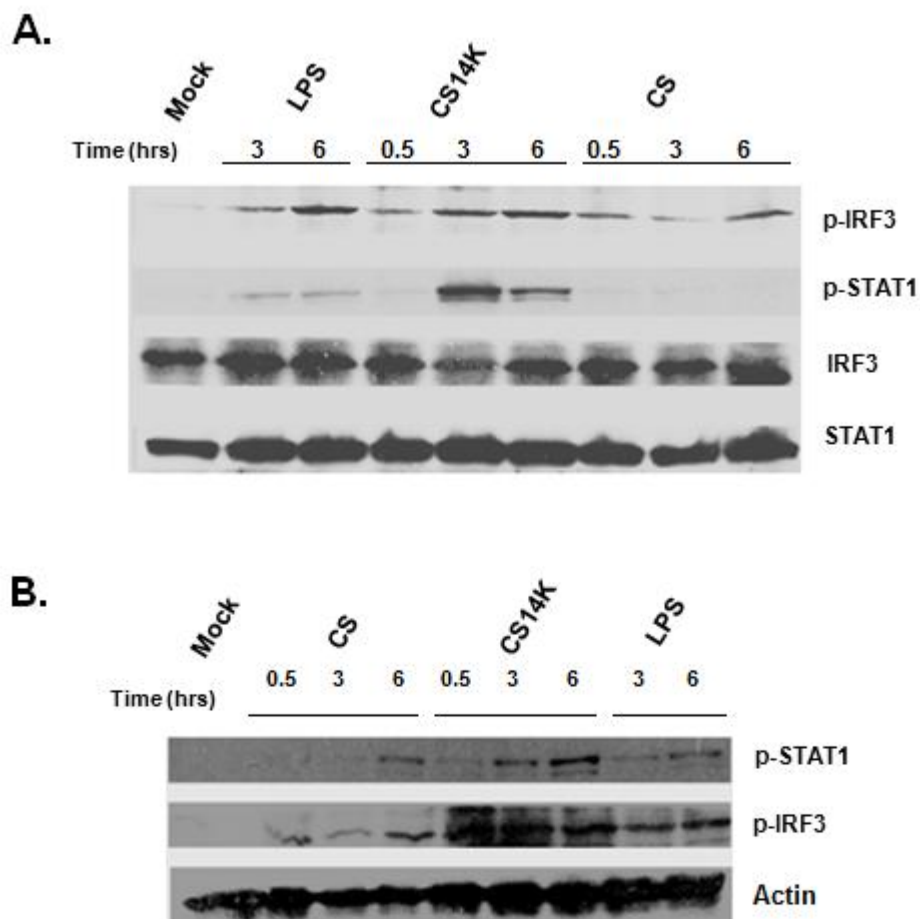


Figure 15: Chimeric protein activates STAT-1 and IRF-3 in macrophages. 2×10^{6} THP-1 cells were stimulated with 5 μg/ml of proteins or as positive control with 1 μg/ml of LPS

following which the samples were collected at different time intervals. ***(A).*** Equal volumes of total cell lysate were run on a 12% SDS-PAGE and were probed with phospho-antibodies against IRF-3 and STAT-1. ***(B).*** Activation of STAT-1 and IRF-3 by CS-14K is independent of dsRNA contamination: Proteins (5 *μg/ml*) and LPS (1 *μg/ml*) were treated with 2 μl of Shortcut®RNase III for 30 minutes at 37ºC. Following incubation the proteins were mixed with RPMI medium containing 10% serum to inhibit RNase III and incubated with 2×10^6 cells of THP-1 for defined time intervals. The levels of phospho IRF-3 and Stat-1 were detected as mentioned in *Materials and Methods.*

Previous study has shown that CS protein does not affect the upstream processes leading to NF-κB activation via IκB degradation, however it prevents the migration of p65 into the nucleus (Singh et al., 2007). Hence we determined if there is any activation of NF-κB by the proteins based on the movement of p65 into the nucleus using confocal microscopy (***Figure 16***)**.** We observed that both CS and CS-14K behaved similarly, i.e both proteins blocked the migration of p65 into the nucleus while leading to IκB degradation.

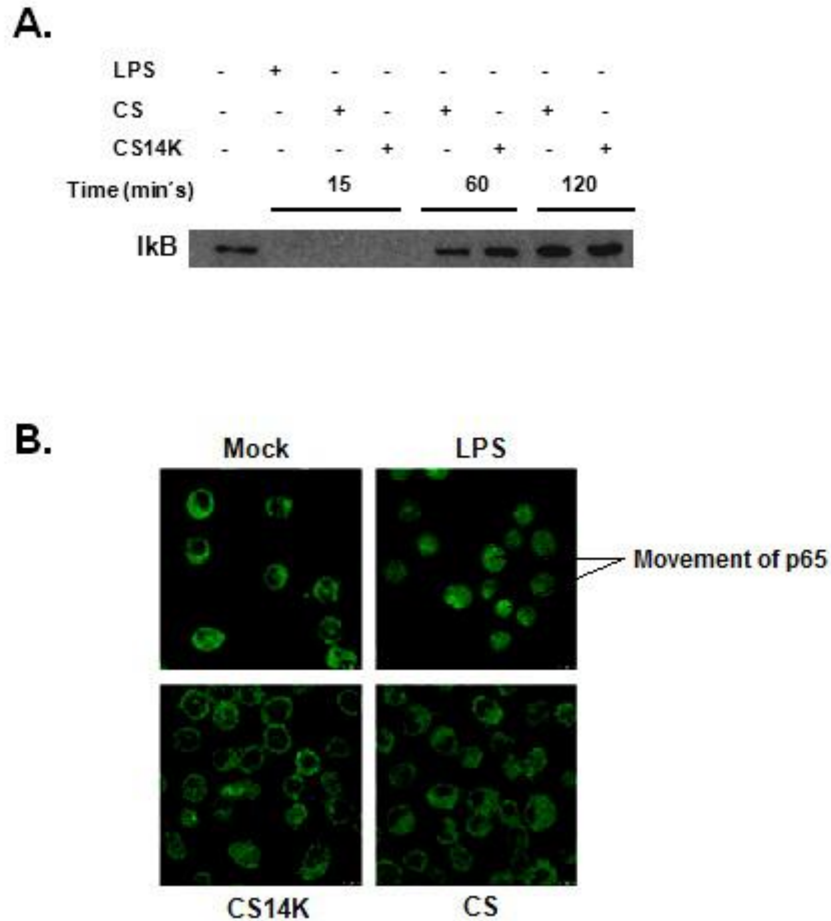


Figure 16: Proteins inhibit NF-κB activation. J774 macrophage cells were stimulated with 5μg/ml of protein or 1 μg/ml of LPS for defined time intervals. ***(A).*** Cells were harvested at 15, 60 and 120 minutes after stimulation and 15 μg of total cell lysate was run on 10% SDS PAGE. WB analysis for IκB degradation was performed. ***(B).*** Confocal microscopy of p65 movement into the nucleus: Following protein treatment for 15 minutes, fixing and permeabilisation of cells was carried out as mentioned in *Materials and Methods*. An antibody against p65 was used to detect the migration of p65 into the nucleus.

Based on the NO and cytokine production in addition to the signaling pathways activated we proposed that CS-14K is able to activate type I IFN response in macrophages in a MyD88 independent pathway (***Figure 17***).

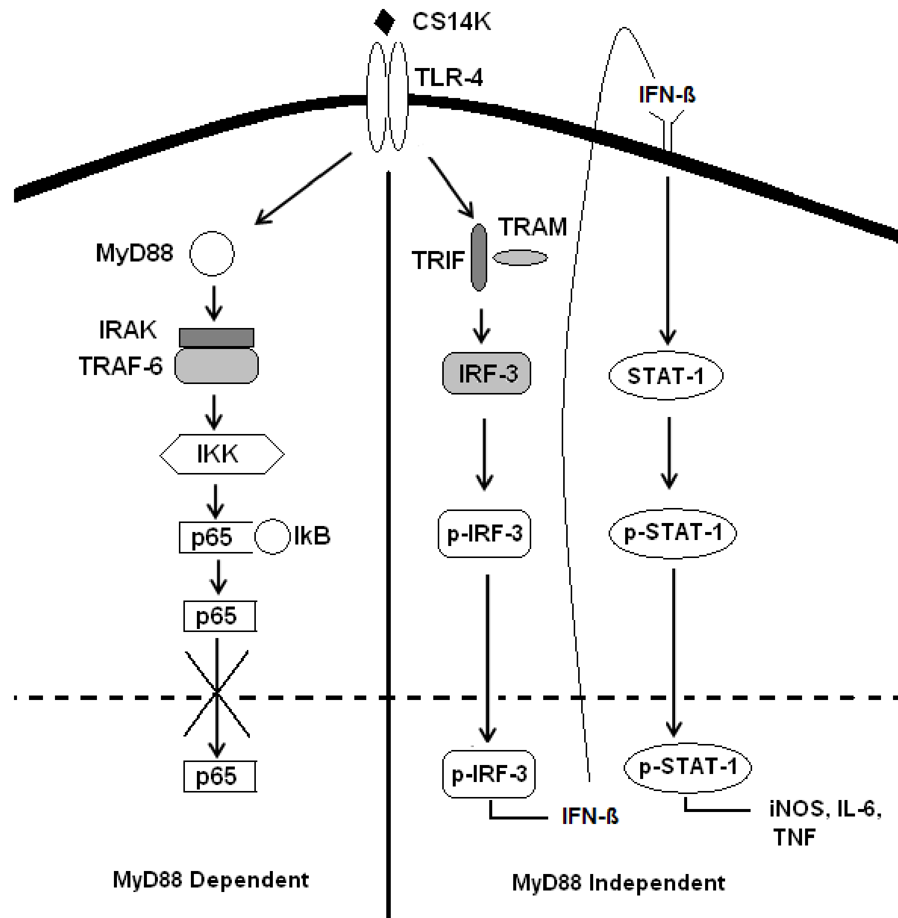


Figure 17: Proposed TLR signaling mechanism by CS-14K. A hypothetical model of TLR-4 signaling, pathway activated by fusion protein, in a MyD88 independent fashion. Inhibition of p65 movement to the nucleus by CS-14K prevents the action of NFkB while activation of IRF-3 and STAT-1 leading to elevated levels of NO, IL-6 and TNF-α which could inhibit the growth of parasite.

4.4 CS-14K PROTEIN VACCINE IMPROVES THE QUALITY AND QUANTITY OF HUMORAL RESPONSE.

It is well established that a strong antibody response against CS correlates with higher protection in many animal and human models. The magnitude, isotype and systemic availability of the antibody produced are important parameters that govern the control of parasite and help to resolve disease (Druilhe et al., 2005; Wipasa et al., 2010). Previous studies have also shown the ability of oligomeric proteins to induce strong humoral responses based on their ability to form large molecular weight aggregates compared to its monomeric counterpart (Kovacs et al., 2012).

Taking into account the imperative role CS antibodies play in controlling malaria, we decided to analyze the quantity and quality of antibodies generated.

Two weeks after the final phase of the prime-boost vaccination protocol described in material and methods, quantitative analysis of the antibodies produced showed that priming with CS-14K chimeric protein followed by MVA-CS induced significantly higher titers of antibodies than mice primed with DNA-CS or CS protein alone (***Figure 18 A***). The antibody titers of CS-14K primed group was 2 times ($p = 0.008$) greater than CS primed group and 8 times more than in the DNA-CS ($p = 0.002$) primed group. Even 53 days post-boost the antibody levels in all the immunized groups were maintained at similar levels except for DNA-CS, which showed a reduction by one log. Further analysis of IgG isotype switching showed that fusion protein induced higher IgG1 response while the DNA vaccinated group showed a pre-dominance of IgG2a response. The levels of IgG1 ($p = 0.012$) and IgG2a ($p = 0.030$) induced by fusion protein primed group were almost double than of CS primed group (***Figure 18 B***).

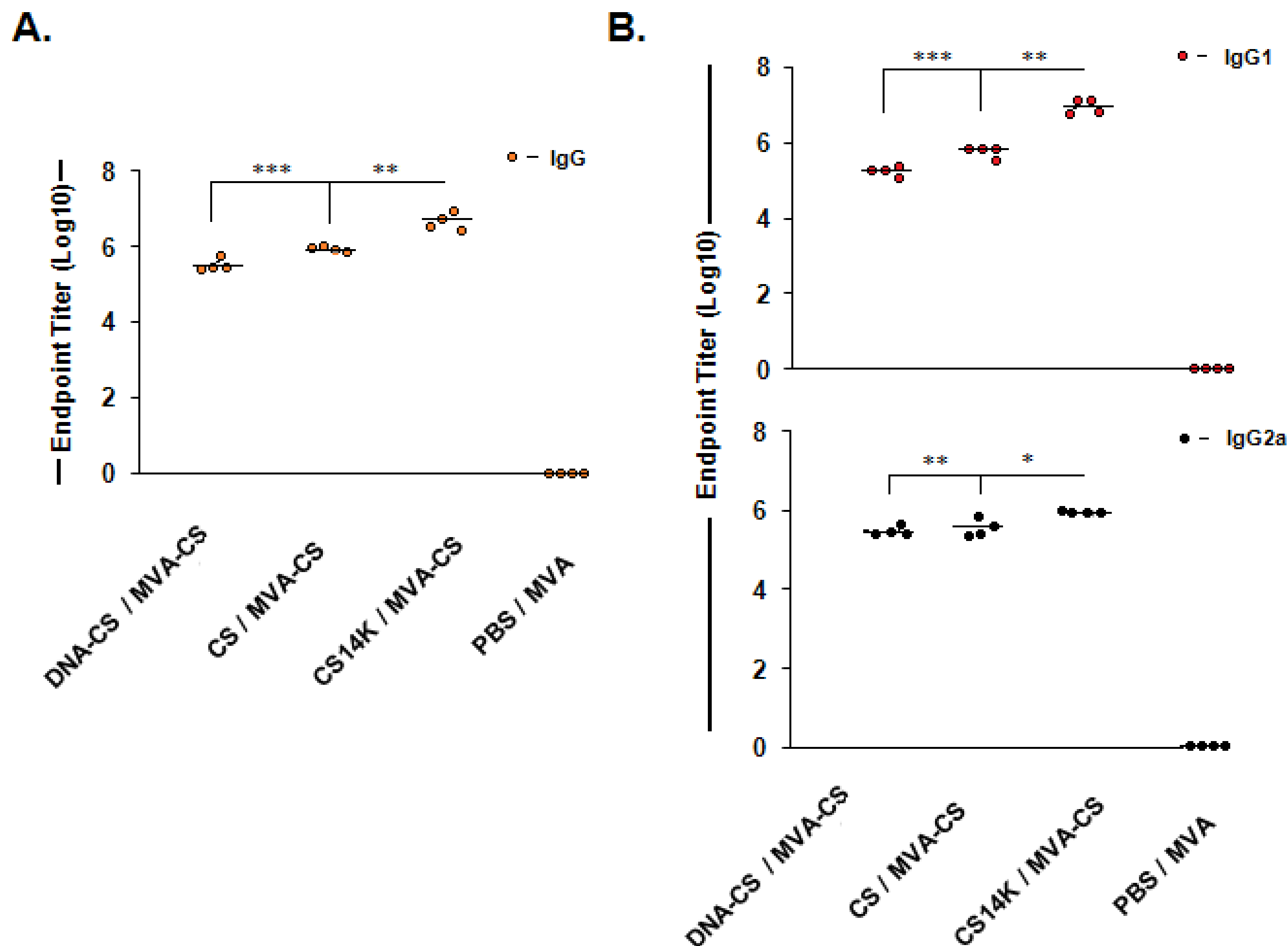


Figure 18: CS-14K priming improves the quality of antibodies *in vivo*. Antibody generated by the vaccine regime was captured using ELISA plates coated with 2 μg/ml of purified CS protein. ***(A).*** Total IgG titers produced by vaccination was determined the last positive dilution which gave an absorbance value three times higher than naïve serum expressed on a log scale. ***(B).*** Represents IgG isotypes induced by immunizations. Levels of IgG1 and IgG2a antibodies generated by the vaccine regimes. Control groups represent the animals receiving empty DNA or PBS for priming and MVA as boost. Data are expressed as mean ± s.e. of triplicate observation (n = 4 mice per group) and are representative of two independent experiments. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

Finally, we sought to determine if the vaccination with chimeric protein affected the avidity of the CS specific antibodies. The antibodies from mice primed with CS-14K showed higher avidity (EC_{50} = 4.2 M Urea) whereas those primed with DNA-CS (EC_{50} = 2.3 M Urea) or CS (EC_{50} = 2.5 M Urea) protein had lower affinity (***Figure 19***).

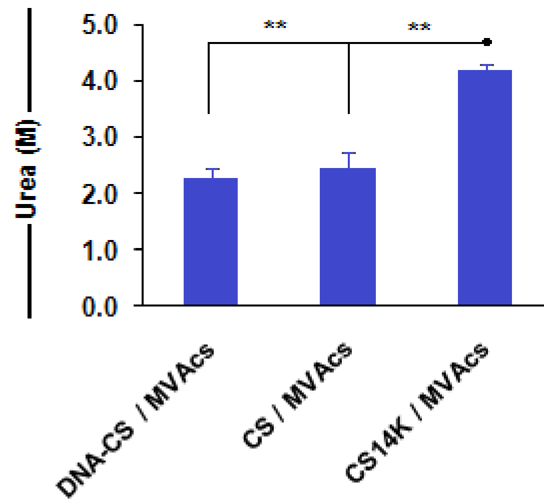


Figure 19: Chimeric protein priming generates elevated levels of high avidity antibodies. Avidity indices of the antibodies against CS protein were estimated as mentioned in *Materials and Methods*. The avidity index was arbitrarily considered as the molarity of urea required to reduce the initial absorbance by 50% i.e. $Log_{10}50\%$ = 1.69. Data are expressed as mean ± s.e. of triplicate observation (n = 4 mice per group) and are representative of two independent experiments. **$p < 0.005$.

Together, these data indicate that priming with proteins, as compared to DNA alone, enhances levels of antibodies against CS. Additionally; priming with CS-14K not only increases the overall production of antibodies but appears to induce a more balanced production of high affinity IgG1 and IgG2a antibodies.

Thus, the fusion of oligomerization domain of 14K protein to CS improves the humoral arm of immune responses against the antigen.

4.5 CS-14K GENERATES DURABLE AND POLYFUNCTIONAL CS SPECIFIC CD8$^+$ T-CELLS.

CD8$^+$ T-cell responses against CS protein are known to be an important factor in the development of sterile immunity using irradiated sporozoites (Weiss et al., 1988). A role of CD8+T cells in subunit vaccine induced protection against malaria was initially established in a heterologus prime-boost approach with flu and vaccinia virus vectors expressing CS CD8 peptide (Gonzalez-Aseguinolaza et al., 2003).Vaccine regimes based on DNA as well as protein prime followed by vaccinia boost have shown to induce CS peptide specific CD8$^+$ T cell responses.

Given the critical role of 14K fusion in aggregating CS protein and the enhancement of innate immune responses, we examined the influence of these factors may have on the development and maintenance of CD8$^+$ T-cells *in-vivo*.

After mice were immunized, they were sacrificed on day 14 to study the adaptive immune response and on day 53 for memory analysis. Efficacy of the vaccination regime was analyzed using IFN-γ based ELISPOT assay and multiparameter flow-cytometry. The fusion protein significantly improved the CS specific IFN-γ secreting cells ($p < 0.05$) compared to other vaccine regimes during adaptive (***Figure 20 A***) as well as memory stage (***Figure 20 B***).

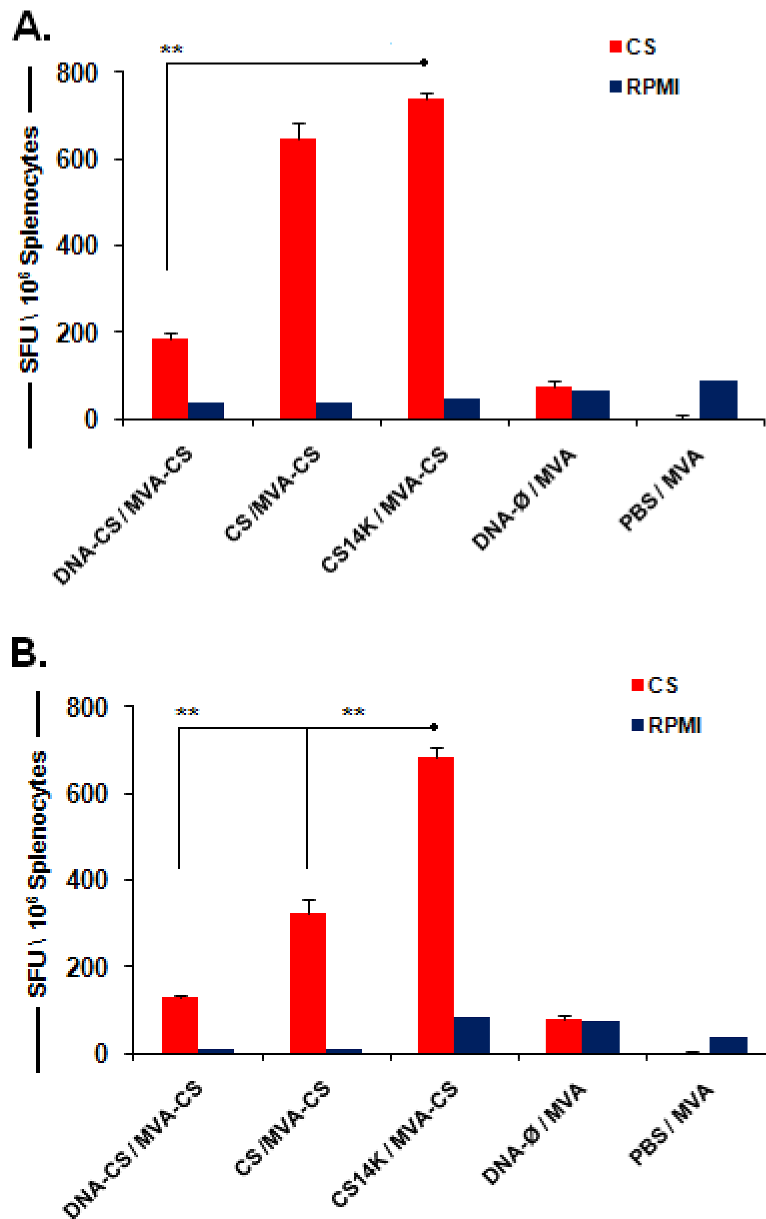


Figure 20: IFN-γ ELISPOT Assay. Splenocytes from vaccinated mice were harvested and 10^6 cells were stimulated with 1μg/ml of CS peptide ``SYVPSAEQI´´. IFN-γ secreted was captured IFN-γ antibody pre-coated plates as mentioned in *Materials and Methods*. ***(A).*** Adaptive response; 14 days post boost. ***(B).*** Memory response; 53 days post boost. Data are expressed as mean ± s.e. of triplicate observation (n = 4 mice per group) and are representative of two independent experiments. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

We characterized the immune responses in terms of polyfunctionality, $CD8^+$ T-cells specific for the peptide 280-288 of *P.yoelii* CS protein, secreting IFN-γ, TNF-α, IL-2, or any combination among these three cytokines. A clear dominance in $CD8^+$ T-cell secreting cytokines was seen in CS-14K primed group of animals over CS protein (2.2 fold) or DNA-CS (4.3 fold) primed groups at day 14 post boost (***Figure 21 A***). Clearly, cells that secreted IL-2 were associated with triple cytokine producing cells. Vaccine regime involving DNA-CS priming resulted in single or double positive cells and did not induce any triple positive population. In contrast, animals receiving protein prime were able to induce IFN-γ^+TNF-α^+IL2^+ secreting cells with CS-14K bringing about 3.6 fold increase over CS. In addition, CS-14K significantly increased double positive ``IFN-γ^+TNF-α^+´´ over both CS protein and DNA-CS priming.

In order to study memory responses the $CD8^+$ T-cells were classified into central memory (TCM) $CD44^{high}CD62L^{high}$, effector cells (TEM) $CD44^{high}CD62L^{low}$, terminally differentiated memory effector memory cells (TEMRA) $CD44^{low}CD62L^{low}$ and naive cells $CD44^{low}CD62L^{high}$. Upon analyzing memory responses on day 53, we observed rapid proliferation of peptide specific effector CD8 T-cell population. Interestingly the frequency of cells secreting IFN-γ was maintained even during the memory stage by CS-14K priming compared to other groups. In terms of polyfunctionality, priming with fusion protein had a significant increase in triple (IFN-γ^+TNF-α^+IL2^+) and double (IFN-γ^+TNF-α^+) population of cytokine secreting cells over both DNA as well as CS protein (***Figure 21 B***). Most of the single positive cells were dominant for TNF-α. A 3.5 fold increase in IFN-γ^+TNF-α^+IL2^+ secreting cells by CS-14K primed group over CS protein primed and nearly 14-fold increase over DNA-CS primed groups was seen. A clear hierarchy in CS peptide specific total CD8 cells secreting cytokines was observed when priming was carried out with CS-14K. Mice immunized with proteins showed significant increase in magnitude of cytokines released over DNA-CS priming. Indeed, priming with CS-14K protein rather that CS protein led to a two fold increase in total IFN-γ ($p < 0.05$) and TNF-α ($p < 0.0005$) in addition to the three fold increase in IL-2 secreting cells ($p < 0.05$) (***Figure 22***). Majority of positive CD8 T-cells produced by the vaccine regime had a phenotype resembling either TEM or TEMRA.

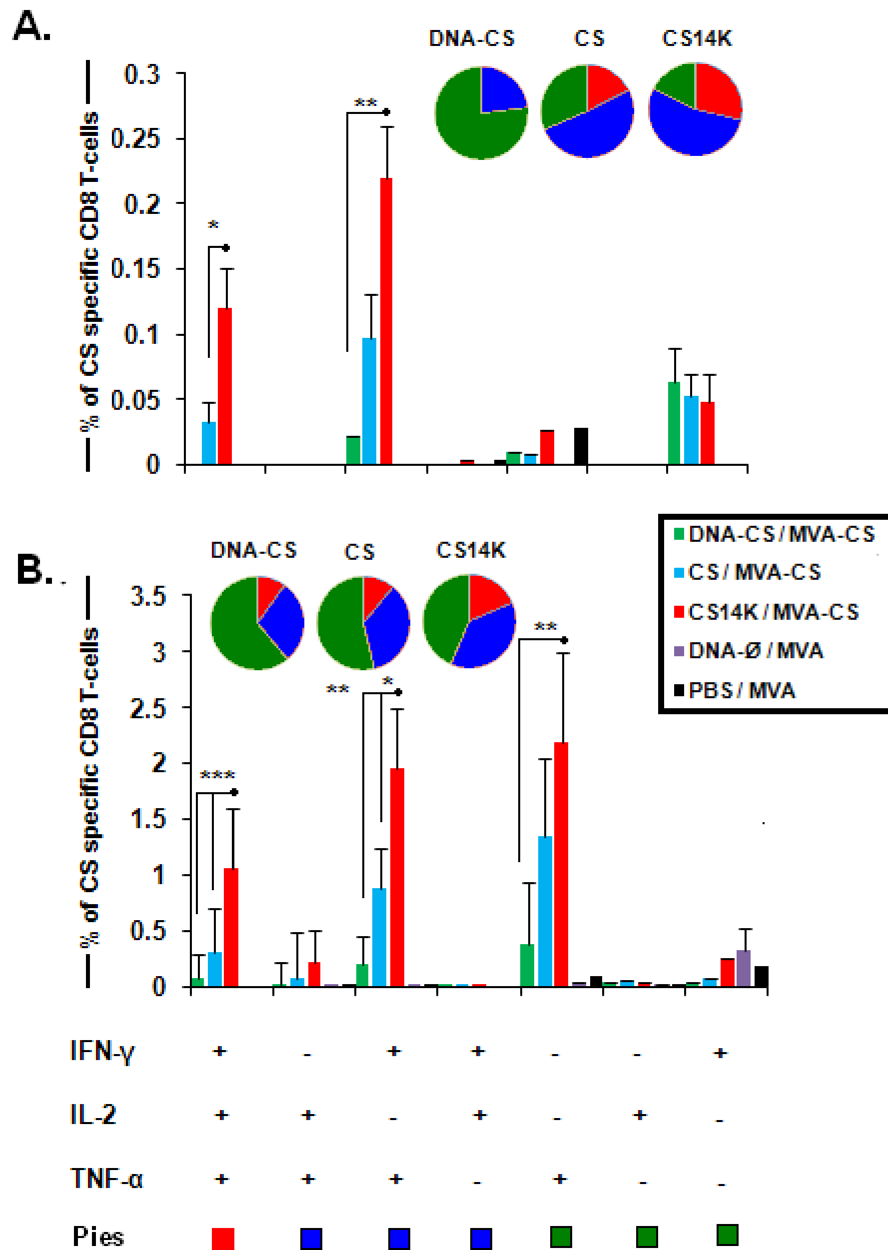


Figure 21: Polyfunctional CD8$^+$ T-cells are produced in CS-14K primed mice. Characterization of CD8$^+$ T-cells secreting IFN-γ, TNF-α and IL-2 by Polychromatic Flow cytometry. ***(A).*** Adaptive response; 14 days after boost animals were sacrificed and the

splenocytes harvested were stimulated with CS peptide ``SYVPSAEQI´´. $CD8^+$ T-cells were selected from CD3 gated lymphocytes and were differentiated into triple, double or single cytokine secreting cells. ***(B).*** Memory response was analysed after 53 days post boost. Memory $CD8^+$ T-cells were classified based on the expression of CD44 and CD62L markers. Pie charts represent the polyfunctionality of $CD8^+$ T-cells secreting single, double and triple cytokines. Data are expressed as mean ± s.e. of triplicate observation (n = 4 mice per group) and are representative of two independent experiments. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

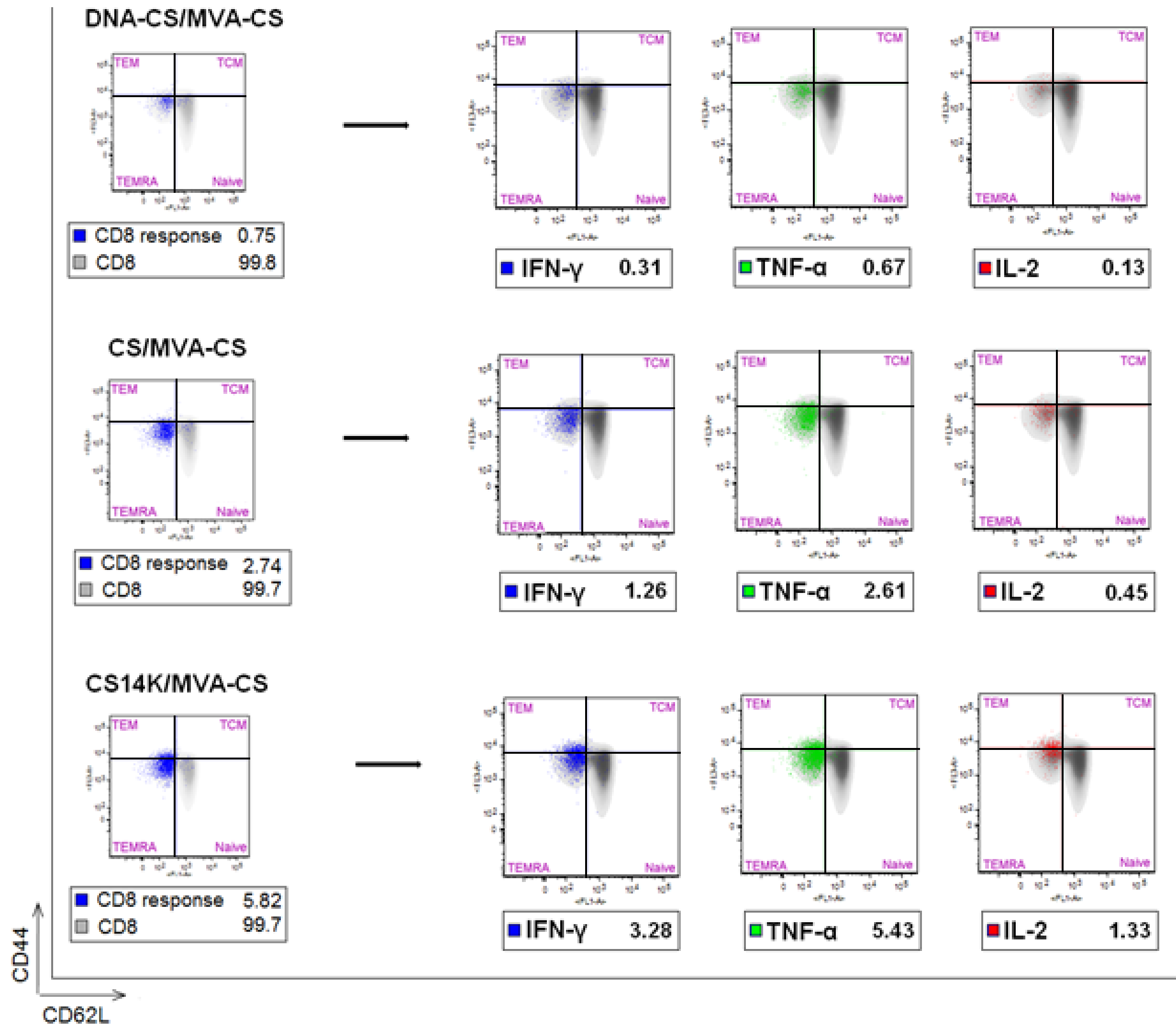


Figure 22: Effective cytokine secretion by CD8^{+} T$_{EM}$ in CS-14K primed mice. Representation of total CS peptide specific CD8 cells were gated on CD44 and CD62L cell surface markers represented on left hand side of the diagram. Total CD8 responses for each group are represented inside the box. Different populations of memory CD8 cells secreting different cytokines are represented on right hand side with their respective total responses as depicted in box.

To extend the immune analysis, we evaluated the amount of IFN-γ or TNF-α secreted by the different populations based on MFI calculation (***Figure 23***). We observed that, irrespective of priming agent, most of the IFN-γ was produced by IFN-γ^{+}TNF-α^{+}IL2^{+} or IFN-γ^{+}TNF-α^{+} cells with similar pattern for TNF-α. The lack of CM CD8 T-cells could be explained by the fact that upon exposure to peptide stimulus most of the CM cells rapidly acquired effector memory characteristics.

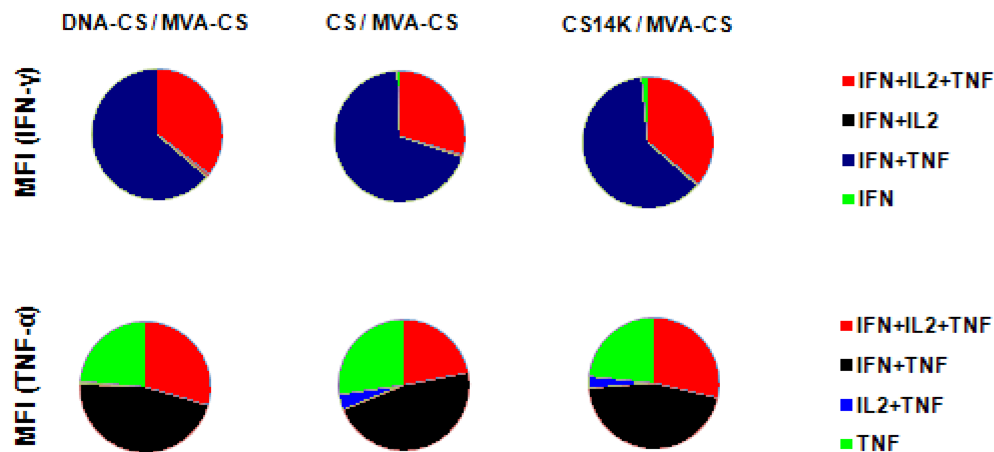


Figure 23: Polyfunctional CD8^{+} T-cells produces more IFN-γ and TNF-α per cell basis. Mean Fluorescence Intensity (MFI) of IFN-γ and TNF-α produced by different polyfunctional population of effector memory CD8^{+} T-cells. Higher amounts of cytokines are produced by polyfunctional triple positive cells compared to double or single positive cells (n=4 mice per group).

These data indicate that proteins are able to prime to a higher extent an effective long-term CD8 T-cell response than DNA. The enhanced CD8 T-cell responses in mice immunized with CS-14K rather that CS is due to the adjuvant-like effect of the unique A27 element.

DISCUSSION

5.1 Theme of current study.

The main objective of the study was to improve the immunogenicity of an antigen by its physical modification brought about by fusing it with the N-terminus of the A27 vaccinia viral protein. This is because A27 contains different domains, a heparin sulfate binding domain essential for the binding of the virus to the cells (21-32 aa), a flexible spacer (33-42 aa), a coiled-coil domain critical for oligomerization (43-65 aa), a disulfide-linked domain (66-84 aa) and a C-terminal leucine zipper domain (85-110 aa). The N-terminus (1-28 aa) was removed from A27 to avoid the induction of neutralizing antibodies. In order to test the hypothesis, we fused the A27 gene with CS protein of *P.yoelii*, a causative agent of malaria in mice. Based on fusion protein priming followed by a recombinant vaccinia viral boost in mice, we analyzed the immune parameters which protected the vaccinated mice against the disease. From the results we concluded that fusing 14K protein to an antigen generates an oligomeric form of the protein that enhances its immunogenicity.

5.1.1 Murine malaria: A perfect model for developing vaccine candidates.

Malaria is a devastating disease against which a vaccine is yet to be developed. There are several strains of malaria parasite infecting different animal species. To test the hypothesis we used a murine malaria model based on *Plasmodium Yoelii*. The only vaccine which provided sterile protection against malaria in humans is by vaccinating with irradiated sporozoites, proof of concept for this vaccine model was first tested in mice. Additionally the importance of $CD8^+$ T-cells secreting cytokines such as IFN-γ and TNF-α was first observed in this model which provided the basis for developing more effective vaccines. Murine malaria model is among the most favored for testing potential malaria vaccine candidates developed against different stages of the disease (Craig et al., 2012; Wykes and Good, 2009). Of several advantages associated with this model, of utmost importance was the ability to assess the efficacy of vaccination in protecting the animals. Furthermore correlates of immune responses which aided protection in animals could be analyzed with ease in this model. Recent advances in molecular biology has

aided the development of humanized transgenic malaria mice models to assess the role of immune system in controlling malaria (Vaughan et al., 2012).

The search for better animal models to study malaria has led to the development of transgenic parasites. Development of *P.berghei* parasite expressing the circumsporozoite protein of *P.falciparum* (3D7 strain), has aided the development of improved pre-erythrocytic stage based vaccine candidates (Persson et al., 2002).

Therefore the use of murine malaria models from pre-clinical evaluation of malaria vaccine candidates is a viable option.

5.1.2 FUSION OF 14K TO AN ANTIGEN RESULTS IN OLIGOMERIZATION.

Bigger antigen results in enhanced immunogenicity, this holds true as shown in many studies (Kovacs et al., 2012). Based on this idea we decided to fuse the oligomeric 14K protein of vaccinia virus to CS, a monomeric protein. Previous studies have demonstrated the differences in CS specific T-cell proliferation using recombinant soluble CS protein, viable sporozoites or heat killed sporozoites (Krzych et al., 1992). This could explain the enhancement of polyfunctional T-cells and antibodies when animals are primed with chimeric protein rather than CS, which contains lower amounts of CS aggregates. An obvious advantage of this chimeric protein is the incorporation of full length CS protein containing both B and T cell epitopes whose presentation would be enhanced as a result of protein aggregation.

When full length CS expressed by MVA was analyzed we did see some aggregate which were absent in presence of tunicamycin, an inhibitor of N-glycosylation. However majority of the protein was monomeric in nature. When 14K protein was fused to CS it formed oligomers/aggregates evident from sucrose gradient and analytical ultracentrifugation. From sucrose gradient analysis it was evident that CS-14K did not exist as a single oligomeric species due to presence of various forms upto the last fraction. However the data from analytical ultracentrifugation suggested the existence of species that is compatible with a moderately elongated protein dimer (calculated frictional ratio $f/f0$ = 1.8). Existence of the protein in its monomeric form during reduced conditions (in presence of ß-mercaptoethanol) suggests that

disulphide bridges formed between cysteine residues present in 14K protein contributes to this phenomenon.

14K protein of vaccinia virus, like HbSAg, is potent target for body's immune system against the virus (He et al., 2007). The current advanced malaria vaccine viz; RTS,S was produced by fusion of CS protein of *P.falciparum* to HbSAg. Hence, by fusing a less immunogenic protein to a potent one a better response was attained even though for a short period of time and only in the presence of AS01 adjuvant. Therefore, in our model the fusion of CS to 14K served dual purpose i.e. formation of large oligomers/aggregates and combining a weak immunogenic CS protein to a potent immunogen, 14K.

5.1.3 Sterile protection using adjuvant free vaccine.

The main purpose behind vaccine development is complete protection against the disease. Many deadly diseases have been eradicated thanks to the development of effective vaccines. Therefore vaccines continue to improve the quality of life around the globe and are considered to be one of the greatest gifts to mankind by science.

A vaccine's success is its ability to provide complete protection for a long time preferably life-long in addition to its stability and ease of transportation. Once we designed CS-14K protein our next step was to analyze whether the protein *per se* could protect the animals without any adjuvants. Additional groups of animals receiving various combination of vaccine were also included as discussed earlier. Even though a homologous CS-14K protein prime-boost decreased the parasitic load in the liver compared to CS prime-boost; it was not as effective as DNA-CS prime MVA-CS boost. However when MVA-CS was used as a boosting agent following CS-14K priming the animals were all protected. Protection was evaluated using a high dose of sporozoites as challenge and the following parasitic burden in the liver was evaluated using qRT-PCR. So even when challenged with high parasite load all the mice that received chimeric protein prime and MVA-CS boost were completely protected. Even though theoretically complete absence of parasite in the liver should lead to sterile immunity, there maybe chances that a single parasite could have evaded detection by qRT-PCR. Therefore to rule out such

possibility we challenged the mice with sporozoites and evaluated the blood stage parasitemia. Animals vaccinated with CS-14K did not develop any blood stage infection which is conclusive evidence of sterile protection. These evidences points out that all the parasites were eliminated within a few minutes before it could establish the liver stage infection.

Although most of protein vaccine´s incorporate a strong adjuvant to enhance the overall immune response, the fusion protein eliminated the requirement of an adjuvant. This is of prime importance considering reports suggesting the side effects of adjuvants in vaccines (Gupta and Siber, 1995; Nohynek et al., 2012). By oligomerizing the protein we were able to achieve sterile protection without adjuvant. LPS contamination of proteins purified from *E.coli* is a major concern for vaccine studies. Being a strong activator of innate immune response, LPS can act as a potent adjuvant. Therefore to avoid the problem of LPS contamination in the protein preparations, we included TritonX-114 in washing steps (Reichelt et al., 2006). TritonX-114 can form micelle with LPS which is easily eliminated during washing step. Quantification of LPS contaminant in the final protein solution by LAL assay was below the accepted levels. This ensured that immune system activated by the protein leading to sterile protection was based solely on protein and not by other factors.

In recent years many experimental CS based vaccines have been developed using novel adjuvants or by modifying the structure of CS protein (***Table 2***). Sterile protection in murine malaria models reported based on protein vaccines often includes strong adjuvant, this study showed how antigen modification alone can improve the immunogenicity of CS antigen leading to sterile protection without any adjuvants.

Table 3: Comparative analysis of immunogenicity of various CS based vaccines.

Priming Agent.	CS (DNA)	CS^GPI (DNA)	CS+GM-CSF (DNA)	CS14K (Protein)	CSgp64 [a] (Protein)	Sb824/pST-TB [b] (Protein) (3)	ADPyCS [d] + 7DW8-5
Boosting Agent (Number of Boost).	MVA-CS (1)	CS^GPI (3)	NYVAC-K1L-CS (1)	MVA-CS (1)	CSgp64 (3)	ACT-CS [c] (1)	-
Antibody Levels.	ND	+++	++	++++	+++	ND	++
Antibody Avidity.	ND	ND	ND	+++	ND	ND	ND
Total $CD8^+$ T-Cells Secreting Cytokines.	ND	ND	+++	+++	ND	+++	ND
ELISPOT (IFN-γ).	+++	ND	+++	+++	+	ND	++++
Nitric Oxide.	ND	ND	ND	Yes	ND	ND	ND
Protection	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a] CSgp64 : Recombinant Baculovirus expressing CS fused with baculovirus envelope protein gp64.

[b] Sb824/pST-TB : *Salmonella* expressing fusion protein between YoPE and CS peptide.

[c] ACT-CS : *Bordetella pertussis* adenylate cyclase toxoid fused with CS peptide.

[d] ADPyCS + 7DW8-5 : Adenovirus expressing CS mixed with a synthetic analogue of Alpha-Galactosylceramide.

+	- Low Levels	(Elispot: Less than 400 spots.)	(CD8 T-cells: Less than 1%.)	(Antibody Levels: < 1:50,000)
++	- Moderate Levels	(Elispot: 450-850 spots.)	(CD8 T-cells: 1% to 3%.)	(Antibody Levels: 1:60,000 to 100,000)
+++	- High Levels	(Elispot: 900-1200 spots.)	(CD8 T-cells: 4% to 6%.)	(Antibody Levels: 1:200,000 to 1:500,000)
++++	- Very High Levels	(Elispot: 1500-2000 spots.)	(CD8 T-cells: 10% and above.)	(Antibody Levels: > 1:800,000)

5.1.4 HIGH QUALITY CS ANTIBODIES: HALLMARK OF A GOOD VACCINE.

A trademark of an effective vaccine is the induction of high levels of neutralizing antibody. However in case of most experimental malaria vaccines even though high titers of antibodies were produced protection could not be achieved. This may be explained by the inability of these vaccines to induce antibodies against critical epitopes on the antigen. There are reports of elevated levels of protective antibodies in people with age in malaria endemic areas. Studies

from murine models helped to elucidate the mechanism involved. During primary infection short-lived B220^{+} plasma B-cells are produced which following secondary exposure develops into long-lived B220^{-} plasma B cells (Stephens et al., 2009). Protective immunity against malaria is associated with high avidity IgG antibodies against CS (Moon et al., 2012). Previous studies have also shown the ability of fusion proteins to induce strong humoral responses based on their ability to form large molecular weight aggregates (Verma et al., 2012). Like the CS-14K priming approach presented in this study, the malaria vaccine in the latest stages of clinical trials, RTS,S, is also dependent on higher magnitude of antibodies belonging to IgG1 subclass. Vaccine studies based only on DNA-CS vaccination lacking GPI anchor improve protection up to 95% based only on antibody titers (Scheiblhofer et al., 2001).

The serum antibody titers obtained by fusion protein priming are much higher than those obtained in presence of strong adjuvants such as Freund´s adjuvant (Wang et al., 1995). However, unlike vaccination with DNA-CS or protein alone, vaccination with the CS-14K fusion protein results in the concomitant induction of high levels of both IgG1 and IgG2a antibodies and this could be beneficial in providing protection against malaria. Increase in cytophilic antibodies against CS protein, such as IgG1, in older people in malaria endemic areas is associated with decreased risk of malaria infection (John et al., 2003). Therefore with constant exposure there is an increase in CS protein IgG1 antibodies. A possible explanation for attaining higher IgG1 antibody titer with chimeric protein could be attributed to its structure. The oligomeric nature of the protein could activate the formation of germinal centre for better production of antibodies compared to its monomeric counterpart. This sort of differences with antigenic structure and the induction of germinal centers are recently reported by many studies in the malaria vaccine field (Moon et al., 2012; Wipasa et al., 2010).

The chimeric protein proved to be superior in inducing high avidity antibodies against CS comparable with those achieved by using nanoparticle based CS peptide vaccination (Kaba et al., 2009). Avidity of an antibody also increases with time. This is usually a result of somatic mutations in the germinal centre and competition with B cell clones. High avidity antibodies produced by the chimeric protein could be due to the unique folding pattern attained by CS-14K

protein resulting in exposure of other potent epitopes. Additionally there is a possibility of the chimeric protein to induce CS specific follicular helper T cells (T_{fh}) which not only improves the avidity but also the titer of antibodies (Moon et al., 2012). These data have important implications in the design of antigens to induce antibody responses against malaria.

5.1.5 CS specific CD8+ T-cells role in malaria: a balance between amount and polyfunctionality.

$CD8^+$ T-cell responses against CS protein are known to be an important factor in the development of sterile immunity using irradiated sporozoites (Overstreet et al., 2008). $CD8^+$ T-cells during the liver stages are predominantly directed against CS protein (Schmidt et al., 2010). A role of $CD8^+$ T-cells in subunit vaccine induced protection against malaria was initially established in a heterologus prime-boost approach with flu and vaccinia virus vectors expressing CS (Li et al., 1993).Vaccine regimes based on DNA as well as protein prime followed by vaccinia boost have shown to induce CS peptide specific $CD8^+$ T cell responses (Sedegah et al., 1998; Stewart et al., 2007). Site of vaccination can affect the CD8 response and since in case of malaria the priming of $CD8^+$ T-cells occurs in the draining lymph node all proteins were given intradermally (Chakravarty et al., 2007). The ability of CS-14K regime to produce significantly elevated levels of IFN-γ and TNF-α single as well as double positive secreting $CD8^+$ T-cells even after 53 days makes it a suitable vaccine candidate. Report of adjuvant-antigen complex promoting CD8 responses compared to an antigen adjuvant mix further strengthens our findings (Olvera-Gomez et al., 2012). We also report a shift in polyfunctionality of CD8 T-cells secreting all the three cytokines produced by chimeric protein which may be effective in controlling the growth of parasite. Furthermore, the ability of CS-14K vaccine regime to induce large amount of TNF-α has an added advantage since it is known to be an important cytokine for the maintenance of memory CD8 T-cells in malaria (Butler et al., 2010). The concomitant increase in TNF-α by CS-14K could also contribute to elevated NO and antibody responses (Li and Langhorne, 2000; Pombo et al., 2002). The need for IL-12 in the development of effective adaptive responses is in agreement with our study which shows the ability of CS-14K protein to prime enhanced T-cell response (Hoffman et al., 1997a). The data from MFI studies shows that double or triple positive

population are better in producing elevated levels of cytokines, both of which are elevated by CS-14K priming. The low levels of CD8 responses against DNA-CS suggest that the protective capability of this regime may be based on the antibodies induced. The maintenance of such durable and polyfunctional memory responses even after 4 months of vaccination taken together with sterile protection, justifies the potent nature of A27 protein fusion and its use for developing better malaria vaccines.

5.1.6 ENGAGING THE INNATE IMMUNE SYSTEM.

Of the first resistance that any foreign body encounters when it enters the body is the innate immune system. A future cataclysmic event that aids in eliminating the pathogen is the direct effect of signaling by the innate immune system (Arnold et al., 2010; Shio et al., 2010). Often the innate immune activation involves signaling via the TLR's leading to the activation of various pathways. In our case we did observe how CS-14K affected the innate immune system based on which we proposed a hypothetical model.

Given that an early activation of innate immune responses curbs the pre-erythrocytic development of parasite, activation of type I interferon signaling by fusion protein further validates its use as a priming agent. Indeed, activation of type I interferon signaling by the chimeric protein did result in elevated levels of cytokines belonging to this family such as IL-6, TNF-α and IL-12. It is believed that the parasiticidal action of TNF-α in hepatocytes is mediated through the synthesis of IL-6 (Nussler et al., 1991). Furthermore, engaging TLR's is critical in developing anti malarial immunity which could be explained by the poor immunogenicity of RTS,S without a strong adjuvant like AS01E, a TLR-4 agonist (Mettens et al., 2008). A recent study also shows the importance of TLR signaling for effective development of protective antibodies to Plasmodium (Wiley et al., 2011). This is in agreement with our study which shows how enhancement in STAT-1 and IRF-3 by CS-14K suggested a role in TLR-4 activation (***Figure 17***), resulting in improved immunogenicity and inhibiting the liver stage development of the parasite (Imada and Leonard, 2000; Spath et al., 2009).

It is known how parasites such as *Leishmania* and *Plasmodium* down regulate synthesis of NO (Liew, 1994). Our study shows the inherent capacity of chimeric protein to enhance the production of NO both *in vitro* and *in vivo*. Since neither CS nor 14K protein alone could induce NO, it seems reasonable to conclude that the CS-14K protein aggregation is responsible for NO induction. The persistently elevated levels of NO at 53 days post-vaccination with chimeric CS-14K protein compared to priming with DNA-CS or protein alone, suggest that differences exists in the processing of these antigens by APCs. In addition, early production of NO has been proposed to be required for proliferation of $CD8^+$ T-cells against the parasite (Scheller et al., 1997). Taking into consideration all of the innate, adaptive, memory and protective efficacy results described in this work, we can infer immune correlates of protection as indicated in Table 4. In conclusion, fusion of 14K to CS leads to a more favorable immunogenic milieu to enhance the efficacy of the vaccine.

Table 4: Comparison of immune correlates defined in this thesis with those observed in humans.

STAGE (Immune Response)	Parameters Upregulated	Correlates	References
INNATE	NO	Inconsistent data	3, 75, 78
	IL-6	ND	55, 85
	IL-12	Surrogate	101, 116, 172
	IRF-3	ND	----
	STAT-1	ND	----
ADAPTIVE	IFN-γ secreting $CD8^+$ T-cells.	Correlate	90, 98, 157
	CS Antibody Titre	Correlate	71, 105, 156, 171
	Antibody Avidity	Surrogate	18, 102
MEMORY	Effector Memory Polyfunctional $CD8^+$ T-cells.	ND	84, 131

CONCLUSIONS

From the study we concluded the following:

- 14K protein (A27) of vaccinia virus when fused at the C-terminus of the CS antigen can form oligomers/aggregates.

- The oligomers/aggregates of CS-14K are of high molecular weight which is evident from sucrose gradient and analytical ultracentrifugation studies.

- CS-14K protein of high purity could easily be purified from *E.coli* facilitated by a GST tag with low levels of LPS contamination.

- Comparative studies of a homologous protein prime-boost in mice showed that CS-14K was more effective in reducing the parasitic load in the liver compared to CS protein when challenged with a high dose of sporozoites.

- A heterologus protein prime followed by MVA-CS boost protected more animals than the homologous treatment, with complete elimination of parasites in the liver when primed with CS-14K protein.

- Sterile protection was achieved only in animals primed with CS-14K and boosted with MVA-CS.

- Elevated levels of antibodies with high avidity were observed in mice which received CS-14K as priming agent. A higher ratio of IgG1:IgG2a was attained in CS-14K vaccinated animals.

- Increased number of IFN-γ secreting $CD8^+$ T-cells was observed in mice primed with CS-14K protein versus CS. The number of cells secreting IFN-γ was maintained during the memory phase, only in animals vaccinated with CS-14K.

- Enhanced levels of polyfunctional $CD8^+$ T-cells secreting cytokines was achieved in animals receiving CS-14K, during the adaptive and memory phase.

- Chimeric CS-14K protein was able to induce IL-12, TNF-α and NO in macrophages via IRF-3 and STAT-1 activation.

From the data observed we concluded that fusion of 14K protein to an antigen improves its immunogenicity. Our findings provide the platform for the development of 14K as an effective adjuvant. In this study, we observed how a fusion protein based on CS antigen affect its immunogenicity and evaluated the correlates of protection in malaria murine model. Moreover, the stability of the fusion protein at room temperature and the ease with which it can be produced and administered makes this a candidate adjuvant for the development of effective vaccines. Signaling of CS-14K via the TLR-4 pathway can shed light in producing better vaccines against malaria. Taken together, this thesis extends previous observations on the role of CS as an immunogenic component against malaria and highlights the development of vaccines based on presentation of antigens as oligomers/aggregates using the immunogenic molecule of A27 from vaccinia virus.

Spanish Version

7.1 RESUMEN.

El desarrollo de vacunas-subunidades para la malaria que inducen una fuerte respuesta a largo plazo de memoria es un área de intensa investigación, enfocado en la mejora de la inmunogenicidad de una vacuna basada en la proteína de circumsporozoite (CS). En este estudio, se encontró que una proteína quimérica, formado por proteína de fusión del virus vaccinia 14K (A27) a la CS de Plasmodium yoelii, induce una fuerte respuesta de memoria de células T $CD8^+$ efectoras, además de anticuerpos de alta afinidad cuando se usa como un agente de ``priming´´ en la ausencia de cualquier adyuvante, seguido por una dosis recuerdo ``boost´´ del virus vaccinia atenuado (MVA) que expresa CS en modelo murino. Por otra parte, el priming con la proteína permite una mejora de la magnitud y la polifuncionalidad de células T $CD8^+$ secretoras de citoquinas. Esta proteína de fusión forma oligómeros/agregados que llevan a la activación de STAT-1 y IRF-3 en los macrófagos humanos, lo que indica una respuesta a IFN de tipo I, dando como resultado la inducción de NO, IL-12, e IL-6. Además, la combinación de CS-14K junto con el vector MVA-CS en protocolo de inmunización inhibió el desarrollo de la etapa hepática del parásito, lo que resulta en una protección completa. En resumen, se propone un nuevo enfoque en el diseño de vacunas pre-eritrocíticas basadas en CS contra el Plasmodium utilizando el efecto adyuvante de la proteína inmunogénica del virus vaccinia 14K.

7.2 INTRODUCCIÒN.

7.2.1 MALARIA LA ENFERMEDAD.

Malaria significa "Aire malo" en italiano debido a su prevalencia en las zonas pantanosas, continúa presentando un importante problema de salud pública y de carga para el desarrollo económico de muchos países. Plasmodium falciparum, el principal agente causal de la malaria en humanos, se sabe que causa aproximadamente 225 millones de casos y alrededor de 781.000 muertes al año. La malaria sigue siendo un factor clave en la mortalidad y morbilidad entre los niños pequeños y las madres en zonas de África y subsaharianos (Informe Mundial sobre el Paludismo, 2010). Las áreas que han sido declaradas previamente libre de malaria también están bajo la constante amenaza de un resurgimiento debido a los cambios en el clima mundial y la globalización. La situación ha empeorado aún más debido a la aparición de parásitos resistentes a los medicamentos y a vacunas ineficaces. Con varios medicamentos en desarrollo, la necesidad de una vacuna eficaz contra la malaria se hace patente.
Hasta la fecha, las vacunas han jugado un papel importante en la eliminación de muchas enfermedades. Sin embargo el desarrollo de una vacuna contra la malaria se ve limitado por la capacidad del parásito para engañar al sistema inmunitario. La inmunidad natural a la malaria se observa en las zonas endémicas, en las que también se requiere una exposición repetida y un tiempo considerable para desarrollarse. Sin embargo, este tipo de rangos de inmunidad parcial a completa protección, generalmente se observa en la población anciana. Por lo tanto el desarrollo de una vacuna eficaz contra el paludismo es esencial. A pesar de que un gran repertorio de antígenos presentes en las diversas etapas de la malaria está disponible, la selección de un candidato es todavía una tarea en proceso. Una vacuna eficaz debe superar varios obstáculos tales como que debería proporcionar una protección de larga duración con independencia de las variaciones genéticas que existen entre las poblaciones humanas así como en el parásito. Además, debería ser fácil de producir y transportar con un coste mínimo. Además, la vacuna puede incorporar antígenos de diferentes etapas del desarrollo del parásito. Con el advenimiento de las nuevas tecnologías en el desarrollo de una vacuna contra el paludismo una vacuna eficaz podría no ser un sueño lejano.

7.2.2 LAS VACUNAS CONTRA LA MALARIA.

Las vacunas son una herramienta esencial en el arsenal para luchar contra la malaria. Teniendo en cuenta la evolución de insecticidas y parásitos resistentes a fármacos, el desarrollo de una vacuna eficaz es imprescindible. Desarrollo de una vacuna rentable está limitada por la capacidad del parásito para manipular el sistema inmune del huésped. A pesar de la amplia gama de los antígenos disponibles, que se expresan por el parásito en varias etapas de su ciclo de vida, la selección de un antígeno específico ha sido bastante intrincada. Dependiendo del ciclo de vida del parásito, las vacunas desarrolladas se pueden clasificar en tres clases (1). Vacunas pre-eritrocíticas (2). Vacunas eritrocíticas y (3). Vacunas para el bloqueo en la transmisión parasitaria.

7.2.2.1 Proteína circumsporozoite: Potente candidate vacunal pre-eritrocítica.

La mayoría de las vacunas contra la etapa pre-eritrocítica se orientan hacia las proteínas que se expresan en la superficie del esporozoito. Respecto de todos los antígenos que se encuentran expresados en esta etapa, sobresalen la proteína CS y la proteína TRAP. Sin embargo, las vacunas basadas en proteína CS han resultado ser más prometedoras y han entrado con éxito en ensayos clínicos de fase III (como es el caso de RTS, S).
La proteína CS es una importante proteína monomérica que se encuentra en la superficie de los esporozoitos. La importancia de la proteína CS como candidato vacunal principal fue reportada por primera vez por Nussenzweig y Nussenzweig (1985). La mayor parte de la protección en el modelo de vacuna de esporocitos γ-irradiados y vacunas de esporozoitos atenuados genéticamente estaba mediada por respuestas humorales específicas de CS y CMI (Kumar et al., 2009). La proteína mantiene similaridades respecto de las propiedades estructurales, bioquímicas e inmunológicas entre las diferentes especies de Plasmodium. El dominio central de todas las especies de Plasmodium contiene una región de repetición que contiene epítopos inmunodominantes para células B y células T (Lal et al., 1987). Esta región está generalmente flanqueada por la región conservada I en el extremo N-terminal y la región III y la región II + en el extremo C-terminal. La unión de los esporozoitos a las cadenas de GAG de HSPG de los hepatocitos está mediada por la región II de la proteína CS (Pinzon-Ortiz et al., 2001). CS

presenta una estructura cilíndrica que está anclada a la superficie de los esporocitos por un dominio GPI que se encuentra la región C-terminal (Plassmeyer et al., 2009). En un estudio se demostró que la CS se une a los ribosomas interrumpiendo la síntesis de proteínas en hepatocitos infectados (Frevert et al., 1998). Proteína CS también ayuda a la supervivencia de los esporozoitos en hepatocitos mediante el bloqueo de la translocación de p65 en el núcleo lo que se traduce en un bloqueo la vía de NFkB. Además, también promueve la expresión de diversos genes vitales en el proceso metabólico para permitir que el parásito pueda prosperar dentro de las células infectadas (Singh et al., 2007). A pesar de ciertas desventajas, el desarrollo de vacunas basadas en la proteína CS están progresando.

7.3 Resultados y Discusión.

Circumsporozoito (CS) es una proteína monomérica de superficie de esporozoitos de Plasmodium, el agente causante de la malaria (Nussenzweig y Nussenzweig, 1985). Sin embargo, el CS nativa de esporozoitos también se conoce para formar algunos agregados/ oligómeros que pueden facilitar la unión de CS a los hepatocitos (Pinzon-Ortiz et al., 2001). Dado que los niveles de agregados de CS / oligómeros son comparativamente mucho menor que sus monómeros, creemos que niveles más altos de agregados CS / oligómeros se requiere para alcanzar una mejor protección.

Basándose en esta hipótesis, decidimos fusionar CS con una proteína inmunogénica del virus vaccinia, 14K, lo que facilita la formación de oligómeros superiores a través de su enrollado de la bobina de dominio. La proteína CS carece de la secuencia señal de GPI y C-terminal fue fusionado con la proteína 14K del virus vaccinia carece de los primeros 28 aminoácidos. CS y CS14K fueron entonces purificados de E. coli. Proteína CS existía como un monómero de 50 kDa ~ mientras CS14K existía como oligómeros grandes / agregados. La forma monomérica de CS14K cuando bajo condiciones reductoras existían como molécula de 60 kDa. Los datos de la ultracentrifugación analítica sugirió la existencia de especies que sean compatibles con un dímero de proteína moderadamente alargado (calculó la razón de fricción f/f0 = 1,8). Existencia de la proteína en su forma monomérica durante condiciones reducidas (en presencia de ß-

mercaptoetanol) sugiere que los puentes disulfuro formados entre restos de cisteína presentes en la proteína 14K contribuye a este fenómeno.
Con el fin de analizar si el cambio en las propiedades físicas de la proteína influye en su inmunogenicidad, se decidió vacunar a los animales sobre la base de una estrategia de sensibilización / refuerzo. Después de la vacunación los animales fueron desafiados con 2 × 104 esporozoitos por vía intravenosa. Después de 42 horas, los animales fueron sacrificados y los niveles de 18S rRNA P.yoelii niveles se evaluaron por qRT-PCR. Hemos observado que una proteína heteróloga prime / vaccinia régimen reactivación del virus se encontró que era más efectiva que una proteína homóloga de cebado / régimen de impulso de proteínas, una reducción del 32% en la carga de parásitos etapa hígado se observó en ratones que recibieron CS-14K (CS-14K/CS-14K) en comparación con CS (CS / CS) de proteínas. Además, una dosis recuerdo con MVA-CS redujo significativamente los niveles de parásitos en el hígado. Por otra parte, el ``priming´´ con proteína CS-14K seguido de una segundo dosis ``booster´´ con MVA-CS resultó en una inhibición casi completa (~ 99,9%, p <0,005) de desarrollo del parásito en el hígado en comparación con la proteína CS y ADN-CS. Para confirmar aún más que la reducción de la parasitemia se traduce en la protección estéril después de dos semanas después de aumentar los ratones se estimularon con 300 esporozoitos y la presencia de iRBCs en la sangre se analizó en los frotis de sangre de 3 a 21 días. Los animales que recibieron la proteína CS-14K como agentes de cebado estaban completamente protegidos en comparación con el grupo control. Aunque la mayor parte de la vacuna de proteína es incorporar un adyuvante fuerte para potenciar la respuesta inmune en general, la proteína de fusión elimina el requisito de un adyuvante. Esto es de gran importancia teniendo en cuenta los informes que sugieren los efectos secundarios de los adyuvantes de las vacunas (Gupta y Siber, 1995;. Nohynek et al, 2012). Mediante la oligomerización de la proteína hemos sido capaces de conseguir una protección estéril sin adyuvante. LPS contaminación de las proteínas purificadas a partir de E. coli es una preocupación importante para los estudios de la vacuna. Siendo un fuerte activador de la respuesta inmune innata, LPS puede actuar como un potente adyuvante. Por lo tanto, para evitar el problema de la contaminación de LPS en las preparaciones de proteína, que incluye Triton X-114 en pasos de lavado (Reichelt et al., 2006). Triton ® X-114 se pueden formar micelas con

LPS, que es fácilmente eliminado durante la etapa de lavado. La cuantificación de LPS contaminante en la solución final de proteína por el ensayo de LAL era inferior a los niveles aceptados. Esto aseguró que el sistema inmune activado por la proteína que conduce a la protección estéril se basa únicamente en la proteína y no por otros factores. Los títulos de anticuerpos de suero obtenidos mediante cebado proteína de fusión son mucho mayores que los obtenidos en presencia de los adyuvantes fuertes, tales como el adyuvante de Freund (Wang et al., 1995). Sin embargo, a diferencia de la vacunación con ADN-CS o proteína por sí sola, la vacunación con los resultados de proteína CS-14K de fusión en la inducción concomitante de altos niveles de anticuerpos IgG1 e IgG2a y esto podría ser beneficioso en la protección contra la malaria. Aumento de los anticuerpos contra la proteína CS citofílicos, como IgG1, en las personas mayores en las zonas endémicas de malaria se asocia con un menor riesgo de infección por malaria (John et al., 2003). Por lo tanto con la constante exposición hay un aumento en la proteína CS anticuerpos IgG1. Una posible explicación para la consecución de mayor título de anticuerpos IgG1 con proteína quimérica podría atribuirse a su estructura. La naturaleza oligomérica de la proteína podría activar la formación de centro germinal para una mejor producción de anticuerpos en comparación con su contrapartida monomérica. Este tipo de diferencias con estructura antigénica y la inducción de centros germinales se informó recientemente por muchos estudios en el campo de vacuna contra la malaria (Luna et al, 2012;.. Wipasa et al, 2010). La proteína quimérica demostró ser superior en la inducción de anticuerpos de alta avidez contra CS comparables con los obtenidos mediante el uso de nanopartículas basadas CS vacunación péptido (Kaba et al., 2009). Avidez de un anticuerpo también aumenta con el tiempo. Este es generalmente el resultado de mutaciones somáticas en el centro germinal y la competencia con los clones de células B. Anticuerpos de alta avidez producidos por la proteína quimérica podría ser debido a el patrón único de plegado alcanzado por CS-14K proteína resultante de la exposición de otros epítopos potentes. Además, hay una posibilidad de que la proteína quimérica para inducir CS específicos foliculares células T auxiliares (TFH) que no sólo mejora la avidez, sino también el título de anticuerpos (Moon et al., 2012). Estos datos tienen implicaciones importantes en el diseño de antígenos para inducir respuestas de anticuerpos contra la malaria.

CD8 + respuestas de células T contra la proteína CS se conocen por ser un factor importante en el desarrollo de la inmunidad estéril usando esporozoitos irradiados (Overstreet et al., 2008). CD8 + células T durante las etapas del hígado son predominantemente dirigido contra la proteína CS (Schmidt et al., 2010). La capacidad de régimen CS14K para producir niveles significativamente elevados de IFN-γ y TNF α-única, así como secreción de doble positivo CD8 + células T, incluso después de 53 días lo convierte en un candidato de vacuna adecuado. La formación del complejo antígeno-adyuvante promoción de respuestas de CD8 en comparación con una mezcla de adyuvante antígeno refuerza nuestras conclusiones (Olvera-Gómez et al., 2012). También el reportar un cambio en la polifuncionalidad de células T CD8 secretora de todas las tres citoquinas producidas por la proteína quimérica que pueden ser eficaces en el control del crecimiento de parásito. Además, la capacidad de régimen de vacuna para inducir CS14K con gran cantidad de TNF-α tiene una ventaja añadida ya que se sabe que es una citocina importante para el mantenimiento de la memoria de las células T CD8 en malaria (Butler et al., 2010). Los datos de los estudios MFI muestra que la población positiva doble o triple son mejores niveles en la producción de citoquinas, ambos de los cuales son elevados por cebado CS14K. Los bajos niveles de respuestas de CD8 anti-DNA-CS sugieren que la capacidad protectora de este régimen puede estar basado en los anticuerpos inducidos. El mantenimiento de tales respuestas de memoria duraderos y polifuncional, incluso después de 4 meses de la vacunación tomado junto con la protección estéril, justifica la potente naturaleza de la proteína de fusión A27 y su uso para el desarrollo de mejores vacunas contra la malaria. Dado que una activación temprana de la respuesta inmune innata impide el desarrollo pre-eritrocítico del parásito, la activación de interferón de tipo I a través de señalización por la proteína de fusión, valida aún más su uso como un agente de inducción de respuesta innata. De hecho, la activación de interferón de tipo I por la proteína quimérica sí resultó en niveles elevados de citocinas pertenecientes a esta familia, tales como IL-6, TNF-α e IL 12-. Se cree que la acción parasiticida de TNF-α en los hepatocitos está mediada por la síntesis de IL-6 (Nüssler et al., 1991). Además, la participación TLR es crítica en el desarrollo de la inmunidad contra la malaria que podría ser explicado por la pobre inmunogenicidad de RTS, S sin un adyuvante fuerte como AS01E, un agonista de TLR-4 (Mettens et al., 2008). Un estudio reciente también

muestra la importancia de la señalización TLR para el desarrollo eficaz de anticuerpos protectores a Plasmodium (Wiley et al., 2011). Esto está de acuerdo con nuestro estudio, que muestra cómo la mejora en STAT IRF-1 y 3-por CS14K sugiere un papel en la activación de TLR-4 (Figura 17), lo que resulta en la inmunogenicidad mejorada y la inhibición de la fase de desarrollo del parásito hepático (Imada y Leonard, 2000; Spath et al, 2009). Se sabe cómo los parásitos, tales como Leishmania y Plasmodium regulan a la baja la síntesis de NO (Liew, 1994). Nuestro trabajo demuestra la capacidad inherente de la proteína quimérica para mejorar la producción de NO tanto in vitro como in vivo. Dado que ni el CS ni proteína 14K por sí sola podrían inducir NO, parece razonable concluir que la agregación de proteínas CS14K produce NO como molécula responsable de la inducción. Los niveles persistentemente elevados de NO a 53 días después de la vacunación con la proteína quimérica CS14K en comparación con el cebado con ADN o proteína-CS sola, sugieren que las diferencias existen en el procesamiento de estos antígenos por las APC. Además, la producción temprana de NO se ha propuesto que se requiere para la proliferación de células T $CD8^{+}$ contra el parásito (Scheller et al., 1997). En conclusión, la fusión de 14K a CS conduce a un medio inmunogénico mucho más favorable para potenciar la eficacia de la vacuna CS-14K.

7.4 CONCLUSIONES.

Del studio se concluye lo siguiente:

- La proteína 14K de vaccinia puede formar oligómeros/agregados cuando se fusiona a un antígeno.
- Los oligómeros / agregados son de alto peso molecular según se evidencia en ensayos de gradiente de sacarosa y estudios analíticos de ultracentrifugación.
- La proteína CS-14K de alta pureza puede ser fácilmente purificada de E. coli empleando una etiqueta de GST con niveles bajos de contaminación por LPS.

- Estudios comparativos empleando protocolos de inmunización homólogos “prime/boost” con proteína in vivo mostraron que CS-14K es más eficaz en la reducción de la carga parasitaria en el hígado en comparación con CS cuando son desafiados con una dosis alta de esporozoitos.
- Un ``prime´´ con proteína CS-14K seguido de un ``boost´´ con MVA-CS mejora la protección en animales, después de un desafio con *Plasmodium yoelii*, con una eliminación total de los parásitos en el hígado.
- La protección completa sólo se logró en los animales vacunados con CS-14K / MVA-CS.
- Se observó, mediante el empleo de ELISPOT-IFN-γ, un aumento del número de células T $CD8^+$ que secretan IFN-γ en ratones que recibieron el prime con proteína CS-14K. El número de células secretoras de IFN-γ en el grupo de los animales vacunados con CS-14K se mantuvo incluso durante la fase de memoria, sin presentar ninguna disminución.
- Mejora en los niveles células T $CD8^+$ polifuncionales secretoras de citoquinas en los animales que recibieron CS-14K, tanto durante la fase la respuesta adaptativa como la de memoria.
- La proteína quimérica era capaz de inducir IL-12, TNF-α y NO en los macrófagos a través de IRF-3 y la activación de STAT-1.

De los datos se observó que la fusión de la proteína 14K a un antígeno puede mejorar su inmunogenicidad. Nuestro hallazgo proporciona la plataforma para el desarrollo de 14K como un adyuvante eficaz. Además se observó cómo una proteína de fusión basada en el antígeno CS , afecta a su inmunogenicidad. De la misma forma se evaluaron las correlaciones de protección de la malaria. Además, la estabilidad de la proteína de fusión a temperatura ambiente y la facilidad con la que pueden ser producidas y administradas, hace de éste un mejor adyuvante para el desarrollo de vacunas eficaces. La señalización de CS-14K a través de la vía TLR-4 puede arrojar luz en la producción de mejores vacunas contra la malaria. En conjunto, esta tesis ofrece una amplia evidencia para el desarrollo de vacunas basado en modificaciones estructurales de los

antígenos mediante la molécula A27 de virus vaccinia y formación de oligómeros/aggregados proteicos como inmunògenos.

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APPENDIX

Adjuvant-like Effect of Vaccinia Virus 14K Protein: A Case Study with Malaria Vaccine Based on the Circumsporozoite Protein

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Development of subunit vaccines for malaria that elicit a strong, long-term memory response is an intensive area of research, with the focus on improving the immunogenicity of a circumsporozoite (CS) protein-based vaccine. In this study, we found that a chimeric protein, formed by fusing vaccinia virus protein 14K (A27) to the CS of *Plasmodium yoelii,* induces strong effector memory CD8⁺ T cell responses in addition to high-affinity Abs when used as a priming agent in the absence of any adjuvant, followed by an attenuated vaccinia virus boost expressing CS in murine models. Moreover, priming with the chimeric protein improved the magnitude and polyfunctionality of cytokine-secreting CD8⁺ T cells. This fusion protein formed oligomers/aggregates that led to activation of STAT-1 and IFN regulatory factor-3 in human macrophages, indicating a type I IFN response, resulting in NO, IL-12, and IL-6 induction. Furthermore, this vaccination regimen inhibited the liver stage development of the parasite, resulting in sterile protection. In summary, we propose a novel approach in designing CS based pre-erythrocytic vaccines against *Plasmodium* using the adjuvant-like effect of the immunogenic vaccinia virus protein 14K. *The Journal of Immunology*, **2012, 188: 6407–6417.**

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Malaria continues to present a major public health challenge and burden on economic development in many countries. *Plasmodium falciparum*, the main causative agent of malaria in humans, is known to cause ~225 million cases and ~781,000 deaths annually (1). Development of a cost-effective vaccine continues to be a daunting task because of the confounding ability of the parasite to manipulate the host immune system. The most advanced malaria vaccines have focused on the use of protein-in-adjuvant formulations, and a phase III clinical trial with RTS,S/AS01E (a liposome-based adjuvant system) is under way (2). First results of the phase II trial of RTS, S/AS01E in Africa revealed vaccine-reduced clinical episodes of malaria and severe malaria by about half during 12 mo after vaccination in children 5–7 mo age (3). The rationale behind the development of RTS,S is its ability to form virus-like particles, which enhances the immunogenicity of monomeric circumsporozoite (CS) protein. The ability of protein aggregates to stimulate strong immune responses compared with their monomeric counterparts has been studied for many decades and is supported by a number of recent studies (4–6).

CS protein is a monomeric protein of ~40–60 kDa covering the surface of infective sporozoites (7). However, the native CS from sporozoites is also known to form some aggregates that may facilitate the binding of CS to hepatocytes (8). The CS protein is GPI-anchored and consists of a central portion of immunodominant repeat regions of B and T cell epitopes (9). Reports of successful vaccination against malaria in humans have been attributed to the development of CS-specific humoral and cell-mediated immune responses (10). Therefore, the strong immunogenic nature of the CS protein and its presence during pre-erythrocytic and liver stages (11) make it a promising candidate for the development of a malaria vaccine.

Control of infection for most pathogens requires different strategies of intervention, including humoral or cell-mediated immune responses at different times in their life cycle. Therefore, an effective vaccination regimen should encompass these aspects to develop sterile immunity. Viral vaccine vectors expressing CS protein and other *Plasmodium* Ags as promising vaccine candidates have been previously described (12, 13). Many of these studies have focused on using DNA as priming agent, which met with success rates as high as 70–100% in mouse models, even though it was short-lived (14, 15). However, DNA-based vaccines have recently come under renewed scrutiny for a number safety factors such as integration of the DNA into host gene (16) and also by their induction of tolerance against the Ag (17). Another disadvantage is the reduced capacity of DNA vaccines to induce a strong Ab response. The quality of immune responses generated by DNA vaccines administered alone is comparatively weaker to the traditional vaccines such as subunit vaccines or attenuated organisms (18). Currently, most licensed vaccines are based on attenuated whole pathogens or subunit

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Abbreviations used in this article: CS, circumsporozoite; IRF, IFN regulatory factor; MFI, mean fluorescence intensity; MVA, modified vaccinia virus Ankara.

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particles. Therefore, as an alternative to DNA vectors, the use of proteins as priming agents is gaining acceptance due to their better efficacy and safety.

In this study, we describe the development of a novel CS-based chimeric protein, which when combined in a heterologus prime–boost vaccine regimen with an attenuated vaccinia virus vector-induced enhanced immune responses. Additionally, this vaccine regimen gave sterile protection in mice when challenged with sporozoites. Our chimeric CS protein (CS14K) makes use of the oligomerization domain of the A27L gene of vaccinia virus. Our previous studies helped in the understanding of the structural organization of the 14-kDa protein encoded by the A27L gene (19). Considering the drawbacks of using a DNA priming strategy and CS protein encoding its C-terminal GPI sequence, we sought to determine the possible differences in the quality and quantity of humoral and T cell responses induced by different vaccine constructs and define how this might influence protection.

We also show how the differences in the induction of $CD8^+$ T cells and Abs could influence the protective capacity of malaria vaccines. In agreement with previous studies with fusion protein (4, 20), we found that priming with CS14K resulted in the development of high avidity and balanced IgG Ab production. An important aspect of this work is the extensive analysis of quantity and quality of $CD8^+$ T cells during primary and memory stages. Comparing our vaccine regimen to a protective vaccination regimen based on DNA priming followed by poxvirus boost, we showed that priming with CS14K followed by a modified vaccinia virus Ankara (MVA)-CS boost triggered strong memory polyfunctional effector T cell responses, an important component for providing long-term protection (21).

These results demonstrate that by fusing the vaccinia virus 14K (A27 protein) to CS we achieved a significant improvement in the quality and quantity of the humoral and T cell responses against CS in addition to protection of mice against liver stage development of malaria parasites. This study demonstrates that fusing an immunogenic protein from vaccinia virus improved the overall immunogenicity of the *Plasmodium* CS Ag in the absence of adjuvants.

Materials and Methods

Cells and viruses

Construction of recombinant MVA virus expressing circumsporozoite protein of *Plasmodium yoelii* 17XNL strain (MVA-CS) has been describe previously (13). The virus was grown and titrated in primary chicken embryo fibroblastcells and in DF-1 cells (22). Murine J774 and human THP-1 macrophages used for the in vitro cell culture studies were maintained at appropriate conditions as specified by the American Type Culture Collection.

Plasmid construction

pCI-Neo-CS The PyCSP gene was amplified MVA-CS using the primers CS-XhoI-F (5′-ACTTACTCGAGATGTGTTACAATGAAGAAAATG-3′) and CS-NotI-R (5′-ATTGCGGCCGCTTTAAAATATACTTGAAC-3′) to yield a 972-bp fragment lacking the N-terminal signal sequence and C-terminal GPI sequence. The gene was inserted into a mammalian expression vector, pCI-Neo, that had been previously digested with XhoI and NotI followed by shrimp alkaline phosphatase treatment. The CS gene in both the virus and plasmid were sequenced (Secugen, Madrid, Spain). The plasmid was purified using a Qiagen Mega Prep kit according to the manufacturer's protocol. Expression of CS from pCI-Neo-CS was confirmed by transfecting DF-1 cells followed by Western blot analysis with CS specific Abs.

pGEX-CS/pGEX-CS14K. The CS gene from pCI-Neo-CS plasmid was amplified using the primers CS-EcoRI-F (5′-ACTTAGAATTCATGTGTTACAATGAAGAAAATG-3′), CS-NotI-R (5′-ATTGCGGCCGCTTTAAAATATACTTGAAC-3′) for pGEX-CS, and with primers CS-EcorI-F and CS-14K-NotI-R (5′-ATTGCGGCCGCTATTAAATATACTTGAAC-3′) for pGEX-CS-14K. The A27L open reading frame from vaccinia strain WR (accession no. YP_233032; www.ncbi.nlm.nih.gov/genbank/) was amplified with the primers A27L-NotI-F (5′-GCTGCTAGCGGCCGCGAGGCTAAACGCGAAG-3′) and A27L-XhoI-R (5′-CCCTCGAGTGGGTTACTCATATGGACG-3′) to generate a 276-bp fragment that lacks the first 28 aa from the original sequence. The chimeric gene fragment was generated by digesting with NotI followed by ligation. The fusion gene fragment was then inserted into pGEX-6p-1 plasmid to produce pGEX-CS14K plasmid.

Recombinant protein construct and protein isolation

The recombinant proteins were purified from *Escherichia coli* strain DH5-α. The starter culture was diluted 1:100 in fresh Luria-Bertani media and allowed to grow at 37°C until the OD_{600} reached 0.7, following which isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM. The culture was then incubated in an orbital shaker at 18°C and 200 rpm for 24 h. After the incubation, the cells were harvested and the pellet was suspended in extraction buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1 mM EDTA and protease inhibitor tablets; Roche). The cells were then lysed with lysozyme, added to a final concentration of 1 mg/ml, and incubated on ice for 20 min. Following lysozyme treatment, 1% Sarkosyl detergent and 1% Triton X-100 was added and incubated at 37°C for 10 min. Clarified supernatant from the lysis was incubated with glutathione-Sepharose 4B beads at 4°C overnight. The beads were then washed using three washes with wash buffer I (extraction buffer with 0.5% Triton X-114) and three with wash buffer II (extraction buffer with 0.1% Triton X-114) followed by two washes with extraction buffer. The purified protein was then eluted with 20 mM reduced glutathione. The protein was desalted using an Amicon centrifugal concentrator and the protein concentration was determined using Bradford reagent. The GST tag from the protein was cleaved using PreScission protease (GE Healthcare) according to the manufacturer's protocol. The proteins were tested for LPS contamination using a chromogenic *Limulus* amebocyte lysate kit (QCL-1000; Lonza), which was maintained at <2 endotoxin units per microgram of protein.

Neutralization assay

Sera from immunized rabbit were inactivated at 56°C for 30 min, following which 2-fold serial dilutions of the serum were made and incubated with 200 PFU MVA at 37°C for 1 h. Afterwards, confluent DF-1 cells were infected in triplicate and were visualized by immunostaining with anti-WR serum after 48 h. As a control, nonimmune serum from nonimmunized animals was used. The number of plaques obtained from each serum dilution was normalized to this control value.

Nitrite measurement

NO synthesis was measured by estimating the levels of nitrite present in the supernatant. Briefly, J774 cells were stimulated with proteins. For in vivo nitrite analysis, 10^6 splenocytes from vaccinated animals, sacrificed 53 d after boost, were treated with 5 μg/ml CS protein. Nitrite accumulation was measured by treating 50 μl supernatant with 50 μl Griess reagent I (1% sulfanilamide solution in 2.4 N HCl) for 10 min in dark followed by the addition of 50 μl Griess reagent II (0.1% naphthylethlyenediamine in 2.4 N HCl) for 10 min. The assay was read by a spectrophotometer at 540 nm.

RNA extraction and RT-PCR

Total RNA was extracted from cells treated with respective proteins for 24 h, using a RNeasy Mini kit according to the manufacturer's instructions. Analysis of RNA was carried out using RT-PCR as described in the kit for reverse transcriptase (Invitrogen). Briefly, 1 μg RNA was reverse transcribed into cDNA using oligo(dT) primers (Invitrogen). For relative quantitative PCR, 2 μl cDNA was used as a template with primers specific for inducible NO synthase, IL-12p40, and GAPDH (23). All the experiments were done in triplicates and the bands from gel electrophoresis were quantified using Adobe Photoshop CS4.

Confocal microscopy

Immunostaining was carried out as described previously (24). Briefly, after fixation (30 min; 4% formaldehyde in PBS; 37°C), permeabilization in 0.1% Triton X-100 (Sigma-Aldrich), and blocking with 10% FCS in PBS, cells were incubated with primary Ab (anti-CS Ab, 1:500; C3-anti-14K, 1:400; NF-κBp65, 1:500) along with DNA staining dye, DAPI (1:200), for 1 h at room temperature. Following extensive washing with PBS secondary Abs (Alexa 546 goat anti-mouse and Alexa 488 goat anti-rabbit, 1:500) were applied for 1 h at room temperature. The slides were washed three

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times with PBS and mounted in ProLong antifade medium and analyzed with a Bio-Rad Radiance 2100 confocal laser microscope.

Animals and immunizations

All animal procedures were approved by the Ethical Committee of Animal Experimentation of Centro Nacional de Biotecnologia (Consejo Superior de Investigaciones Cientificas). Female BALB/C mice (H-2^d), 6–8 wk old, were obtained from Harlan U.K. A standard immunization protocol based on a heterologus prime–boost approach designed in the laboratory was followed (25). In short, animals were primed with DNA (100 μg; DNA-CS or empty DNA-ϕ) or protein (20 μg; CS or CS-14K) via intradermal route and were boosted after 2 wk with 2×10^7 PFU respective sucrose-purified viruses (MVA or MVA-CS) through i.p. injection. All the preparations were made in endotoxin-free PBS.

P. yoelii *sporozoite challenge study*

Challenge experiments were performed as previously described (26). Briefly, sporozoites were obtained from the salivary glands of *Anopheles stephensi* mosquitoes. Two weeks after boost, mice were challenged with 2×10^4 sporozoites via i.v. route through the tail vein. After 42 h, animals were sacrificed and levels of *P. yoelii* 18S rRNA levels were assessed by quantitative RT-PCR. To determine sterile protection 2 wk after immunization, mice were challenged i.v. with 300 *P. yoelii* sporozoites. Parasitemia was monitored by performing daily blood smears from days 3 to 21.

ELISA and Ab avidity

Abs present in the serum of immunized animals were determined using ELISA as previously described (25). Purified CS protein was coated to the 96-well Nunc MaxiSorp plates at a concentration of 2 μg/ml in coating buffer ($NaHCO_3/Na_2CO_3$) at 4°C overnight. Bound Abs were detected using 1:2000 dilution of alkaline phosphate-conjugated goat anti-mouse Ab total IgG or IgG1 or IgG2a (SouthernBiotech, Birmingham, AL). Plates were developed by adding TMB substrate (Sigma-Aldrich) and stopping the reaction with 1 M H_2SO_4. OD was read at 450 nm. Endpoint titer values were determined as the last positive dilution of serum giving an absorbance value 3-fold higher than naive serum. For analyzing the avidity of Abs, an initial serum dilution that gave an absorbance of 2.7 ELISA units was selected. Following incubation, with serum, the Ag/Ab interaction was disrupted using a range of dilutions from 0 to 5 M urea (Invitrogen) in Tris-HCl (pH 8.0) for 15 min before the addition of secondary Ab. EC_{50} was then calculated by linear regression ($R^2 = 0.99$) between 1 and 5 M urea concentration (log of 50% reduction, 1.699).

IFN-γ ELISPOT analysis

Fresh IFN-γ ELISPOT analysis was carried out as previously described (27).

Multiparameter flow cytometry

Flow cytometry and intracellular cytokine staining analysis were performed to detect the different phenotype of lymphocytes secreting cytokines, as reported previously (25). Briefly, 4×10^6 splenocytes were stimulated with 1 μg/ml $CD8^+$ peptide, $PyCS_{280–288}$ (SYVPSAEQI), with GolgiPlug (BD Biosciences) for 6 h in a 96-well plate. The cells were then washed and Fc receptors were blocked using anti-CD16/CD32, following which the cells were stained with surface-specific mouse Abs, namely CD4-Alexa 700, CD3-FITC, and CD8-PerCP for adaptive response studies, or CD8-FITC, CD44-PeCy5, and CD62L-PE for memory studies. Cells were permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) and were stained for intracellular cytokines IFN-γ/allophycocyanin, IL-2/PE, and TNF-α/PeCy7 for adaptive response or with IFN-γ/PeCy7, IL-2/allophycocyanin, and TNF-α/PE for memory response. A million cells were then passed through an LSRII flow cytometer (BD Biosciences) and the data were analyzed with FlowJo (Tree Star) and Spice (version 5.0). Appropriate controls were used and the values from unstimulated samples were subtracted.

Statistical Analysis

Statistical analysis was performed using Minitab for Windows. For ELISA, to determine the differences between groups we performed a linear regression of the logarithmic absorbance versus the logarithmic dilution, removing those samples clearly not following a linear trend ($R^2 > 0.98$). We then computed the logarithmic dilution at which this regression line was crossing the threshold given by twice the absorbance of the control values for that mouse. Doing this for the four mice we have four estimates of the logarithmic dilution beyond which the absorbance is smaller than twice the absorbance given by the control. We will refer to this value as the critical logarithmic dilution. Finally, we compared with a Student *t* test the hypothesis that the critical logarithmic dilution between the groups. For intracellular cytokine staining and ELISPOT, statistical analysis was done based on a previously described method (25). Briefly, we developed a novel method for analysis by correcting the control values (RPMI 1640) for determining standard deviations and *p* values for the samples.

Results

14K fusion aids CS protein aggregation

To investigate the effects of 14K conjugation with CS, we determined the structural organization of CS protein and its reactivity against Abs generated from *P. yoelii* sporozoites. With the aim of improving the immune response against CS, we removed the GPI motif (28) and signal sequence in the CS and fused it with the 14K protein after deleting the first 28 aa from A27, which is responsible for producing neutralizing Abs against vaccinia virus (Supplemental Fig. 1A). Abs generated against CS14K did not neutralize the infectivity of MVA as seen by neutralization assay (30–36 spots/well) (Supplemental Fig. 1B). Using SDS-PAGE analysis under reducing and nonreducing conditions, we evaluated the oligomerization/aggregation status of recombinant proteins and their reactivity with a CS-specific mAb obtained after immunization with sporozoites (Fig. 1A, 1B). Under reducing conditions CS14K has a molecular mass of ~60 kDa, compared with ~50 kDa for CS, whereas under nonreducing conditions CS14K has a size apparently >250 kDa, in contrast to 50 kDa for CS. Additional experiments by analytical ultracentrifugation revealed that CS14K protein has a main sedimenting species with a standard *s* value of 4.3 ± 0.1, which is compatible with a moderately elongated protein dimer (calculated frictional ratio *f/f0* = 1.8). The CS14K has high tendency to form oligomers/aggregates (data not shown). To determine the kinetics of CS expression by the vector MVA-CS, we performed time-course analysis of cells infected with the MVA recombinant expressing CS (Fig. 1C). We observed that after infection with MVA-CS, CS was detectable 2 h postinfection, and these expression levels remained elevated up to 6 h postinfection. Next, to determine whether CS expressed by MVA undergoes posttranslational modifications, we incubated infected cells in the presence of tunicamycin and observed that most of the CS expressed in the virus-infected cells was glycosylated, as indicated by the reduced intensity of the protein bands (Fig. 1D). We also investigated whether there was any difference in the localization of proteins in macrophages expressed by virus or upon transient transfection. To study the subcellular localization of full-length CS during infection with MVA-CS, J774 cells were infected at a multiplicity of infection of 5 PFU/cell for 18 h. We observed that MVA-CS expresses CS as punctuated spots with complete cytoplasmic spread (Supplemental Fig. 1C). In contrast, when macrophages were transfected with DNA encoding CS and CS14K, we observed that CS and CS14K were strongly localized with the nuclear envelope of the cell.

Taken together, these data show that the 14K (A27L) protein of vaccinia virus when fused to CS protein of *Plasmodium* is able to form oligomers/aggregates displaying an apparent molecular mass of >250 kDa compared with its monomeric CS counterpart of 50 kDa. A moderately elongated protein forming oligomers/aggregates was observed when CS14K protein was run under nonreducing conditions by analytical ultracentrifugation. The lower band that we see in the immunoblot under reducing condition is the cleaved CS14K protein. We therefore sought to investigate

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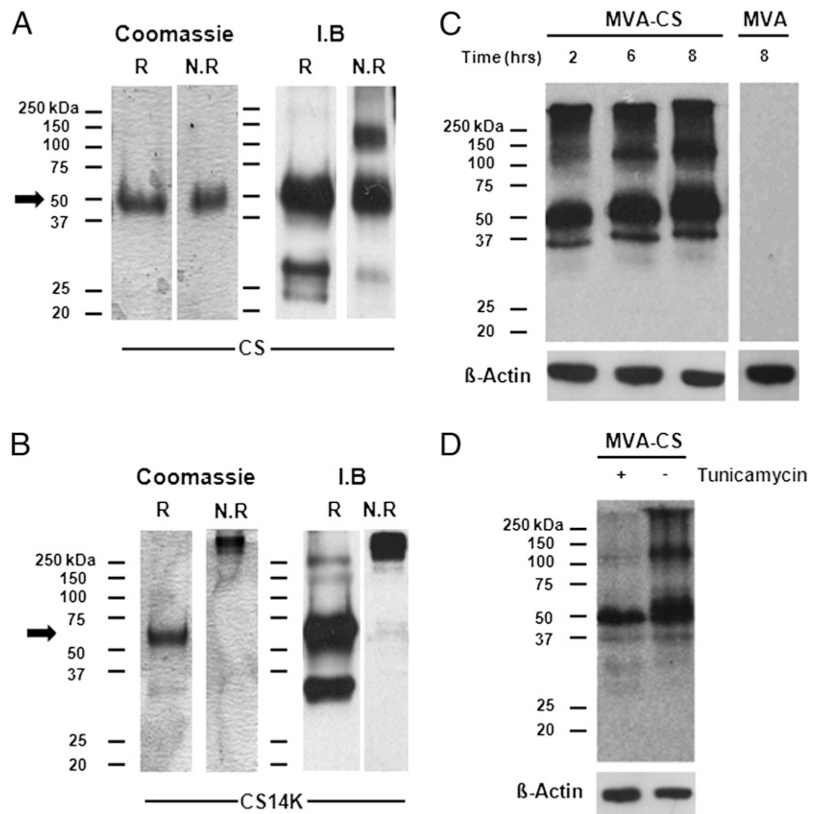

FIGURE 1. Biophysical and biochemical properties of recombinant proteins: Coomassie blue-stained SDS page and immunoblot with mAb against CS (NYS1; 1:1000 dilution) of 1 μg recombinant (**A**) CS protein or (**B**) CS14K protein, under reduced (R) and nonreduced (NR) conditions. Western blots of DF-1 cells infected with MVA-CS virus at 1 MOI for (**C**) 2, 6, and 8 h or for (**D**) 16 h in the presence and absence of 10 μg/ml tunicamycin and probed with NYS1 Ab.

a possible biological effect on the immunogenicity of CS protein when fused with A27.

CS14K protein modulates innate immune responses in macrophages

Fusion of Ags to immunomodulatory fragments such as TLRs (5) or to complement proteins (29), which is aimed at improving immunogenicity by aggregation of proteins or by exploiting the innate immune signaling of macrophages, is an intense area of research. Because fusion of 14K protein to CS facilitates its aggregation, we sought to investigate whether this could also modulate the innate immune responses in macrophages.

Ability of macrophages to induce NO to inhibit the growth of parasite has been well documented (30); therefore, we sought to evaluate the ability of the fusion protein to induce NO production. When comparing the NO levels in supernatant of J774 cells treated with fusion protein or normal protein, it was apparent that only CS14K resulted in NO production (Fig. 2A). However, when the cells were stimulated with proteins along with recombinant IFN-γ, elevated levels of NO were found in all samples, regardless of the presence of additional proteins (data not shown). Further analysis revealed a significant increase in NO ($p = 0.044$) and IL-12p40 ($p = 0.05$) mRNA levels in CS14K-treated macrophages compared with CS protein treatment (Fig. 2B). We next investigated the effects of proteins on IL-6 and TNF-α secretion, given their possible role in controlling pre-erythrocytic stages of malaria. Chimeric protein was able to induce a significant increase in IL-6 in macrophages (Fig. 2C). Although not statistically significant, we also observed increased TNF-α production at 24 h with CS14K treatment.

Given the significance of TLR activation and the induction of type I IFN, experiments focused on the analysis of the downstream molecules involved in IFN signaling such as IFN regulatory factor (IRF)-3 and STAT-1 were performed. To determine whether there are differences between CS and CS14K, human macrophage THP-1 cells were stimulated and harvested at different time intervals and probed with phospho-Abs against STAT-1 and IRF-3 (Fig. 2D). It is notable that CS14K was particularly effective in activating STAT-1 and IRF-3. To ensure that the activation of STAT-1 and IRF-3 is not influenced by any dsRNA contamination, the cells were stimulated with proteins previously digested with RNAse III (Supplemental Fig. 2).

NF-κB, an important transcription factor responsible for activating several genes involved in innate immune signaling in macrophages, has been shown to be blocked by CS protein (11). Therefore, we analyzed whether CS14K could overcome this constraint. Although both CS and CS14K proteins led to early degradation of IkB (Fig. 2E), the movement of p65 into the nucleus was inhibited (Fig. 2F).

In summary, these data establish that the fusion of A27 protein to CS could alter the innate immune responses in macrophages by STAT-1 and IRF-3 activation without the involvement of NF-κB, which possibly resulted in increased levels of NO, IL-6, and IL-12 production, suggesting signaling via the TLR4 pathway. CS14K was also able to activate higher levels of CD54, a marker for macrophage activation (data not shown). We therefore sought to investigate whether such a response could alter the immunogenicity of the protein in vivo.

CS14K fusion protein priming arms the humoral responses in vivo

It is well established that a strong Ab response against CS correlates with higher protection in many animal and human models (31, 32). The magnitude, isotype, and systemic availability of the Ab produced are important parameters that govern the control of parasites and help to resolve disease (33, 34).

Two weeks after the final phase of the prime–boost vaccination protocol described in *Materials and Methods*, quantitative analysis

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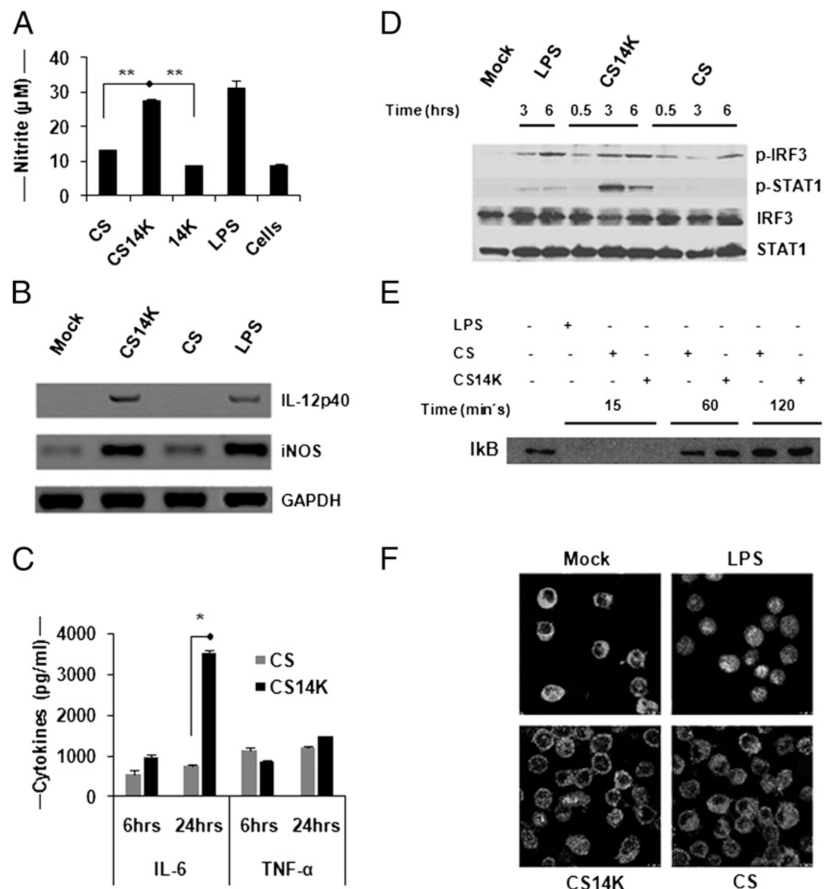

FIGURE 2. Modulation in innate immune sensing of CS14K by macrophages: fusion protein can activate innate sensing in macrophages. THP-1 or J774 macrophage cells were stimulated with 5 μg/ml proteins or as positive control with 1 μg/ml LPS. (**A**) Induction of NO by proteins in macrophages. Nitrite accumulation in the supernatant was determined after 48 h protein treatment in J774. (**B**) Semiquantitative RT-PCR analysis of NO and IL-12p40 expression. RNA was extracted and the corresponding cDNA was obtained as mentioned in *Materials and Methods*. Proportionate volume of amplified DNA was run on 8% agarose gel. (**C**) Cytokine analysis by Luminex assay. Supernatants were collected after 6 and 24 h treatment. Cytokine levels in the supernatant were measured by Luminex. (**D**) CS14K induces IRF-3 and STAT-1 phosphorylation: 2×10^6 THP-1 cells were stimulated with proteins, following which the samples were collected at different time intervals and probed with phospho-Abs against IRF-3 and STAT-1. Normalization was carried out using total STAT-1 and IRF-3 Abs (1:1000). (**E** and **F**) Proteins inhibit NF-κB activation: proteins block the movement of p65 (mouse anti-p65 NF-κB Ab p65, 1:500) into the nucleus in J774 macrophages inspite of IkB degradation. Images were acquired at ×63 objective magnification. Data are expressed as mean ± s.e. of triplicate observation and are representative of two independent experiments. $*p < 0.05$, $**p < 0.005$.

of the Abs produced showed that priming with CS14K chimeric protein followed by MVA-CS induced significantly higher titers of Abs than mice primed with DNA-CS or CS protein alone (Fig. 3A). The Ab titers of CS14K-primed group was 2-fold greater than the CS-primed group ($p = 0.008$) and 8-fold more than in the DNA-CS–primed group ($p = 0.002$). Even 53 d after boost, the Ab levels in all of the immunized groups were maintained at similar levels except for DNA-CS, which showed a reduction by 1 log (data not shown).

Further analysis of IgG isotype switching showed that fusion protein induced a higher IgG1 response whereas the DNA-vaccinated group showed a predominance of IgG2a response. The levels of IgG1 ($p = 0.012$) and IgG2a ($p = 0.030$) induced by the fusion protein-primed group were almost double those of the CS-primed group (Fig. 3B). Finally, we sought to determine whether the vaccination with chimeric protein affected the avidity of the CS-specific Abs. The Abs from mice primed with CS14K showed higher avidity (EC_{50} = 4.2 M urea) whereas those primed with DNA-CS (EC_{50} = 2.3 M urea) or CS (EC_{50} = 2.5 M urea) protein had lower affinity (Fig. 3C). Taken together, these data indicate that priming with proteins, as compared with DNA alone, enhances levels of Abs against CS. Additionally, priming with CS14K not only increases the overall production of Abs but appears to induce a more balanced production of high-affinity IgG1 and IgG2a Abs.

Having established that CS14K can mediate the induction of NO in cultured macrophages, we sought to assess the ability of splenocytes from vaccinated mice to respond to purified CS protein by evaluating NO production. Consequently, splenocytes harvested from animals receiving a different prime–boost vaccine regimen 53 d after boost were stimulated with 5 μg/ml purified CS protein for 48 h. Mice that were primed with DNA-CS showed no detectable NO. Alternatively, mice that received proteins as priming agents were able to induce NO production (Fig. 3D). Whereas CS protein was able to increase NO by low levels, CS14K increased NO production by 4-fold ($p = 0.001$). These data indicate a reduced capability of CS-based vaccination to induce NO compared with CS14K. The unique nature of CS-based malaria vaccines to induce NO has not been reported before. Thus, the fusion of oligomerization domain of 14K protein to CS improves the humoral arm of immune responses against the Ag along with increased production of NO.

CS14K generates durable and polyfunctional CS-specific $CD8^+$ T cells in mice

Given the critical role of 14K fusion in aggregating CS protein and the enhancement of innate immune responses, we examined the influence of these factors may have on the development and maintenance of $CD8^+$ T cells in vivo.

After mice were immunized, they were sacrificed on day 14 to study the adaptive immune response and on day 53 for memory analysis. Efficacy of the vaccination regimen was analyzed using an IFN-γ–based ELISPOT assay and multiparameter flow cytometry. The fusion protein significantly improved the CS-specific IFN-γ–secreting cells ($p < 0.05$) compared with other vaccine regimes (Fig. 4A, 4B). We characterized the immune responses in terms of polyfunctionality, $CD8^+$ T cells specific for the peptide 280–288 of *P. yoelii* CS protein, secreting IFN-γ, TNF-α, IL-2, or any combination among these three cytokines. A clear dominance in $CD8^+$ T cell-secreting cytokines was seen in CS14K-primed group of animals over CS protein (2.2-fold)- or DNA-CS (4.3-fold)-primed groups (Fig. 4B) at day 14 after boost. Clearly, cells that secreted IL-2 were associated with triple cytokine-producing cells. Vaccine regimen involving DNA-CS priming resulted in single- or

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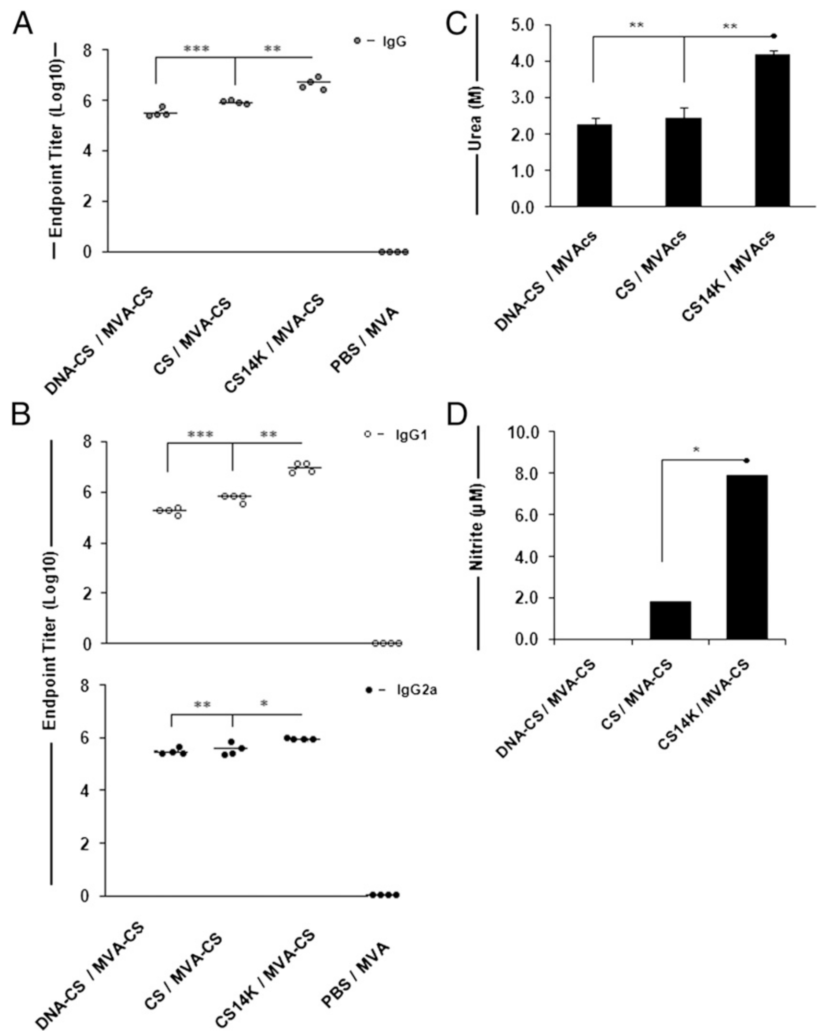

FIGURE 3. Chimeric protein generates elevated levels of high-avidity Abs in addition to NO. (**A**) Total IgG serum Ab responses against CS protein as measured by ELISA. (**B**) IgG isotypes induced by immunizations. Levels of IgG1 and IgG2a Abs generated by the vaccine regimes are shown. Control groups represent the animals receiving empty DNA or PBS for priming and MVA as boost. (**C**) Avidity indices of the Abs against CS protein. The avidity index was arbitrarily considered as the molarity of urea required to reduce the initial absorbance by 50% (i.e., $\log_{10}$ 50% = 1.69). (**D**) Splenocytes from vaccinated groups harvested after 53 d after boost were incubated with 5 µg/ml recombinant CS protein for a period of 48 h. Amount of NO in control groups was deducted from DNA-CS– and protein-primed groups. Data are expressed as means ± SEM of triplicate observation (n = 4 mice/group) and are representative of two independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

double-positive cells and did not induce any triple-positive population. In contrast, animals receiving protein prime were able to induce IFN-γ^+TNF-α^+IL-2^+–secreting cells with CS14K bringing an ~3.6-fold increase over CS. Additionally, CS14K significantly increased double-positive IFN-γ^+TNF-α^+ over both CS protein and DNA-CS priming.

To study memory responses, the $CD8^+$ T cells were classified into central memory ($CD44^{high}CD62L^{high}$), effector cells ($CD44^{high}CD62L^{low}$), terminally differentiated memory effector memory cells ($CD44^{low}CD62L^{low}$), and naive cells ($CD44^{low}$ $CD62L^{high}$) (35, 36). Upon analyzing memory responses on day 53 and also on day 120 (data not shown), we observed rapid proliferation of the peptide-specific effector CD8 T cell population. Interestingly, the frequency of cells secreting IFN-γ was maintained even during the memory stage by CS14K priming compared with other groups (Fig. 4C). In terms of polyfunctionality, priming with fusion protein had a significant increase in the triple-positive (IFN-γ^+TNF-α^+IL-2^+) and double-positive (IFN-γ^+TNF-α^+) population of cytokine-secreting cells over both DNA as well as CS protein (Fig. 4D). Most of the single-positive cells were dominant for TNF-α. A 3.5-fold increase in IFN-γ^+TNF-α^+IL-2^+–secreting cells by the CS14K-primed group over the CS protein-primed group and a nearly 14-fold increase over the DNA-CS–primed groups was seen. A clear hierarchy in CS peptide-specific total CD8 cells secreting cytokines was observed when priming was carried out with CS14K (Fig. 5A). Mice immunized with proteins showed significant increase in magnitude of cytokines released over DNA-CS priming. Indeed, priming with CS14K protein rather that CS protein led to a 2-fold increase in total IFN-γ ($p < 0.05$) and TNF-α ($p < 0.0005$) in addition to the 3-fold increase in IL-2–secreting cells ($p < 0.05$). Interestingly, all of the positive CD8 T cells produced by the vaccine regimen had a phenotype resembling either T effector cells or terminally differentiated effector memory cells. To extend the immune analysis, we evaluated the amount of IFN-γ or TNF-α secreted by the different populations based on the mean fluorescence intensity (MFI) calculation (Fig. 5B). We observed that, irrespective of priming agent, most of the IFN-γ was produced by IFN-γ^+TNF-α^+IL-2^+ or IFN-γ^+TNF-α^+ cells with a similar pattern for TNF-α. The lack of CM CD8 T cells could be explained by the fact that upon exposure to peptide stimulus most of the CM cells rapidly acquired effector memory characteristics. These data indicate that proteins are able to prime to a higher extent an effective long-term CD8 T cell response than does DNA. The enhanced CD8 T cell responses in mice immunized with CS14K rather that CS is due to the adjuvant-like effect of the unique A27 element.

CS14K fusion protein abrogates the liver stage development of parasites when challenged with sporozoites

Given that polyfunctional $CD8^+$ T cell responses in addition to high-avidity Abs are crucial in enhancing protection against parasite infection, and also that CS14K improved both adaptive and memory immune responses, compared with CS or DNA-CS

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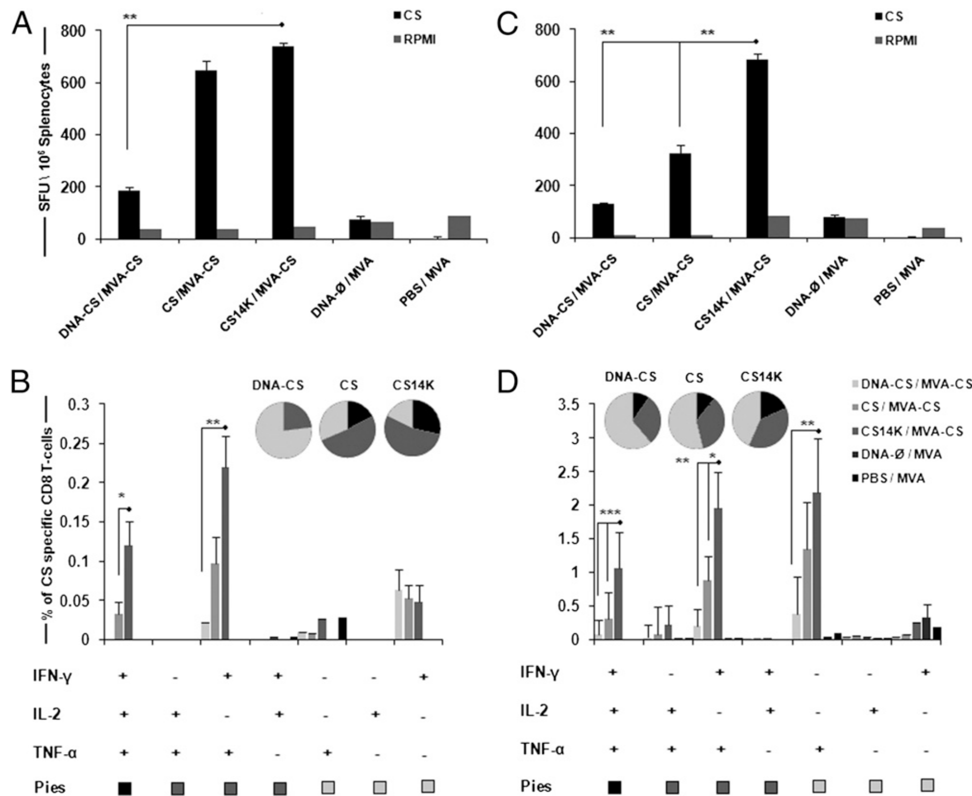

FIGURE 4. Enhanced immunogenicity of vaccine regimen based on chimeric protein. BALB/c mice were primed intradermally with DNA or proteins and boosted with i.p administration of 2×10^7 PFU MVA-CS after 2 wk. Splenocytes were stimulated with CS peptide SYVPSAEQI. The primary immune responses were analyzed on day 14 by (**A**) IFN-γ ELISPOT assay and (**B**) $CD8^+$ cells secreting IFN-γ, TNF-α, and IL-2 by polychromatic flow cytometry. Memory responses were analyzed on day 53 after boost (**C**) IFN-γ ELISPOT assay and (**D**) memory CD8 analysis using CD44 and CD62L memory markers. Pie charts represent the polyfunctionality of $CD8^+$ T cells secreting single, double, and triple cytokines. Data are expressed as means ± SEM of triplicate observations (n = 4 mice/group) and are representative of two independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

priming, we sought to investigate the efficacy of immunization against sporozoite challenge. Protection was evaluated by measuring levels of *P. yoelii* 18S rRNA in liver and also by determining parasitemia in sporozoite-challenged animals. To investigate the protective capacity of this subunit vaccine, groups of mice were immunized in various prime–boost protocols (Fig. 6). We show that a heterologus protein prime/vaccinia virus boost regimen was found to be more effective than a homologous protein prime/protein boost regimen, and a 32% reduction in the liver stage burden of parasites was observed in mice receiving CS14K (CS14K/CS14K) compared with CS (CS/CS) protein. Furthermore, a MVA-CS boost significantly lowered the parasite levels in the liver. Moreover, the CS14K protein priming followed by MVA-CS resulted in a near complete inhibition (~99.9%; $p < 0.005$) of parasite development in liver compared with CS protein and DNA-CS (Fig. 6).

To evaluate whether protection from liver-stage parasites induced by CS14K/MVA prime–boost can prevent the development of blood stages, mice were challenged with 300 live *P. yoelii* sporozoites and monitored until patency. Importantly, none of the vaccinated animals developed blood stages from days 3 to 21 of daily follow-up after challenge with sporozoites (Table I).

Thus, priming with CS14K protein in combination with a boost by MVA-CS is a highly effective vaccine regimen against murine malaria liver stage parasites. A proposed mechanism is shown in Fig. 7.

Discussion

In this study, we report a novel approach in designing effective protein vaccines based on the fusion of 14K (A27) protein of vaccinia virus with a model Ag, CS of *P. yoelii*, for developing an effective vaccination regimen based on protein prime/vaccinia virus boost against murine malaria. An obvious advantage of this vaccine strategy is the concomitant increase in both CS-specific humoral and $CD8^+$ T cells up to 4 mo. By comparing CS14K protein with other priming agents such as CS- and DNA-CS–based vaccines, we showed the correlation between the immune responses generated and protective efficacy generated in the murine malaria model.

In this study, we showed that the fusion of 14K protein with CS results in a protein with high tendency to form oligomers/aggregates of CS14K, which, in turn, enhanced the immune response profile leading to protection against murine malaria. Previous studies have demonstrated the differences in CS-specific T cell proliferation using recombinant soluble CS protein, viable sporozoites, or heat-killed sporozoites (37). This could explain the enhancement of polyfunctional T cells and Abs when animals are primed with chimeric protein rather than CS, which contains lower amounts of CS aggregates. An obvious advantage of this chimeric protein is the incorporation of near full-length CS protein containing both B and T cell epitopes whose presentation would be enhanced as a result of aggregation. Additionally, the use of viral vaccinia vectors for boosting could further enhance the development of long-term effector CS-specific T cells. In Fig. 6, we show how the vaccine regimen based on CS14K prime/MVA-CS boost inhibits the liver stage development of the parasite in mice when challenged with a high dose of sporozoites. When we calculated the C_T value of individual mice in each group we found that for the CS14K/MVA-CS group the values were well >35, the reference value in naive mice (indicated as dotted lines in Fig.

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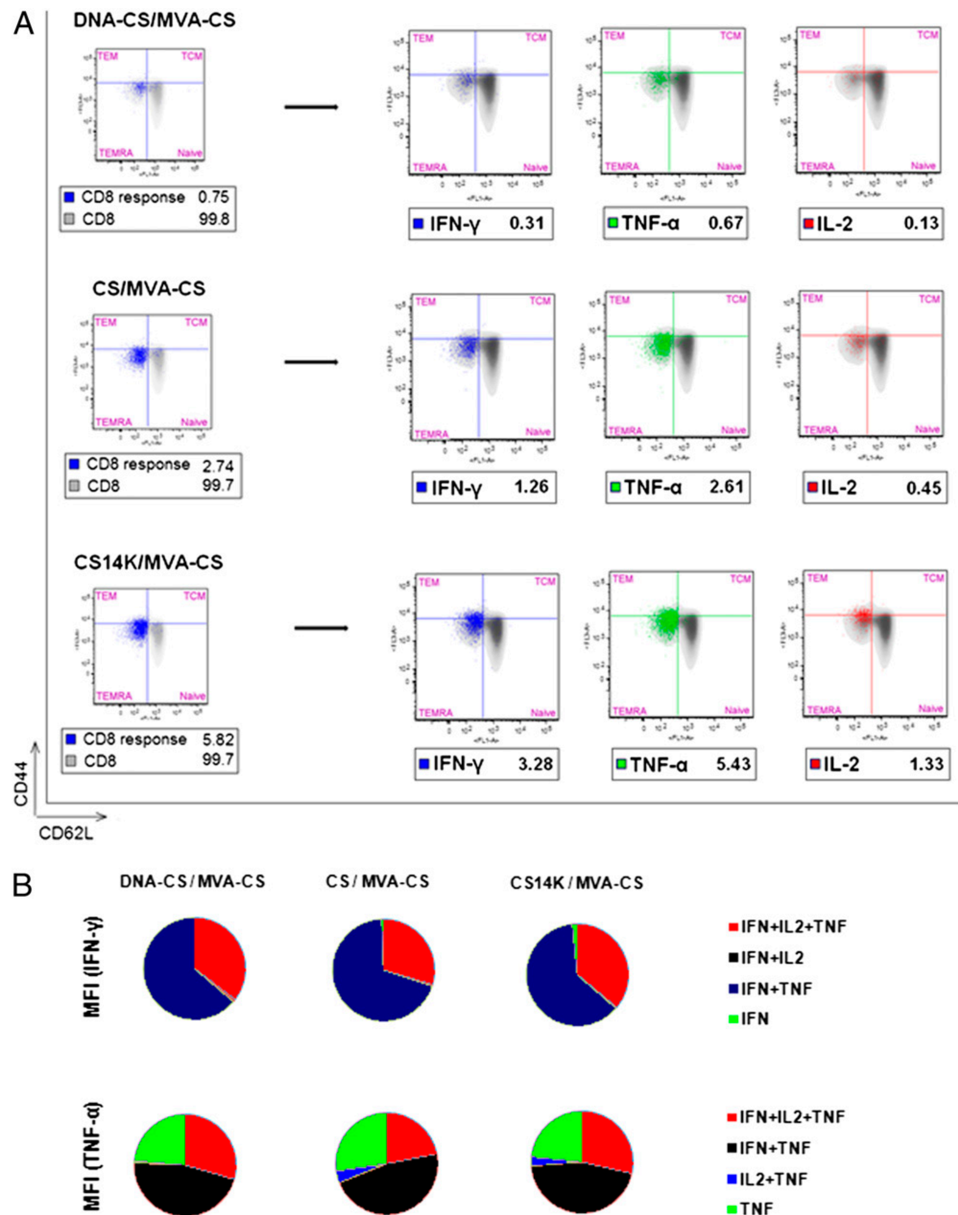

FIGURE 5. Polyfunctionality related to increased cytokine production by CS14K induced memory $CD8^+$ T cells: characterization of memory $CD8^+$ T cells. (**A**) Representation of total CS peptide specific CD8 cells were gated on CD44 and CD62L cell surface markers represented on *left-hand side* of the diagram. Total CD8 responses for each group are represented inside the box. Different populations of memory CD8 cells secreting different cytokines are represented on the *right-hand side* with their respective total responses as depicted insdie the box. (**B**) MFI of IFN-γ and TNF-α produced by different polyfunctional population of effector memory $CD8^+$ T cells (n = 4 mice/group).

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6). This suggests that the mice in this group did not have any parasite in the liver. Moreover, given that sterile protection is the target of pre-erythrocytic vaccines, we showed that CS14K/MVA-CS prime–boost prevented the development of blood stage parasites (Table I). The maintenance of both cellular and humoral responses even after 4 mo indicated the strong nature of the vaccine regimen used in this study.

Besides protein aggregation, another mechanism that could explain the enhanced immunogenicity of CS14K is its ability to activate type I IFN signaling. Given that an early activation of innate immune responses curbs the pre-erythrocytic development of parasites (38, 39), activation of type I IFN signaling by fusion protein further validates its use as a priming agent. Indeed, activation of type I IFN signaling by the chimeric protein did result in elevated levels of cytokines belonging to this family such as IL-6, TNF-α, and IL-12. It is thought that the parasiticidal action of TNF-α in hepatocytes is mediated through the synthesis of IL-6 (40). Furthermore, engaging TLRs is critical in developing antimalarial immunity, which could be explained by the poor immunogenicity of RTS,S without a strong adjuvant such as AS01E, a TLR4 agonist (41). A recent study also shows the importance of TLR signaling for effective development of protective Abs to *Plasmodium* (42). This is in agreement with our study, which shows how enhancement in STAT-1 and IRF-3 by CS14K suggested a role in TLR4 activation (Fig. 7), resulting in improved immunogenicity and inhibiting the liver stage development of the parasite (43, 44).

It is known how parasites such as *Leishmania* and *Plasmodium* downregulate synthesis of NO (45). Our study shows the inherent capacity of chimeric protein to enhance the production of NO both in vitro and in vivo. Because neither CS nor 14K protein alone could induce NO, it seems reasonable to conclude that the CS14K

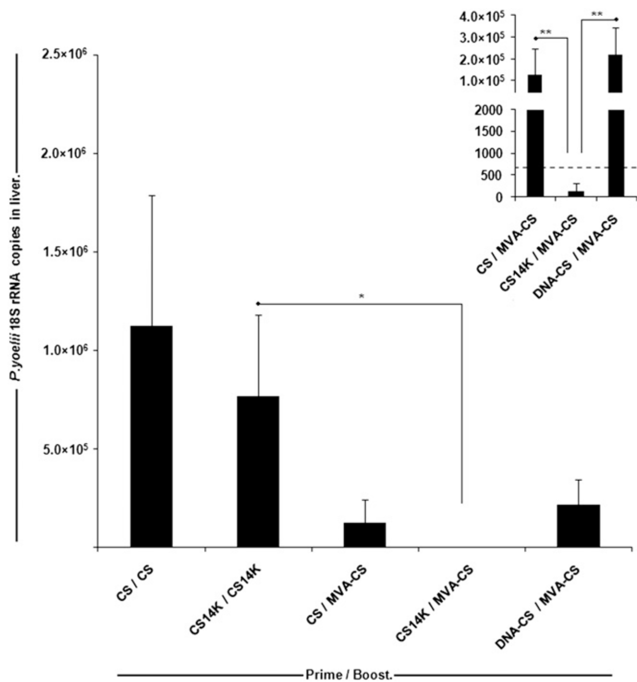

FIGURE 6. Strong inhibition of liver stage parasite development by CS14K. Mice were immunized as described in *Materials and Methods*. Two weeks after boost animals were challenged with 2×10^4 *P. yoelii* sporozoites through i.v. route via tail vein. After 42 h, the amounts of parasites in the liver were estimated by measuring the number of copies of 18S rRNA by RT-PCR. The dotted line represents the minimum detectable levels by the highly sensitive quantitative RT-PCR (26). Data are expressed as means ± SEM (n = 6 mice/group). $*p < 0.05$, $**p < 0.005$.

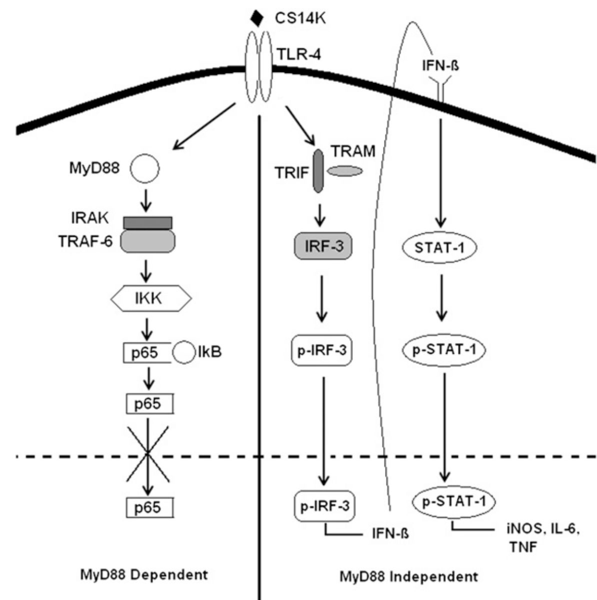

FIGURE 7. Proposed TLR signaling mechanism by CS14K: a hypothetical model of TLR4 signaling, pathway activated by fusion protein, in a MyD88-independent fashion. Inhibition of p65 movement to the nucleus by CS14K prevents the action of NF-κB while activation of IRF-3 and STAT-1 leads to elevated levels of NO, IL-6, and TNF-α, which could inhibit the growth of parasites.

protein aggregation is responsible for NO induction. The persistently elevated levels of NO at 53 d after vaccination with chimeric CS14K protein compared with priming with DNA-CS or protein alone suggest that differences exist in the processing of these Ags by APCs. Additionally, early production of NO has been proposed to be required for proliferation of $CD8^+$ T cells against the parasite (46). In conclusion, fusion of 14K to CS leads to a more favorable immunogenic milieu to enhance the efficacy of the vaccine.

Protective immunity against malaria associates with circulating IgG Abs against CS (47). Previous studies have also shown the ability of fusion proteins to induce strong humoral responses based on their ability to form large molecular mass aggregates (20). Similar to the CS14K priming approach presented in this study, the malaria vaccine in the latest stages of clinical trials, RTS,S, is also dependent on a higher magnitude of Abs belonging to the IgG1 subclass (48). Vaccine studies based only on DNA-CS vaccination lacking the GPI anchor improve protection up to 95% based only on Ab titers (49). Also, the serum Ab titers obtained by fusion protein priming are much higher than those obtained in presence of strong adjuvants such as Freund's adjuvant (50).

Table I. CS14K priming induces sterile protection

Priming[a]	Boosting[b]	Challenge[c]	Protected/Challenged (% Protected)	Prepatent Period
CS14K	MVA-CS	300 sporozoites	10/10 (100)	—
PBS	PBS	14 d after boost	0/10 (0)	4 d

[a]Protein (20 μg) administered intradermally.
[b]Virus (2×10^7 PFU) administered i.p.
[c]Challenge via i.v. route.

However, unlike vaccination with DNA-CS or protein alone, vaccination with the CS14K fusion protein results in the concomitant induction of high levels of both IgG1 and IgG2a Abs, and this could be beneficial in providing protection against malaria. The chimeric protein proved to be superior in inducing high-avidity Abs against CS comparable to those achieved by using nanoparticle-based CS peptide vaccination (51). High-avidity Abs produced by the chimeric protein could be due to the unique folding pattern attained by CS14K protein resulting in exposure of other potent epitopes. These data have important implications in the choice of Ags designed to induce Ab responses against malaria.

$CD8^+$ T cell responses against CS protein are known to be an important factor in the development of sterile immunity using irradiated sporozoites (52). A role of $CD8^+$T cells in subunit vaccine-induced protection against malaria was initially established in a heterologus prime–boost approach with flu and vaccinia virus vectors expressing CS (53).Vaccine regimes based on DNA as well as protein prime followed by vaccinia boost have shown to induce CS peptide-specific $CD8^+$ T cell responses (15, 54). The ability of the CS14K regimen to produce significantly elevated levels of IFN-γ and TNF-α single-positive– as well as double-positive–secreting CD8 T cells even after 53 d makes it a suitable vaccine candidate. We also report a shift in polyfunctionality of CD8 T cells secreting all three cytokines produced by chimeric protein, which may be effective in controlling the growth of parasites. Furthermore, the ability of CS14K vaccine regimen to induce a large amount of TNF-α has an added advantage because it is known to be an important cytokine for the maintenance of memory CD8 T cells in malaria (55). The concomitant increase in TNF-α by CS14K could also contribute to elevated NO and Ab responses (56, 57). The need for IL-12 in the development of effective adaptive responses is in agreement with our study, which shows the ability of CS14K protein to prime an enhanced T cell response (58). The data from MFI studies show that double- or triple-positive populations are better in producing elevated levels

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of cytokines, both of which are elevated by CS14K priming. The low levels of CD8 responses against DNA-CS suggest that the protective capability of this regimen may be based on the Abs induced. The maintenance of such durable and polyfunctional memory responses even after 4 mo of vaccination, taken together with sterile protection, justifies the potent nature of A27 protein fusion and its use for developing better malaria vaccines.

In recent years, many experimental CS-based vaccines have been developed using novel adjuvants or by modifying the structure of CS protein (Supplemental Fig. 3). The assessment of protective capacity of vaccine regimen gave a good platform to study the efficacy of chimeric protein generated by 14K fusion. Chimeric protein prime–boost alone could inhibit the liver stage development better than those attained by DNA-CS prime and MVA-CS boost under a high challenge dosage. This could be explained by the potent nature of the attenuated vaccinia viral boost (59).

Developing an effective pre-erythrocytic vaccine against malaria is difficult because it requires high levels of both humoral and cell-mediated immune responses. The role of CSP Abs in providing protection is a much debated topic. However, the latest trial of RTS,S vaccine does show that the protection could be mediated by CSP Abs (60, 61). In this study, we describe an optimal prime–boost approach using modified CS protein fused to the 14K protein of vaccinia virus as priming and MVA-CS as a boost. This unique approach is able to improve the magnitude and polyfunctionality of both humoral as well as cell-mediated immune responses, resulting in complete protection of vaccinated animals compared with other experimental CS based vaccines (62–65). Additional studies in primates could be performed using the chimeric protein in prime–boost protocols. These data establish a ground for the development of vaccines based on structural modifications of Ags using the immunogenic molecules from vaccinia virus.

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Disclosures

The authors have no financial conflicts of interest.

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Vaccine Efficacy against Malaria by the Combination of Porcine Parvovirus-Like Particles and Vaccinia Virus Vectors Expressing CS of Plasmodium

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Abstract

With the aim to develop an efficient and cost-effective approach to control malaria, we have generated porcine parvovirus-like particles (PPV-VLPs) carrying the CD8$^+$ T cell epitope (SYVPSAEQI) of the circumsporozoite (CS) protein from *Plasmodium yoelii* fused to the PPV VP2 capsid protein (PPV-PYCS), and tested in prime/boost protocols with poxvirus vectors for efficacy in a rodent malaria model. As a proof-of-concept, we have characterized the anti-CS CD8$^+$ T cell response elicited by these hybrid PPV-VLPs in BALB/c mice after immunizations with the protein PPV-PYCS administered alone or in combination with recombinant vaccinia virus (VACV) vectors from the Western Reserve (WR) and modified virus Ankara (MVA) strains expressing the entire *P. yoelii* CS protein. The results of different immunization protocols showed that the combination of PPV-PYCS prime/poxvirus boost was highly immunogenic, inducing specific CD8+ T cell responses to CS resulting in 95% reduction in liver stage parasites two days following sporozoite challenge. In contrast, neither the administration of PPV-PYCS alone nor the immunization with the vectors given in the order poxvirus/VLPs was as effective. The immune profile induced by VLPs/MVA boost was associated with polyfunctional and effector memory CD8+ T cell responses. These findings highlight the use of recombinant parvovirus PPV-PYCS particles as priming agents and poxvirus vectors, like MVA, as booster to enhance specific CD8+ T cell responses to Plasmodium antigens and to control infection. These observations are relevant in the design of T cell-inducing vaccines against malaria.

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Introduction

The development of a malaria vaccine is a major goal in the fight against infectious diseases, as infection with *Plasmodium falciparum* causes an estimated 225 million cases of malaria and 781,000 deaths from the disease worldwide, mostly among children in Africa [1]. Progress in this direction has been obtained with the demonstration in phase 2 clinical trials that the administration of an anti-sporozoite vaccine based on the parasite CS protein fused with the hepatitis B antigen (referred as RTS,S) in combination with the adjuvants AS01 and AS02 generates in children and infants around 50% clinical efficacy against malaria [2,3,4] [5]. Immunological analysis indicates that the likely main protective mechanism is the induction of antibodies against the CS protein, while CD8+ T cell responses might play a minor role as they are very modest [4,6]. The combination of anti-CS antibody concentrations titers and CS-specific TNFα(+) CD4(+) T cells could account for the about 50% level of protection against clinical malaria conferred by RTS,S/AS01(E) [4,7]. Based on the CS-protective efficacy, a phase III clinical trial is ongoing in 11 centers in Africa with RTS,S (NCT00866619). Thus far, initial results of this trial at 12 months provided about 50% reduction of clinical episodes of malaria and severe malaria in vaccinated children 5 to 17 months of age [8]. The limited protection thus far obtained with the RTS,S vaccine, suggest the need to improve the efficacy of this malaria vaccine.

Because both B and T cell responses might be required for the control of Plasmodium infection, it could be beneficial for the current RTS,S vaccine to also enhance CD8+ T cell responses. Numerous protocols have been developed that trigger specific B and T cell responses to Plasmodium antigens with different degrees of protection in animal models, using a combination of viral and non viral vectors [9]. Among vaccine protocols, the heterologous prime/boost immunization using poxvirus vectors as booster has been shown to be quite effective at inducing T cell responses with protection in animal model systems. In fact, influenza and VACV vectors both expressing the CS antigen of *Plasmodium yoelii* (*P. yoelii*), provided evidence in mice that the

combination of influenza/poxvirus vectors was highly effective at inducing specific CD8+ T cell responses and high levels of protection against rodent malaria, and that the order of vector immunization was critical [10]. The question of which are the most appropriate vectors for use in human trials is still unclear and is based on several assessments in which nature of antigens, immune potency, durability, efficacy, safety and cost play important roles. Recently, a combination of poxvirus vectors (fowlpox and MVA) expressing different Plasmodium antigens has shown lack of protection in phase I/IIa clinical trials [11], and this could be related to the complex nature of the six pre-erythrocytic malaria antigens linked together in a single protein. The use of single protein components as immunizing agents that can prime an anti-malaria immune response which is expanded after a boost with a poxvirus vector could be a favorable option to avoid antigen competition. Proteins can be delivered either alone, conjugated with adjuvants, in the form of fusion polypeptides or as part of virus-like particles (VLPs).

The use of VLPs might be preferable due to the ease of production and of presentation of the malaria antigen, as the VLPs behave as native virus particles during entry and processing into antigen-presenting cells. It has been previously demonstrated that chimeric PPV-VLPs carrying heterologous epitopes, when injected intraperitoneally (i.p) into mice, activate strong CD4+ and CD8+ T-cell responses specific for the foreign epitopes, and these responses are mediated by dendritic (DC) cells and influenced by the flanking sequences [12,13,14]. PPV-VLPs are formed by the assembly of 60 copies of the major virus capsid protein (VP2) of PPV [15]. PPV-VLPs have been engineered to deliver CD8+ T-cell epitopes [12] or CD4+ T cell helper epitopes [16] inserted into the N terminus of the PPV VP2. Cytotoxic T lymphocyte (CTL) response was characterized by a high frequency of specific T cells of high avidity [17]. Moreover, the CTL activation does not require CD4+ T cell help [12]. PPV-VLPs are captured by DC through macropinocytosis and these cells are the only antigen presenting cells (APCs) for PPV-VLPs [13]. VLPs can be found in the endosome of DC, where processing of the epitopes inserted in the VLPs takes place [13]. In addition, B cell epitopes can be engineered on the surface of the parvovirus VLPs by manipulating the VP2 loops to elicit a potent antibody response [18].

Thus, to enhance the CD8+ T cell response of a CS-based immunization procedure, in this investigation we compared in mice the immunogenicity and anti-malaria efficacy of protocols based on priming with parvovirus PPV-VLPs carrying a CD8+ T-cell epitope from the *P. yoelii* CS protein, restricted by the H-2K^d molecule (SYVPSAEQI) and boosted with either a recombinant replication competent (WR strain) or replication restricted (MVA strain) of VACV, both vectors expressing the entire *P. yoelii* CS protein.

Results

The CS-T cell priming effect induced by PPV-PYCS VLPs is markedly enhanced by boosting with a replication competent VACV vector

Recombinant PPV-VLPs have been shown to be effective delivery systems of heterologous sequences that trigger specific Th1 type of immune responses to the foreign epitopes when inoculated in animal models [12,16]. With the aim to define the priming capacity of PPV-VLPs in prime/boost combination of immunogens, we generated recombinant PPV-VLPs by the insertion into PPV VP2 of the specific CD8+ T cell epitope (SYVPSAEQI) from *P. yoelii* CS protein, restricted by the H-2K^d molecule [10]. A DNA fragment coding for the CS epitope was fused to the 5′-end of the gene coding for PPV VP2 capsid protein. This chimeric gene, when expressed by the baculovirus system, produces a CS epitope-VP2 fusion protein that self assembles generating PPV-PYCS VLPs that can be easily purified (Figure 1). Production and purification of PPV-PYCS from baculovirus infected insect cells was performed as described under Materials and Methods. Figure 1A shows a coomassie blue stained SDS-PAGE of the purified PPV-PYCS. A main single band of about 67 kDa was observed on the gel, indicating high purity of the recombinant protein. This was also confirmed by electron microscopy as shown by a photomicrograph of the purified VLPs in Figure 1B. To study the ability of PPV-PYCS to induce a specific anti-CS T cell immune response, and to test whether the achieved immune response could be enhanced after booster with a replication competent VACV recombinant from WR strain expressing the complete CS protein (VV-PYCS), we immunized groups of BALB/c mice (5 per group) with PPV-PYCS given subcutaneously (s.c) (10 or 50 µg/ per mouse), and boosted 14 days later with 10^7 pfu/mouse of VV-PYCS by the same route. At 14 days after the booster, splenocytes from immunized animals were subjected to a standardized and quantitative ELISPOT assay, measuring IFN-γ secreting cells [19]. We used MHC-class I P815 cells (H-2^d) as APC, pulsed with 10^{-6} M of the synthetic peptide SYVPSAEQI, corresponding to the *P. yoelii* CS protein. Previous studies have shown the CD8+ T cell responses of the *P. yoelii* CS-peptide (SYVPSAEQI), which is specific for IFN-γ secreting cells [10] [20]. As shown in Figure 2, animals immunized with two doses of 10 µg of PPV-PYCS did not develop a significant CS-specific CD8 T cell response. However, when animals were boosted with a VV-PYCS a significant ($p<0.001$; one-way ANOVA) increase in the response was observed. This increment was dose-dependent, since animals primed with the highest dose of PPV-PYCS (50 µg) developed the strongest response. In addition, this response was specific, as a non-related recombinant VACV expressing luciferase when used for booster (VV-LUC) had no effect on T cell responses. These findings revealed that while PPV-PYCS *per se* is low inducer of IFN-γ secreting cells, however, is an effective priming component when booster is done with the replication competent VV-PYCS recombinant vector.

Priming with PPV-PYCS followed by booster with replication competent VV-PYCS protects mice against liver stage parasites after challenge with *P. yoelii*

To determine whether PPV-PYCS prime/VV-PYCS boost immunization could induce protective immunity against pre-erythrocytic stages of the parasite, BALB/c mice (5 per group) were immunized with 10 or 50 µg of PPV-PYCS administered s.c and 2 weeks later were boosted with one dose of 10^7 pfu of VV-PYCS administered by the s.c route. Thereafter, immunized mice were challenged i.v with 5x10^5 *P. yoelii* highly infective sporozoites. Parasite development was monitored at 42 h after challenge by measuring plasmodial rRNA in the liver of the challenged mice by semi-quantitative RT-PCR assay, a well defined and standardized protocol to evaluate protection [10,21]. We found that mice immunized following the prime-boost immunization scheme had very low levels of the parasite load in the liver, with an 86% percent reduction when the dose of PPV-PYCS was 50 µg and 81% reduction in parasitemia with a dose of 10 µg (Figure 3; $p<0.001$ by one-way ANOVA). Mice immunized with either two doses of PPV-PYCS or VV-PYCS showed a partial decrease of the parasite load in the liver in comparison with non-vaccinated control mice. These findings showed that administration of PPV-VLPs alone does not protect against sporozoite challenge while the

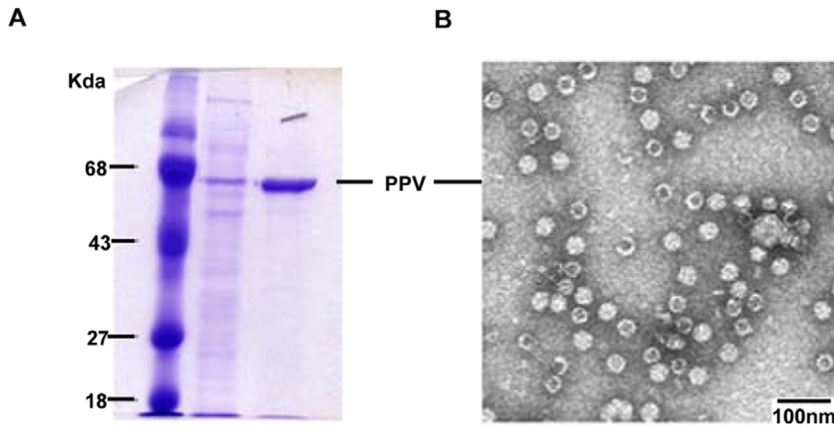

Figure 1. Characterization of PPV-PYCS VLPs. Conditions for generation of the PPV vector expressing the specific CD8+ T cell epitope of *P. yoelii* CS, growth in baculovirus-infected insect cells and purification of the PPV-VLPs is described under Materials and Methods. (**A**). Proteins were resolved by 9% SDS-PAGE and visualized after coomassie-blue staining. Molecular masses of standard proteins (lane 1) are indicated at the left. Lane 2, shows partial purification and lane 3, purified protein with the size corresponding to the PPV VP2. (**B**). Electron microscope image of negatively stained PPV-PYCS VLPs.
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combination of PPV-VLPs/VACV is an effective protocol to control *P. yoelii* infection.

Booster with a replication-restricted MVA-PYCS vector triggers strong CD8+ T cell responses to CS after priming with PPV-PYCS

While studies shown in Figures 2 and 3 were performed with replication competent VACV, it was of interest to know to what extent the use of a replication restricted and highly attenuated vector like MVA [22] could boost PPV based anti-malaria immune response. Thus, groups of BALB/c mice (5 per group) were primed s.c with different doses of PPV-PYCS (10, 50 and 100 μg) and two weeks later those mice were boosted s.c with 10^7 pfu of the attenuated MVA-PYCS vector. A group of mice immunized with 50 μg of PPV-PYCS and boosted with 10^7 pfu of VV-PYCS was included as comparison for replication competent vector. Also groups of mice primed/boosted with 100 μg of PPV-PYCS, or primed with PPV-VLPs lacking the CS-specific CD8+ T

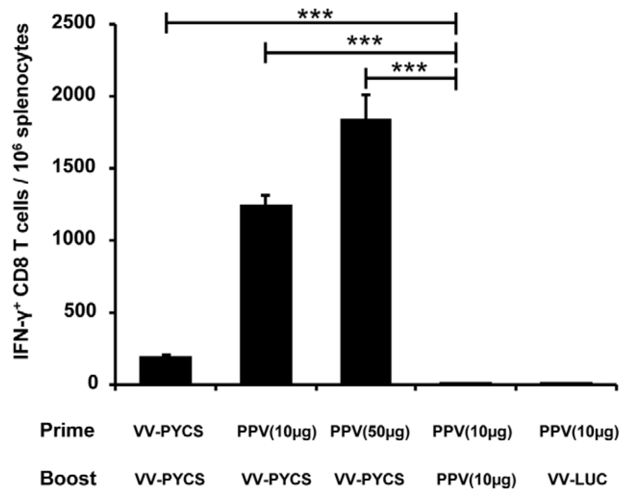

Figure 2. The CS-specific CD8+ T-cell priming effect of PPV-PYCS is markedly enhanced by boosting with replication competent VACV vector expressing CS. Different groups of BALB/c mice (5 per group) were immunized s.c with 10 or 50 μg of PPV-PYCS [PPV(10 μg) and PPV(50 μg) respectively] or with VACV vectors in prime/boost protocols. Fourteen days after the boost CS-specific IFN-γ secreting cells for the plasmodial epitope SYVPSAEQI in splenocytes were measured by ELISPOT as described under Materials and Methods. The results are expressed as the mean of triplicate assays using cultured pooled splenocytes. Statistical values were determined by one-way ANOVA; P values, *P<0.05, **P<0.01, ***P<0.001.
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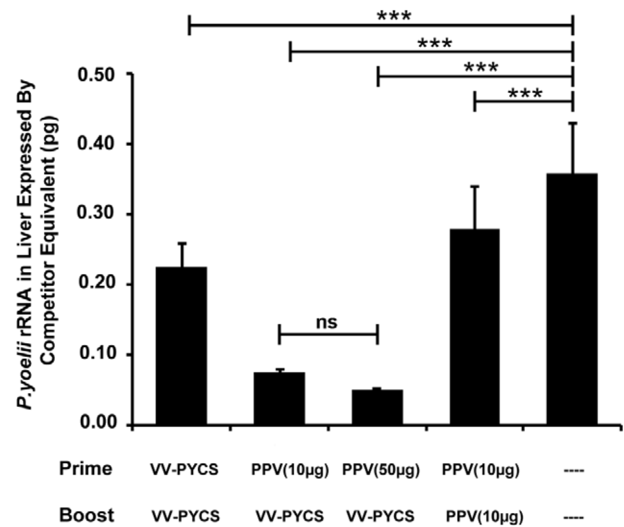

Figure 3. Protective capacity of PPV-PYCS prime followed by booster with replication competent VACV vector expressing CS. Groups of BALB/c mice (5 per group) were immunized with different amounts of PPV-PYCS [PPV(10 μg) and PPV(50 μg)] or non-vaccinated and two weeks later animals were boosted with the replication competent VACV vector from the WR strain (referred as VV-PYCS) or with PPV-PYCS. Thirteen days later, animals were challenged with sporozoites and the amount of parasite rRNA in the liver of each animal was estimated 42 h after the challenge, as described under Materials and Methods. Results are expressed as the mean with standard deviation. Statistical values were determined by one-way ANOVA; *P* values, **P*<0.05, ***P*<0.01, ****P*<0.001. Lane (–), non-vaccinated.
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cell epitope and boosted with MVA-PYCS were included for reference. The frequencies of *ex vivo* splenocytes producing IFN-γ upon MHC class-I restricted peptide stimulation was determined by ELISPOT assay two weeks later. As shown in Figure 4, the groups of animals receiving PPV-PYCS/MVA-PYCS had the highest values of IFN-γ secreting cells ($p<0.001$ by one-way ANOVA). These values were even higher than those observed in the group PPV-PYCS/VV-PYCS. Significantly, a low immune response was obtained when single dose of MVA-PYCS was used, or when it was given after priming with non-recombinant VLPs. In addition, a minor immune response was obtained when PPV-PYCS was used at 100 µg/per dose (Figure 4).

These findings clearly demonstrate that priming with PPV-PYCS is necessary to activate specific T cells, but to obtain large expansion of these T cells it required MVA-PYCS boost.

Protection against parasites after prime/boost with PPV-PYCS/MVA-PYCS protocol

Next we examined if the increase in immune response observed after MVA-PYCS boost is translated into a high degree of protection. In this experiment we compared the protective immunity elicited against parasite challenge after prime-boost immunization with PPV-PYCS followed by VV-PYCS or MVA-PYCS, with that obtained in a group of mice immunized with a recombinant influenza virus expressing *P. yoelii* CS-CD8+ T cell epitope (Flu-PYCS) and boosted with VV-PYCS. As shown in Figure 5 the degree of protection, based on reduced parasitic burden in the liver, was higher in the group of mice primed with PPV-PYCS and boosted with MVA-PYCS than in the group of mice boosted with VV-PYCS ($p<0.001$ by one-way ANOVA. On the other hand the 97% inhibition of parasite development in the group of mice boosted with MVA was similar to that obtained in the group of mice immunized with Flu-PYCS/VV-PYCS which serves as a reference, as it is a well established and standardized protocol previously described to induce high level protection after parasite challenge [10]. These findings demonstrate the high degree of protection against liver stage parasites obtained by a PPV/MVA regime.

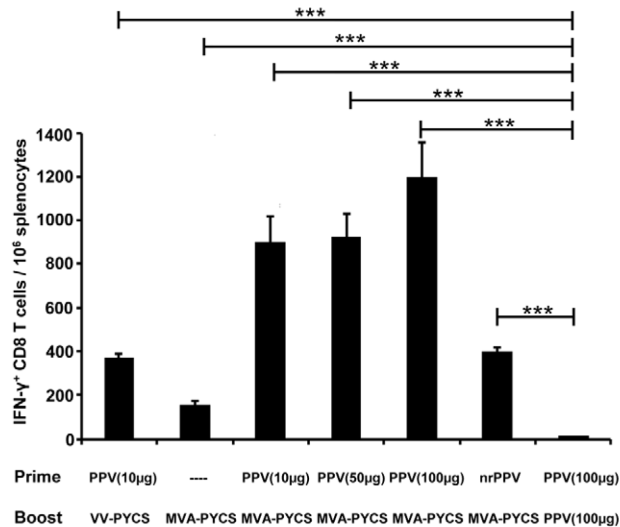

Figure 4. Booster with replication restricted MVA-PYCS vector triggers strong CS CD8+ T-cell response after priming with PPV-PYCS. Groups of BALB/c mice (5 per group) were immunized s.c with different amounts of PPV-PYCS [PPV(10 µg), PPV(50 µg) PPV(100 µg)], with non-recombinant PPV-VLPs (nrPPV) or with VACV vectors (VV-PYCS or MVA-PYCS) in prime/boost protocols and 14 days after the boost CS-specifc IFN-γ secreting cells for the plasmodial epitope SYVPSAEQI in splenocytes were measured by ELISPOT as described under Materials and Methods. Purified PPV-VLPs without the peptide insert were used as control. The results are expressed as the means of assay triplicates of cultured pooled mouse splenocytes. Statistical values were determined by one-way ANOVA; P values, *P<0.05, **P<0.01, ***P<0.001.
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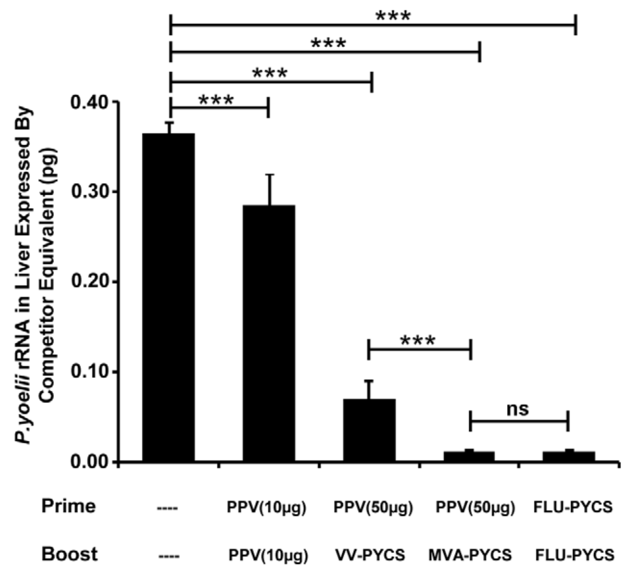

Figure 5. Protection against malaria after prime/boost with PPV-PYCS/MVA-PYCS protocol. Groups of BALB/c mice (5 per group) were immunized with different amounts of PPV-VLPs [PPV(10 µg) and PPV(50 µg)] and two weeks later animals were boosted with the replication restricted MVA-PYCS or the replication competent VV-PYCS. A positive control group primed with an influenza virus recombinant expressing the CD8+ T cell epitope and booster with VV-PYCS, a protocol previously shown to induce high protection against the parasite, was included [10]. Thirteen days later, animals were challenged with sporozoites and the amount of parasite rRNA in the liver of each animal was estimated 42 h after the challenge, as described under Materials and Methods. Results are expressed as the mean with standard deviation. Statistical values were determined by one-way ANOVA; *P* values, *$P<0.05$, **$P<0.01$, ***$P<0.001$.
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PPV-PYCS priming followed by MVA-PYCS boost improved the polyfunctionality and magnitude of memory CD8 T-cell responses

Enhancement in the quality and magnitude of memory CD8+ T-cell response is known to correlate with better protection in malaria [23,24]. Therefore, to define the CD8+ T cell responses induced by VLP-PYCS/MVA-PYCS prime-boost, we performed intracellular cytokine staining (ICS) study with splenocytes obtained from mice immunized by various protocols and collected 53 days post-boost to evaluate memory T cells. The vaccination regime based on VLP-PYCS prime/MVA-PYCS boost generated a robust response in mice when compared to VLP-PYCS prime/boost or DNA-PYCS/MVA-PYCS prime/boost, as revealed after separation of CD8 T-cells based on memory markers CD127 and CD62L (Figure 6). This approach helped to differentiate $T_{Effector}$; T_E ($CD127^{Lo}CD62L^{Lo}$), $T_{Effector\ Memory}$; T_{EM} ($CD127^{Hi}CD62L^{Lo}$) and $T_{Central\ Memory}$; T_{CM} ($CD127^{Hi}CD62L^{Hi}$). Considering the negligible CD8+ T-cell responses generated when using DNA-PYCS as priming agent, a VLP-PYCS/MVA-PYCS vaccine regime elevated the CS specific

CD8+ T cell response by 1.5 fold ($p<0.001$ determined as in ref 33) (Fig 6). Interestingly, the majority of the CD8+ T cells, following stimulation, rapidly acquired T_E phenotype. However a significant increase in the T_{EM} population was also observed (Fig 6). Furthermore, the polyfunctional nature of the CD8 T-cells generated, suggested the enhanced capability of VLP priming followed by VACV boost. The majority of the CD8 cells produced were double positive for IFN-γ and TNF-α (IFN-γ^+TNF-α^+) ($p<0.001$) (Figure 7). In addition, there was high surge in the TNF-α secreting cells ($p<0.005$). Although, at lower levels, there was a significant induction of triple positive cytokine secreting cells (IFN-γ^+TNF-α^+IL-2^+) ($p<0.001$). Thus, the data revealed how VLP-PYCS prime/MVA-PYCS boost improves the polyfunctionality and the population of memory CD8+ T cells in immunized mice, immune parameters which might be relevant in protection against malaria.

Discussion

While one of the main interests in the malaria vaccine field is to develop immunogens based on proteins for safety considerations, however, the immune response triggered by proteins is generally weak with a bias for Th2 type. Thus, efforts have been directed to combine proteins with adjuvants and immunostimulatory molecules, to enhance specific innate immune responses and to drive the response to a Th1 type. In fact, the current phase III clinical trial with malaria vaccine RTS,S is based on CS protein fused to hepatitis B antigen and combined with a potent adjuvant AS01. Because previous studies with the RTS,S vaccine have shown

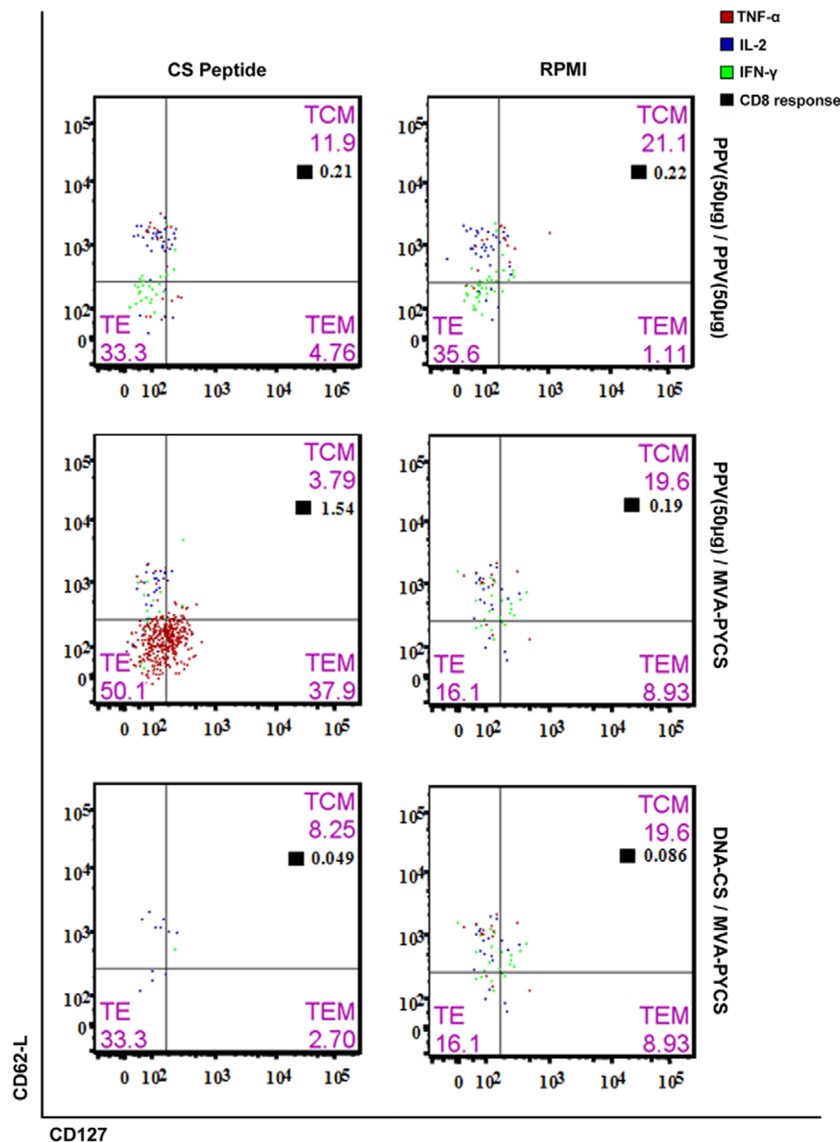

Figure 6. PPV-PYCS/MVA-PYCS prime/boost improves cytokine secretion by memory CD8$^+$ T-cells. Groups of BALB/c mice (4 per group) were immunized in prime/boost as indicated in Table 1 and 53 days post boost splenocytes were processed for ICS as described under Materials and Methods. Phenotypic differentiation of CD8$^+$ T-cells based on memory markers CD127 (V450) and CD62L (FITC). Each quadrant represents different memory population of CD8 cells with its respective percentages. The distribution of antigen specific CD8+ T-cells secreting cytokines in response to CS peptide stimulation, within the different memory population is also shown. The total CD8+ T-cell response is indicated by the black boxes. The boxes on left indicate the responses towards CS peptide stimulation while the ones on right represent its respective RPMI controls.
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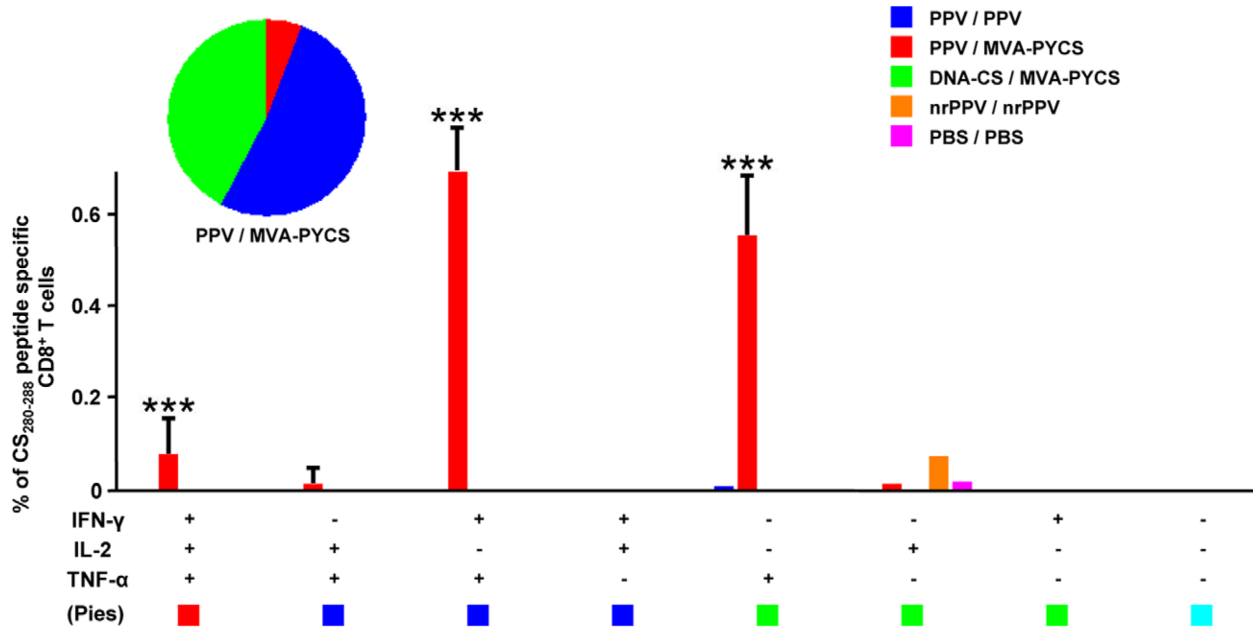

Figure 7. Polyfunctionality of CD8+ T-cells is improved by heterologous prime/boost. Groups of BALB/c mice (4 per group) were immunized in prime/boost as indicated in Table 1 and 53 days post boost splenocytes were processed for ICS as described under Materials and Methods. Memory CD8 T-cells were differentiated into single, double or triple positive cells secreting IFN-γ (PeCy7), TNF-α (PE) and IL-2 (APC). Polyfunctionality is indicated by the pie chart.The data was then analyzed using SPICE software. Data are expressed as mean ± s.e. (n = 4 mice per group). Statistical values were determined by a novel approach previously described (33). P values were calculated between PPV/MVA-PYCS and PPV/PPV groups. ** $p<0.01$; *** $p<0.001$.
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weak CD8+ T cell responses in vaccinees, in this investigation we have asked the question as to what extent CS antigen presentation by chimeric VLPs obtained from PPV is able to prime CD8+ T cell responses, and how these responses can be enhanced when combined with poxvirus vectors expressing CS. In addition, we have asked in which grade specific CD8+ T cell immune responses are associated with protection against malaria and what are the CD8+ T cell populations induced. This proof-of-concept has been tested in mice. In this work we have generated recombinant parvovirus-derived VLPs expressing the CTL epitope from the *P. yoelii* CS protein.

The PPV-VLP system presents several advantages such as the long term stability of VLPs in addition to high levels of protein expression. Although the system has not been rigorously tested for the largest insert size that can be accommodated without disrupting the particle, there would be two ways to overcome the limitation of the system to accept large insertions. First, it is relatively simple to build different PPV-VLPs containing different CD8+ epitopes that can be mixed during administration. Second, PPV VP2 containing different epitopes can be co-expressed in the baculovirus system to prepare VLPs with mixtures of different epitopes. These possibilities would facilitate the use of multiple CD8+ epitopes, as required for human vaccines, or their combination with CD4+ or B cell epitopes. These combinations should result in a more efficient vaccine design.

Groups of mice immunized in a prime/boost regimen with two doses of PPV-PYCS did not develop significant anti-CS cellular immune response, determined by the ELISPOT assay. However, when animals were immunized with different doses of PPV-PYCS, followed by the recombinant VV-PYCS, expressing the full CS protein from *P. yoelii*, a strong $CD8^+$ T cell response was generated against this antigen. This response was about 10 times higher that the one obtained with two doses of VV-PYCS. When we used for priming two different doses (10 and 50 µg) of PPV-PYCS followed by a booster with 10^7 PFU of VV-PYCS there was a dose response effect, obtaining the highest CD8+ T cell response with the highest dose of PPV-PYCS. In this experiment we observed again that this response was much higher than that obtained with two doses of VV-PYCS. We have also included as control a non-related recombinant VACV for booster, which showed that the CD8+ T cell response primed by the recombinant VLPs is boosted in an antigen dependent manner, and cannot be boosted by a non-related VACV recombinant.

Importantly, when we analyzed protection after challenge with the parasite in animals immunized as described above, quantified as reduction in the amount of Plasmodium rRNA in the liver with respect to non-immunized animals, we observed maximal protection in mice primed with PPV-PYCS followed by a booster with VV-PYCS or MVA-PYCS. Because safety is a major concern for developing poxvirus based vaccines, we included for boosting a recombinant vector expressing the full-length CS protein based on the highly attenuated MVA strain instead of the recombinant based on replicating WR strain. When mice were immunized with 10, 50 or 100 µg of PPV-PYCS followed by the MVA recombinant (10^7 pfu), we obtained a stronger cellular immune response that was about 2 to 3 times higher than that obtained when we used for boosting a recombinant based on the WR strain. While two doses of PPV-PYCS did not elicit significant cellular immune responses, these PPV-VLPs were able to prime a specific anti-CS CD8+ T cell response that could be subsequently be boosted by MVA-PYCS. When PPV-PYCS was substituted by PPV-VLPs without the CS epitope during priming, the magnitude of the response after booster with the virus was similar to that obtained with only one dose of MVA-PYCS.

The magnitude of the specific cellular immune response was associated with protection, defined by markedly reduced parasites

in the liver two days after challenge. Thus, when groups of mice immunized as before were challenged with the Plasmodium parasite, maximal protection was observed in animals boosted with the MVA-PYCS recombinant. The high protection (about 95%) obtained after priming with PPV-PYCS and boosting with MVA-PYCS was comparable to that obtained after immunization in similar prime/boost approach but using instead two live recombinant viral vectors, i.e influenza expressing the $CD8^+$ T cell epitope followed by VV-PYCS, that we have previously reported to be protective [10]. This is an important consideration, since by using PPV-VLPs and MVA immunogens we are able to achieve similar protective immune response than previously attained with two replicating viral vectors.

How can we explain the enhanced response obtained by PPV-VLPs/poxvirus prime/boost approach and its association with protection against the malaria parasite? Based on current understanding of prime/boost protocols, and due to the induction of anti-vector immunity by the VACV prime, we suggest that during priming with PPV-PYCS the particles which are able to enter the cells, are processed by antigen presenting cells and trigger an innate immune response that activate T cells; after booster the primed T cells are expanded as a result of infection of antigen presenting cells by MVA-PYCS and cross-priming effects by induction of apoptosis [25]. In addition, the innate immune response triggered by MVA [26] can also be responsible for T cell expansion. Significantly, the T cell responses observed in the two protocols PPV/VV and PPV/MVA were associated with high levels of protection against the parasite, while low levels of T cells were ineffective to control the parasite. While it is difficult to compare immune correlates between studies performed with different vectors, the magnitude of CD8+ T cell responses after the PPV-VLPs prime/pox boost and degree of protection against the parasite in this study are remarkable. In fact, analysis of the memory CD8+ T cells after prime/boost revealed that the protocol VLP/MVA induced a polyfunctional response with activation of effector memory CD8+ T cells, both immune parameters that could be relevant in protection against malaria [24].

While a number of different prime/boost combination of vectors expressing malaria antigens have shown activation of CD8+ T cell responses and different degrees of protection against murine malaria [9,27], [28] there are several advantages in the use of PPV-VLPs and MVA vectors expressing the CS antigen for T cell activation. First, CS has shown good immunogenicity profile with efficacy in preclinical assays whether from DNA, Ty-particles, a fusion protein or delivered by viral vectors, but most importantly when fused with hepatitis B antigen plus an adjuvant it induced significant protection in children exposed to malaria, indicating the CS protective capacity if formulated properly for immune B and T cell activation. Second, expression of CS as part of PPV-VLPs is able to prime the antigen presenting cell in a way that further booster with the poxvirus vector MVA triggered high levels of CD8+ T cells, which are polyfunctional and of effector memory phenotype, that are associated with high degree of protection after challenge when evaluated by liver stage parasite inhibition. Third, both vectors PPV-VLPs [29] and MVA recombinants [22] can be easily grown to large scale for vaccination purposes. Future studies on potential clinical applicability of the the PPV-VLP platform will require to define if these capsids are capable of carrying large regions of antigen or carrying multiple T-cell epitopes which diverse humans will respond.

Overall, the results reported here demonstrate that prime/boost immunization with PPV-VLPs and MVA expressing CS is a logical vaccine approach to optimize cellular immunity and protection against malaria. These findings are relevant in the design of vaccine strategies against Plasmodium.

Materials and Methods

Ethics Statement

The animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of Centro Nacional de Biotecnologia (CNB-CSIC, Madrid, Spain) in accordance with national and international guidelines and with the Royal Decree (RD 1201/2005), permit number: 130/07.

Cells

African green monkey kidney cells (BSC-40; ATCC CRL-2761) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS). Chick embryo fibroblast (CEF) cells were obtained from sterile pathogen-free eggs provided by INTERVET (Salamanca, Spain), and primary cultures were prepared according to standard procedures and maintained in DMEM supplemented with 10% fetal calf serum (FCS) (Gibco®, Grand Island, NY, USA). P815 (ATCC TIB-64) mastocytoma cells that express MHC class I molecules H-2d were cultured in DMEM containing 10% FCS.

Spodoptera frugiperda clone 9 (Sf9, CRL 1711, ATCC) cells were grown and maintained in suspension at 27°C using Grace's insect tissue culture medium (Gibco®, Grand Island, NY, USA) supplemented with 10% FCS, 0.2% Pluronic F-68 (Sigma-Aldrich, Steinheim, Germany) and antibiotics.

Growth and purification of VACV expressing the CS protein from *P. yoelii*

The recombinant VACV virus VV-PYCS carrying in the TK locus the entire CS gene of *P. yoelii*, has been previously described [10]. To generate the recombinant virus MVA-PYCS, the CS gene was inserted by homologous recombination into the TK region of the genome of the MVA strain, by using the VV transfer plasmid pSC-PYCS containing the CS DNA sequence under the control of the VACV p7.5 promoter. β-galactosidase-producing plaques were picked, cloned three times, and amplified in CEF cells as previously described [21]. VV-PYCS and MVA-PYCS were propagated and titrated in monkey BSC-40 and CEF cells respectively [30]. Both viruses were purified by banding in sucrose gradients, as previously described [31].

Construction of a recombinant baculovirus expressing PPV-PYCS

Oligonucleotide 5′-TCGAGATGTCATACGTTCCCTCGG-CCGAACAAATCC-3′ and its complementary 5′-TCGAG-GATTTGTTCGGCCGAGGGAACGTATGACATC-3′ were designed in order to regenerate the PYCS CD8 epitope SYVPSAEQI plus an initiation codon and two cohesive *XhoI* sites. The oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed at 70°C for 15 min and ligated into *XhoI*-digested pPPV29mod, which contains a unique XhoI restriction site immediately downstream of the initiation codon of the PPV VP2 gene, as previously described [12]. Then, the chimeric VP2 sequence containing the PYCS epitope was isolated by *BamHI* digestion and subcloned into *BamHI*-digested pAcYM1. The recombinant baculovirus transfer vector was called pAcYM1-PPV-PYCS.

The recombinant viruses were obtained by cotransfection of *Spodoptera frugiperda* (Sf9) insect cells with a mixture of 2 μg of purified transfer vector DNA plus 500 ng of parental BacPAK6

baculovirus DNA, previously linearized with *Bsu36I*, in the presence of cationic liposomes [18]. Transfected cultures were collected when the cells started to show signs of infection, usually 5–6 days later. Recombinant baculoviruses were plaque-purified in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) until no more blue plaques (wild-type) were detected. Then, high-titers stocks ($>10^8$ pfu/ml) of the recombinant baculovirus AcPPV-PYCS were obtained.

Characterization and purification of PPV-PYCS VLPs

Sf9 cells were infected with AcPPV-PYCS at a multiplicity of infection of 0.5 plaque-forming units (pfu) per cell. Cells were collected at 72 h post-infection with a clear cytophatic effect. Purification of PPV-VLPs was carried out as previously described [12,16]. Briefly, infected Sf9 cells were lysed by hypotonic shock with 25 mM bicarbonate solution at 4°C. Cell debris was removed by centrifugation and the PPV-VLPs present in the supernatant were precipitated with 20% ammonium sulfate, resuspended in PBS and dialysed. The purity of the preparation of PPV-PYCS VLPs was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was estimated by densitometric assay with the Bio-Rad protein assay reagent, and using BSA as reference.

Recombinant baculovirus, AcPPV-PYCS, which express under the polyhedrin promoter the PPV VP2 capsid protein fused at its amino-terminus with the PYCS CD8+ epitope, was used for antigen preparation.

Plasmid construction

pCI-Neo-CS. The gene coding for *P. yoelii* CS protein was amplified from MVA-PYCS using the primers CS-XhoI-F (5′-ACTTA*CTCGAG*ATGTGTTACAATGAAGAAAATG-3′) and CS-NotI-R (5′-ATT*GCGGCCGC*TTTAAAATATACTTGAAC-3′) to yield a 972 bp fragment lacking the N-terminal signal sequence and C-terminal GPI sequence. The gene was inserted into a mammalian expression vector, pCI-Neo, that had been previously digested with *Xho*I and *Not*I followed by SAP treatment (Shrimp Alkaline Phosphatase). The CS gene in both the virus and plasmid were sequenced (Secugen; Spain). The plasmid was purified using Qiagen Mega Prep Kit according to manufacturer's protocol. Expression of CS from pCI-Neo-CS was confirmed by transfecting DF-1 cells followed by western blot analysis with CS specific antibodies.

Parasites

P. yoelii (17XNL strain) was maintained by alternating passages in *Anopheles stephensi* mosquitoes and Swiss Webster mice. Sporozoites were collected through infected mosquito's salivary glands dissection.

Immunization of mice and challenge

Six to eight weeks old female BALB/c mice (Harlan), were used for immunization purposes. Both, PPV-PYCS and recombinant viruses, resuspended in sterile PBS, were injected subcutaneously (s.c). Immunization schedules (dose, route, number of mice) are indicated in Table 1. Non-immunized and immunized mice were challenge 13 days after the booster by inoculation of 5×10^5 *P. yoelii* sporozoites by the intravenous (i.v) route into the mouse tail vein.

ELISPOT assay

The ELISPOT assay was used to detect epitope-specific IFN-γ-secreting cells [19]. Briefly, nitrocellulose-bottomed 96-well plates were coated with anti-mouse IFN-γ mAb R4-6A2 (8 mg/ml, Pharmingen, San Diego, CA). After overnight incubation at room temperature, wells were washed three times with RPMI 1640, then 100 μl of medium supplemented with 10% FCS were added to each well, and plates incubated at 37°C for 1 h. Triplicate cultures were prepared with serial doubling dilutions of immunized splenocytes, beginning with 10^6 cells/well. P815 cells (H-2^d), used as antigen-presenting cells (APC), were pulsed with 10^{-6} M of the synthetic peptide SYVPSAEQI, corresponding to the *P. yoelii* CS protein, and treated with mitomycin C (30 μg/ml, Sigma). After several washes with culture medium, 10^5 P815 cells were added to each well. Control P815 cells were not pulsed with the peptide. Plates were incubated for 26–28 h at 37°C, washed with PBS containing 0.05% Tween-20 (PBS-T) and incubated overnight at 4°C with biotinylated anti-mouse IFN-γ mAb XMG1.2 (2 μg/ml, Pharmingen) in PBS-T. Plates were washed with PBS-T and peroxidase-labeled avidin (Sigma; 100 μl, 1/800 dilution in PBS-T) was added to each well. One hour later, wells were washed with PBS/T and PBS. Spots were developed by adding 50 mM Tris-HCl, pH 7.5 containing 1 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.015% H_2O_2. When the plates were completely dry, the number of spots was determined with the aid of a stereomicroscope.

Intra-cellular Cytokine Staining (ICS) Analysis

Multiparameter flow cytometry was performed to study the efficacy of vaccination regimes in altering the magnitude and polyfunctionality of CD8$^+$ T-cells, as previously described [32]. Groups of mice (4 per group) were inoculated with different combination of immunogens, VLP-PYCS/VLP-PYCS (50 μg each time), VLP-PYCS (50 μg)/MVA-PYCS (10^7 pfu), DNA-PYCS (100 μg)/MVA-PYCS (10^7 pfu), non-recombinant VLP/VLP (50 μg each) and PBS/PBS. Following immunization, the animals were sacrificed after 56 days post-boost to study memory responses. Splenocytes (4×10^6) from the sacrificed animals were stimulated with 1 μg/ml of CS specific CD8 peptide (SYVPSAEQI) along with Brefeldin (1 μg/ml) (BD Bioscience) for 6 hours in a 96 well plate. After incubation, the cells were washed twice with PBS and stained with LIVE/DEAD fixable dead cell stain kit, following which, the cells were washed and blocked with CD16/CD32 antibody (Fc Block; BD Bioscience). The cells were then stained for surface markers with CD4-Alexa 700, CD8-V500, CD127-V450 and CD62L-FITC (BD Bioscience). This was followed by permeabilisation using BD Cytofix/Cytoperm™ Kit (Becton Dickinson) and staining for intracellular cytokines using IFN-γ PeCy7, TNF-α PE and IL-2 APC. Nearly 50,000 cells were then passed through LSRII flow cytometer (Becton Dickinson). The data generated were analyzed using Flo-Jo (Tree Star. Inc) and SPICE (ver 5.0).

Quantitation of liver stages

At 42 h following sporozoite challenge, livers were removed and *P. yoelii* liver-stage parasites were measured by quantification of parasite-specific 18S rRNA in total liver RNA as described elsewhere [20]. Livers were homogenized in a Ten Broeck tissue grinder (VWR Scientific) in 4 ml of a denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% sarcosyl) made fresh as a working solution (50 ml of denaturing solution, 0.2 M -2-mercaptoethanol). Total liver RNA was then isolated from the liver homogenate by using the TRIzol reagent (Gibco/BRL, Grand Island, N.Y.) as outlined in the product insert. One microgram of RNA was treated with 1.0 U of DNase I (Boehringer Mannheim, Mannheim, Germany) and was then converted to cDNA by the Superscript preamplification system for

Table 1. Dosage of different vaccination regimes: A summary of priming and boosting agents used in the study with the corresponding dosages.

Experiment	Priming		Boosting		Number of mice
	Agent	Dose	Agent	Dose	
IFN-γ ELISPOT	VV-PYCS	10^7 PFU	VV-PYCS	10^7 PFU	5
	PPV[1]	10 µg	VV-PYCS	10^7 PFU	5
	PPV	50 µg	VV-PYCS	10^7 PFU	5
	PPV	10 µg	MVA-PYCS	10^7 PFU	5
	PPV	50 µg	MVA-PYCS	10^7 PFU	5
	PPV	100 µg	MVA-PYCS	10^7 PFU	5
	PPV	10 µg	VV-LUC	10^7 PFU	5
	–		MVA-PYCS	10^7 PFU	5
	nrPPV[2]	50 µg	MVA-PYCS	10^7 PFU	5
	PPV	10 µg	PPV	10 µg	5
	PPV	100 µg	PPV	100 µg	5
Intra-cellular Cytokine Staining (ICS)	PPV	50 µg	PPV	50 µg	4
	PPV	50 µg	MVA-PYCS	10^7 PFU	4
	DNA-PYCS	100 µg	MVA-PYCS	10^7 PFU	4
	nrPPV	50 µg	nrPPV	50 µg	4
	PBS		PBS		4
Challenge Studies	VV-PYCS	10^7 PFU	VV-PYCS	10^7 PFU	5
	PPV	10 µg	VV-PYCS	10^7 PFU	5
	PPV	50 µg	VV-PYCS	10^7 PFU	5
	PPV	50 µg	MVA-PYCS	10^7 PFU	5
	FLU-PYCS	10^7 PFU	MVA-PYCS	10^7 PFU	5
	PPV	10 µg	PPV	10 µg	5
	–		–		5

All agents were administered s.c except for DNA-PYCS which was injected intradermally.
[1]PPV refers to PPV-VLPs containing the CD8 epitope of *P.yoelii* CS protein.
[2]nrPPV refers to non-recombinant PPV-VLPs.
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first-strand cDNA synthesis (Gibco/BRL), using random hexamers in a 21-µl total volume as outlined in the product insert.

For quantification of parasite-specific rRNA by quantitative-competitive reverse transcription-PCR (RT-PCR)[21], parasite-specific rRNA was amplified from 5 µl of the cDNA mixture in a PCR master mix containing 46 µl of PCR Supermix (Gibco/BRL), 1 µl each of parasite-specific primers PB1 and PB2 (1) (12 µM, final concentration), 0.2 µl (1 U) of Taq DNA polymerase (Sigma, St. Louis, Mo.), and 1 µl of a known concentration of competitor plasmid. Then 35 cycles of amplification in a PCR Express (Hybaid, Middlesex, United Kingdom) thermocycler were performed under the following conditions: 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min. An initial denaturation step at 94°C for 2 min and a terminal elongation step at 72°C for 10 min were also included. Target and competitor amplicons were resolved on ethidium bromide-stained 2% agarose gels and photographed by the Eagle Eye II still video system (Stratagene, La Jolla, California), and the image was stored electronically. Target-to-competitor ratios were then determined using the NIH Image software program. We performed a series of amplifications with different competitor concentrations and used target-to-competitor ratios from each competitor concentration in linear regression analysis to determine the competitor concentration where target and competitor amplicon ratios were equivalent. This concentration was used as a relative measure of liver parasite burden. Equal cDNA synthesis between samples was ensured by amplification of the housekeeping ß-actin gene at PCR conditions below saturation.

Statistical analysis

Statistical analysis of ELISPOT and protection assays was performed by a one-way ANOVA with Tukey post test and Bonferroni correction to all groups. For the statistical analysis of ICS data we used a novel approach that corrects measurements for the medium response (RPMI) and at the same time allows the calculation of confidence intervals and p-values of hypothesis tests [33]. The background for the different cytokines in the unstimulated controls never exceeded 0.05%. The data analysis program, Simplified Presentation of Incredibly Complex Evaluations (SPICE, version 4.1.5, Mario Roederer, Vaccine Research Center, NIAID, NIH), was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. All values used for analyzing proportionate representation of responses are background-subtracted. In all cases, P values of less than 0.05 were considered statistically significant. All statistic tests were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, C).

Author Contributions

Conceived and designed the experiments: ME DR JR. Performed the experiments: DR GGA JR AV MMG PR JIC. Analyzed the data: ME DR GGA. Contributed reagents/materials/analysis tools: PR JIC. Wrote the paper: ME DR.

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A Human Multi-Epitope Recombinant Vaccinia Virus as a Universal T Cell Vaccine Candidate against Influenza Virus

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Abstract

There is a need to develop a universal vaccine against influenza virus infection to avoid developing new formulations of a seasonal vaccine each year. Many of the vaccine strategies for a universal vaccine target strain-conserved influenza virus proteins, such as the matrix, polymerase, and nucleoproteins, rather than the surface hemagglutinin and neuraminidase proteins. In addition, non-disease-causing viral vectors are a popular choice as a delivery system for the influenza virus antigens. As a proof-of-concept, we have designed a novel influenza virus immunogen based on the NP backbone containing human T cell epitopes for M1, NS1, NP, PB1 and PA proteins (referred as NPmix) as well as a construct containing the conserved regions of influenza virus neuraminidase (N-terminal) and hemagglutinin (C-terminal) (referred as NA-HA). DNA vectors and vaccinia virus recombinants expressing NPmix (WR-NP) or both NPmix plus NA-HA (WR-flu) in the cytosol were tested in a heterologous DNA-prime/vaccinia virus-boost vaccine regimen in mice. We observed an increase in the number of influenza virus-specific IFNγ-secreting splenocytes, composed of populations marked by $CD4^+$ and $CD8^+$ T cells producing IFNγ or TNFα. Upon challenge with influenza virus, the vaccinated mice exhibited decreased viral load in the lungs and a delay in mortality. These findings suggest that DNA prime/poxvirus boost with human multi-epitope recombinant influenza virus proteins is a valid approach for a general T-cell vaccine to protect against influenza virus infection.

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Introduction

Millions of people worldwide are infected with influenza virus every year. Although most yearly outbreaks are characterized by fewer than 40,000 deaths in the United States, highly virulent strains can evolve that cause worldwide pandemics, resulting in a dramatically increased incidence of death. While ribavirin and oseltamvir can be used to combat infection, there has been recent emergence of strains resistant to these drugs [1], demonstrating the need for better therapeutics or vaccine strategies against influenza virus infection.

The major cause of influenza pandemics involves the combination of the two major glycoproteins on the virion surface [2]. These two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), contribute to the considerable antigenic variation of influenza virus because they have 16 and 9 subtypes, respectively. When a new glycoprotein subtype appears on the virion surface, the population is immunogenically naïve to this new strain, raising the possibility of a pandemic. The worst influenza pandemic to date occurred in 1918, by the so-called "Spanish flu," an H1N1 virus, which led to over 40 million deaths worldwide [3]. In 2005, there were outbreaks of an H5N1 virus in Southeast Asia and Europe. While this strain caused death in humans, it has been unable to be transmitted from person to person [4].

Besides increased mortality and morbidity, how else would an influenza pandemic impact society? Compounded with the fact that influenza-associated hospitalizations have increased substantially over the last two decades [5], the economic loss due to another pandemic would be unimaginable. Based on a model which takes into account variables such as different types of vaccination strategies and illness percentages, Thompson et al. proposed that another pandemic would cause up to 207,000 and 734,000 hospitalizations, and 18 to 42 million outpatient visits in the United States alone. Based upon these numbers, they estimate that due to loss of life and medical care, the economic cost of a pandemic to the United States would be up to $167 billion dollars [6].

The current vaccination strategy against influenza virus consists of a live-attenuated or killed virus vaccine regimen containing the three strains of virus (two A subtypes and one B subtype) that are thought to be most prevalent in the upcoming influenza season.

Determining which strains the vaccine will contain is based on bioinformatics analysis of epidemiological data from the previous season. Since the current vaccine strategy requires changing of the vaccine formulation every year, there is a push to develop a universal vaccine [7]. Development of these next generation influenza vaccines is based on technologies utilizing recombinant proteins, virus-like particles, viral vectors, and DNA-based vaccines [8]. Another strategy is prime-boost, using DNA for priming and virus vectors expressing antigens of interest as a boost [9]. Attenuated poxvirus vectors, such as MVA and NYVAC, have been used successfully to induce a greater immune response towards HIV antigens [10,11,12]. In fact, one of the more successful HIV vaccine trials in humans to date utilized a poxvirus vector [13], and there are ongoing phase I/II clinical trials using the MVA poxvirus vector [12,14,15,16].

Regarding influenza virus, many vaccination strategies have used the nucleoprotein (NP) as an antigen to induce immune responses since it is well-conserved across influenza virus subtypes [17,18,19]. However, in some cases, vaccines developed around NP have failed to provide protection [20,21]. Recently, MVA vectors expressing influenza virus antigens have been shown to provide protection against virus challenge, even in human clinical trials [22,23,24]. Novel design strategies using viral antigens in combination with NP may be able to improve immunogenicity to influenza virus and provide more universal protection.

In this study, we designed recombinant influenza virus antigens for use in a DNA prime/vaccinia virus boost vaccination strategy, and studied the ability of these proteins to induce an adaptive immune response and protective response to heterologous challenge. One immunogen was designed around influenza virus NP, which included human epitopes from the M1 (matrix), NS1 (non-structural), PB1 (basic polymerase), and PA (acidic polymerase) viral proteins. The vaccinia virus construct containing NPmix was referred to as WR-NP. The other immunogen contained conserved sequences from H5N1 viruses: the N-terminal NA fused to the C-terminal HA and was combined with the NPmix to generate the viral vector WR-flu. We also generated plasmid DNA vectors from pCIneo expressing independently NPmix and HA-NA. We show that our recombinant vaccinia virus constructs grow well in cell cultures and produce the recombinant products (NPmix or both NPmix and NA-HA) in the cytoplasm of infected cells, similarly as for the DNA vectors. After the DNA prime/poxvirus boost vaccination protocol in mice we observed increased IFNγ-secreting cells, along with an increased $CD4^+$ and $CD8^+$ T cell response with regard to $IFN\gamma^+$ and $IFN\gamma^+TNF\alpha^+$ cells. Upon challenge with influenza virus, lung viral titers were decreased in animals vaccinated with the viral vectors expressing recombinant influenza virus proteins. Taken together, this study demonstrates how a T cell vaccine based on a DNA prime/poxvirus boost strategy containing multiple human influenza virus epitopes can reduce viral load during heterologous challenge and provides a rational design for the generation of universal influenza vaccines.

Results

Immunogen design and characterization of vaccinia virus vectors expressing influenza virus antigens

The goal in developing our T cell vaccine against influenza virus was first to design an immunogen utilizing the conserved regions of influenza virus proteins as antigens and then to produce a recombinant vaccinia virus that can be used as a broad-spectrum vaccine to induce specific immune response to influenza. For this aim, we generated two types of immunogens. One was based on the nucleoprotein (NP) as a backbone, which is well-conserved among H1N1, H2N3, H5N1, H9N2, and H7N7 strains of influenza virus (Figure S1). Within the NP backbone, we added conserved human T cell epitopes for other influenza virus proteins, specifically M1, NS1, PB1, and PA (Figure 1B; NPmix). Briefly, we were guided by Epstein, et al., which lists the influenza virus gene products and peptide sequences that are presented by certain MHC molecules [25]. We narrowed down this list to four M1, one NS1, one PA, and two PB1 epitopes that were the most conserved among the virus strains listed in Figure S1. These, in addition to the NP epitopes, were included in the NPmix recombinant gene shown in Figure 1B. In choosing the amino acids to swap, we were careful in selecting regions without significant secondary structure, and we avoided swapping hydrophilic for hydrophobic regions. We also did not remove any known NP T cell epitopes. The second construct involved fusing the conserved regions of neuraminidase (NA) and hemagglutinin (HA) from H5N1 influenza viruses (Figure 1B and S2; NA-HA). These HA and NA subtypes are not homologous those in the H3N2, H9N2, or H7N7 strains listed in Figure S1; however, the NP protein shown in Fig. S1 is conserved in the H5N1 strains. The NPmix construct alone or together with the NA-HA construct was inserted in the TK locus of the Western Reserve strain of vaccina virus (Figure 1A). We termed the vaccinia virus recombinants containing NPmix insertion alone "WR-NP" and the insertion of NPmix and NA-HA "WR-flu."

We also produced plasmid vectors for their use in the priming stages of the vaccination protocol. The NPmix and NA-HA immunogens were inserted into the pCIneo mammalian expression vector, which could efficiently express the proteins upon transient transfection in cell culture, either transfected alone or in tandem (Figure 2A). Lower levels of NP and HA were observed during double transfections since only 5 µg of each vector was used, as opposed to 10 µg in single transfections. We also verified that the influenza insert was maintained in the recombinant viruses, as shown after staining viral plaques from purified viral stocks for NP and WR protein and observing that the recombinant viruses produced the same number of plaques expressing NP and WR proteins (Figure 2B); the H5 HA antibody did not strongly stain viral plaques. We further tested how the expression of influenza virus proteins from the recombinant viruses was dependent on MOI. We observed that the NA-HA and NPmix protein is strongly expressed by 24 h p.i. during both WR-NP and WR-flu infection (Figure 2C). Finally, all viruses replicate to similar levels (Figure 2D). Together, these results indicate that the recombinant viruses are correctly expressing the influenza virus proteins and that the expression of these proteins does not alter the infection profile as compared to wild-type vaccinia virus.

To further characterize the viruses, we examined the subcellular localization of the influenza virus proteins. The recombinant proteins were expressed mainly in the cytoplasm of cells during either WR-NP or WR-flu infection. HA showed a higher degree of co-localization with viral factories (Figure 3A), as marked by staining for the 14K vaccina virus protein (A27 gene), as compared to colocalization of the NP protein with 14K (Figure 3B). NPmix also exhibits greater distribution throughout the cell.

Immunogenicity of vaccinia virus recombinants during DNA prime/poxvirus boost vaccination

We next examined the influenza virus-specific immune responses induced in mice by the WR-NP and WR-flu recombinant viral constructs. Four BALB/c mice for each group, WR, WR-NP, or WR-flu, were vaccinated according to the schedule in Figure 4A and sacrificed to evaluate the adaptive immune response elicited. Mice were primed with 100 µg DNA by

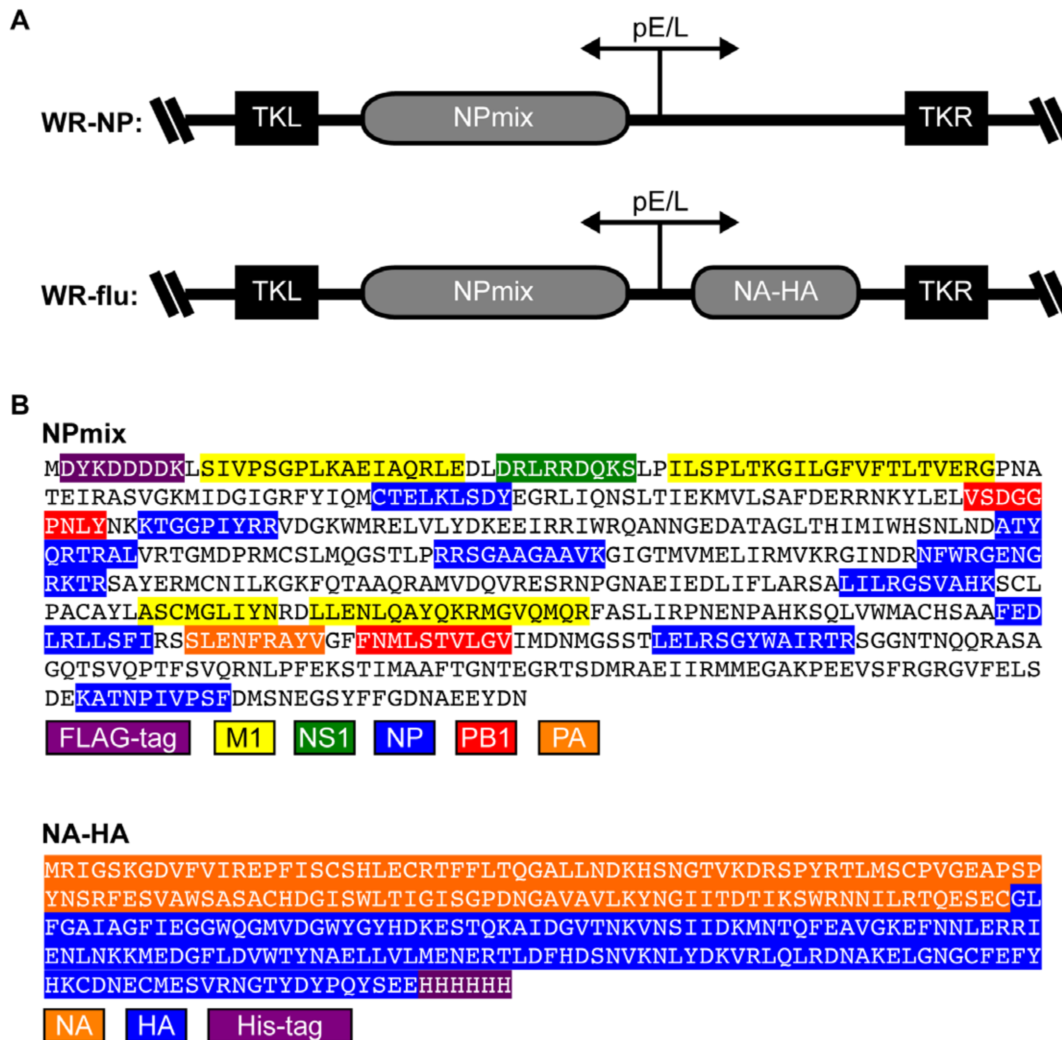

Figure 1. Design and characterization of vaccine constructs. (A) Recombinant influenza virus gene constructs (NPmix or NA-HA) were inserted at the TK locus of the Western Reserve (WR) of vaccinia virus and are driven by the synthetic early/late promoter (pE/L). The cloning vectors (shown) were introduced into the wild-type WR virus by homologous recombination and iterative plaque purification. (B) Amino acid sequences of NPmix and NA-HA recombinant influenza virus protein constructs. The backbone for the NPmix construct is influenza virus NP into which was inserted other influenza virus protein human T cell epitopes. Human T cell epitopes for influenza virus M1, NS1, NP, PB1, and PA proteins are indicated. The NA-HA construct consists of the conserved regions of H5N1 influenza virus neuraminidase (N-terminal amino acids 108–231) and hemagglutinin (C-terminal amino acids 347–511).

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intramuscular injection. 100 µg of empty DNA (pCIneo-Ø) was used for the WR group; 100 µg DNA containing the NPmix vector (pCIneo-NPmix) was used for the WR-NP group; 50 µg of NPmix DNA and 50 µg of DNA containing the NAHA vector (pCIneo-NAHA) was used for the WR-flu group. Two weeks post-prime, the animals were boosted by intraperitoneal infection of 10^7 PFU of WR, WR-NP, or WR-flu. Eleven days post-boost, the adaptive immune response was evaluated using a fresh IFNγ ELISPOT assay with splenocyte stimulation using a peptide corresponding to influenza virus NP (TYQRTRALV) or vaccinia virus E3 (VGPSNSPTF).

As shown in Figure 4B, animals that were vaccinated with WR-NP or WR-flu exhibited a significant increase in splenic T cell responses against the NP peptide. Vaccination with WR-NP lead to a ~7.6-fold increase in IFNγ-secreting cells while vaccination with WR-flu lead to a ~6.5-fold increase, as compared to vaccination with WR alone. All vaccination protocols exhibited similar response levels to the E3 peptide, indicating that all viruses are replicating similarly in the vaccinated animals. We also performed experiments to determine the levels of neutralizing antibodies in the serum of vaccinated mice. However, we did not observe any significant increase in neutralizing antibodies against influenza virus nor against NA or HA in mice vaccinated with WR-NP or WR-flu versus WR alone (data not shown).

Functional profile and polyfunctionality of WR-NP and WR-flu induced CD4$^+$ and CD8$^+$ T cell responses

To determine the phenotypic characteristics of the T cell populations activated after immunization with the DNA-prime/poxvirus-boost protocol, we utilized multiparameter intracellular flow cytometry staining (ICS) analysis to identify influenza virus-specific T cell responses. Splenocytes from four mice per group were cultured overnight and then stimulated with the NP-specific peptide in the presence of brefeldin for 6 h.

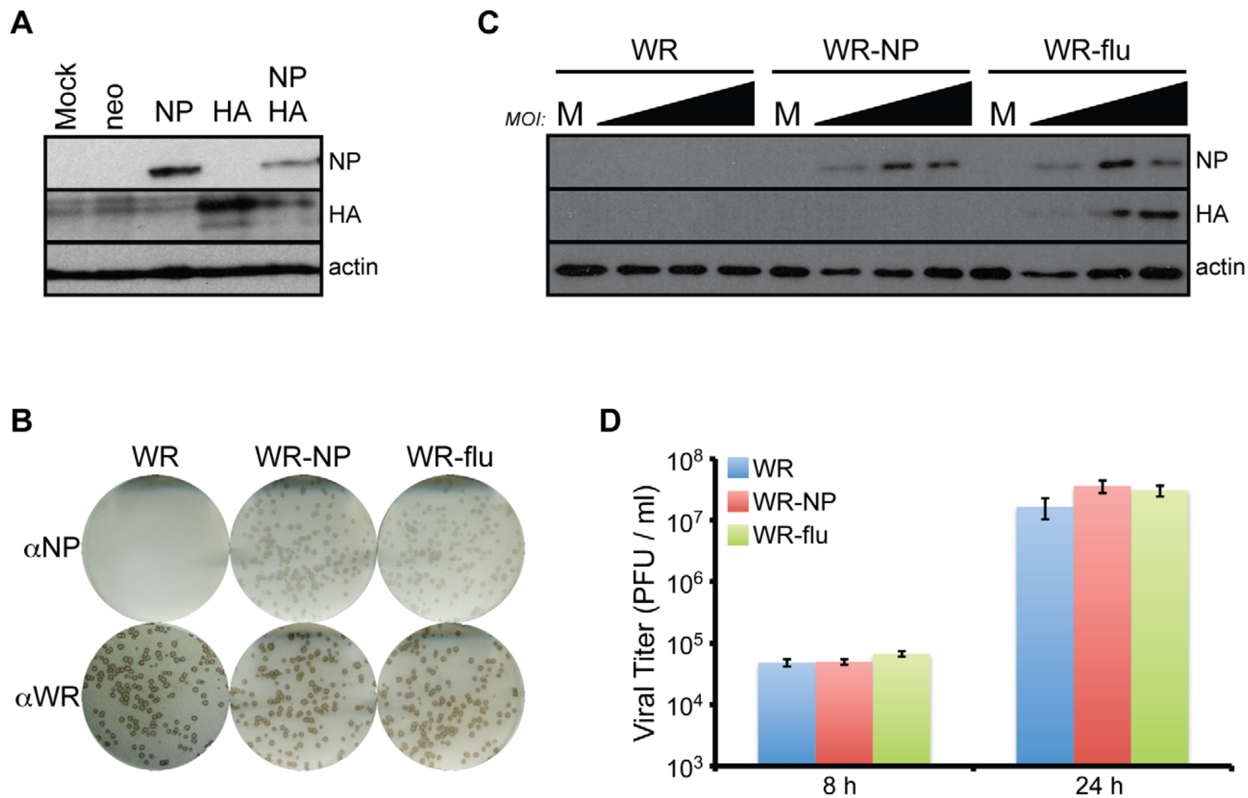

Figure 2. Recombinant viruses synthesize NP and HA proteins and replicate to similar levels. (A) 10 µg of pCIneo (neo) vectors containing the NPmix (NP) or NA-HA (HA) inserts were transfected into BSC40 cells. For double transfections, 5 µg of each vector was used. 48 h post-transfection, cells were lysed and levels of influenza virus proteins were determined using antibodies for NP and HA. (B–D) BSC40 cells were infected with WR, WR-NP, or WR-flu at an MOI of 0.01 (B, D), 0.1, 1, or 10 (C) PFU/cell. (B) At 24 h p.i., cells were fixed and plaques were stained with NP antibody. (C) At 24 h p.i., the levels of NP and HA in the lysates were determined by immunoblot analysis. (D) At 8 or 24 h p.i., infectious virus present in the cells was measured in triplicate standard plaque assay on BSC40 cells.
doi:10.1371/journal.pone.0025938.g002

As shown in the pie charts of Figure 5, both the WR-NP and WR-flu vaccination protocols induced a greater magnitude of T cell responses as compared to WR alone. WR-NP induced a ~2.9-fold increase in T cell response as compared to WR, and WR-flu induced a ~9.1-fold increase. While T cells secreting cytokines following WR-NP vaccination were mostly $CD8^+$, WR-flu induced both $CD4^+$ and $CD8^+$ T cells. However, the $CD8^+$ T cells activated following WR-NP infection secreted both IFNγ and TNFα, while the T cells following WR-flu vaccination secreted mainly IFNγ.

The simultaneous measurements of three secreted cytokines allows for the assessment of the quality of the vaccine-induced $CD4^+$ and $CD8^+$ T cell responses. Upon analyzing concurrent secretion of IFNγ, TNFα, and IL2 by T cells, seven distinct influenza virus-specific $CD4^+$ and $CD8^+$ T cell populations can be identified. To further characterize the immunogenicity triggered in each immunized group, we assessed polyfunctional T cell responses. Regarding $CD4^+$-secreting T cells (Figure 6A), we observed only a significant response following WR-flu vaccination, and this profile was not polyfunctional; $CD4^+$ T cells secreted only IFNγ. However, WR-flu vaccination increased the overall magnitude of the $CD4^+$ T cell response ~19-fold (Figure 6B). With regard to $CD8^+$ T cells, we did observe polyfunctionality following vaccination with WR-NP, but not WR-flu (Figure 6C). While vaccination with WR-flu elicited significantly higher levels of $CD8^+$ T cells secreting IFNγ as compared to WR, vaccination with WR-NP exhibited significant levels of IFNγ/TNFα-secreting $CD8^+$ T cells. While not statistically significant, WR-NP vaccination also lead to high levels of triple, IFNγ/TNFα/IL2-secreting, $CD8^+$ T cells. As compared to vaccination with WR, the magnitude of the $CD8^+$ T cell response was ~3.5-fold higher for WR-NP and ~5.7-fold higher for WR-flu (Figure 6D).

Overall, these results indicated that immunization with WR-NP induced a polyfunctional influenza virus-specific T cell response, while immunization with WR-flu was monofunctional with $CD4^+$ and $CD8^+$ T cells only producing IFNγ. Nevertheless, we show that WR-NP and WR-flu immunization improved the magnitude and quality of the anti-influenza virus response compared to WR. However, even though we observed an adaptive immune response characterized by polyfunctional T cells, we did not observe a memory response 53 days post- vaccination with the single NP peptide used as a test system (data not shown). This apparent lack of a memory response could be associated with the fact that we did not observe the production of neutralizing antibodies.

Virus load is reduced in the lungs of mice upon challenge with influenza virus following vaccination

Upon measuring the adaptive immune response induced by vaccination with the recombinant DNA and vaccinia virus constructs, next we determined what effect vaccination had on challenge with influenza virus. Two weeks following the end of the vaccination protocol, mice from each vaccination group, along with 9 mice that had received PBS alone, were infected intranasally with $10 \times LD_{50}$ of the A/WSN/33 (WSN), A/PR/8/

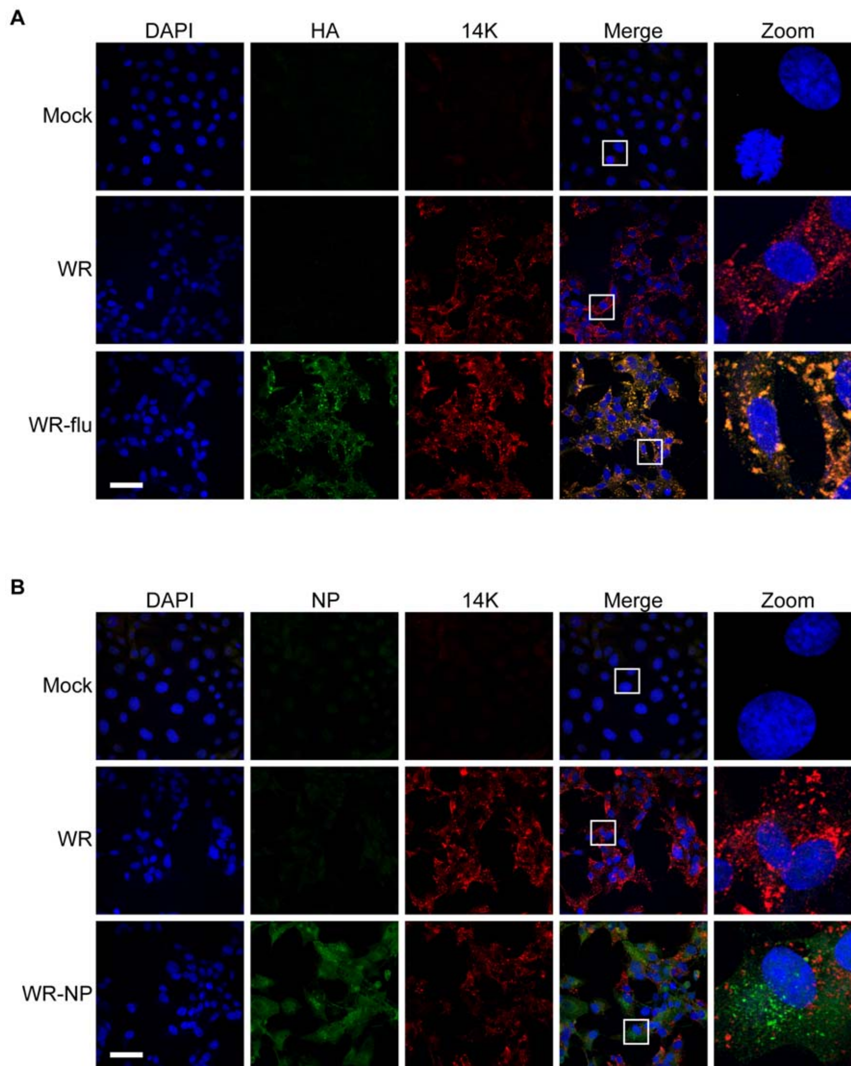

Figure 3. Recombinant influenza virus proteins are present in the cytosol of infected cells. BSC40 cells were infected with WR, WR-flu (A), or WR-NP (B) at an MOI of 1 PFU/cell. At 24 h p.i., cells were fixed with 2% paraformaldehyde, permeabilized, and stained with DAPI or antibodies recognizing influenza virus HA (A), NP (B), or vaccinia virus 14 K (A27 gene). Bar = 25 μm.
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34 (PR8), or A/California/07/09 (CA) strains of influenza virus, corresponding to 10^4, 10^3, or 10^5 PFU/mouse, respectively. Each day following challenge, mice were weighed and sacrificed when body weight reached at least 75% of their starting weight. While vaccination with WR-NP or WR-flu did not protect mice from death or weight loss, mortality was generally delayed by 1–2 days (Figure 7). However, we did observe a significant decrease in lung viral titers following vaccination with WR-NP and WR-flu as compared to WR (Figure 8). WR-NP vaccination decreased lung viral titers by ~5.2-, ~55-, and ~8.6-fold upon challenge with WSN, PR8, and CA, respectively. Vaccination with WR-flu lead to a significant decrease in lung viral titer only upon challenge with PR8 (~33-fold). We hypothesize that vaccination with WR-NP resulted in viral titers lower than vaccination with WR-flu because we observed a higher quality, polyfunctional T cell response during WR-NP vaccination.

Taken together, our study indicates that recombinant vaccinia viruses containing human T cell epitopes for influenza virus proteins embedded in NP or the conserved N-terminal of NA fused with the conserved C-terminal of HA can elicit specific T cell immune responses following heterologous vaccination utilizing a DNA-prime/poxvirus-boost protocol. While we observed significant levels of T cells secreting cytokines in a polyfucntional manner, this was not sufficient to protect mice from mortality; however, we did observe a decrease in lung viral replication upon vaccination with the recombinant viruses.

Discussion

The vaccination strategy of heterologous prime-boost vaccination to elicit specific protective T cell responses has been widely established ever since its ground-breaking conception almost two decades ago [26], in which an influenza virus prime followed by vaccinia virus boost provided protection against *Plasmodium* infection in mice. Since this first evidence that vaccinia virus boost was required for strong activation of specific T cells, many different techniques have arisen, including the use of different viral vectors, DNA, and protein, in the presence and absence of adjuvant. In the present study, we constructed two different vaccinia virus vectors expressing recombinant influenza virus

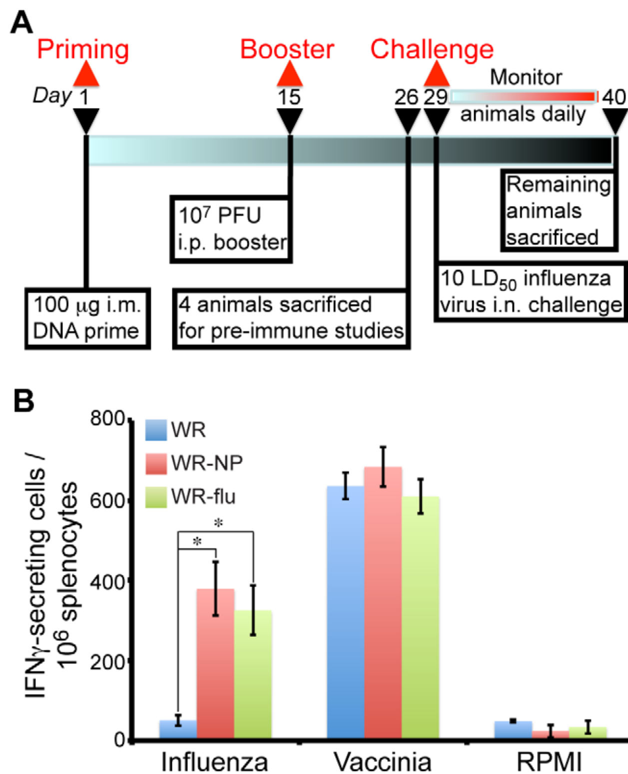

Figure 4. Immunogenicity of WR-NP and WR-flu in mice. (A) Immunization schedule. BALB/c mice were primed with 100 µg of DNA (either 100 µg pCIneo-NPmix or empty vector, or 50 µg pCIneo-NPmix+50 µg pCIneo-HANA) intramuscularly (i.m.) at the start of the vaccination protocol. Two weeks later, the mice were boosted by intraperitoneal (i.p.) infection with 10^7 PFU of WR, WR-NP, or WR-flu. Eleven days post-boost, four mice were sacrificed to analyze the adaptive immune response. The remaining mice were challenged with influenza virus A/WSN/33, A/PR/8/34, or A/California/07/09. (B) Vaccine-elicited T cell responses of splenocytes 25 d after the start of the immunization protocol were measured in triplicate for each immunization group by fresh IFNγ ELISPOT assay following stimulation with influenza virus NP peptide TYQRTRALV, vaccinia virus E3 peptide VGPSNSPTF, or RPMI media alone. The results represent the mean number of IFNγ-secreting cells per 10^6 splenocytes from three biological replicates ± standard deviations. *P* values from a two-tailed t test assuming nonequal variance are indicated (*, $P<0.05$).
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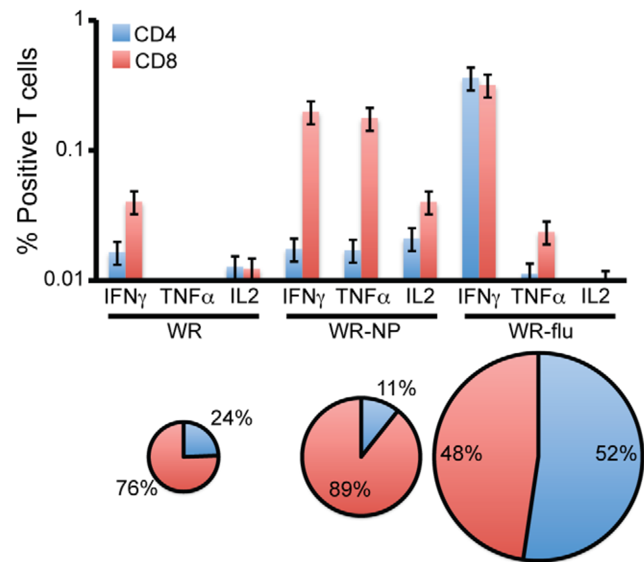

Figure 5. Phenotypic analysis of vaccine-induced $CD4^+$ and $CD8^+$ T cell responses. The same groups of splenocytes as described in Figure 4 were stimulated with the influenza virus NP-specific peptide and analyzed using polychromatic flow cytometry. The results represent the mean number of $CD4^+$ and $CD8^+$ T cells secreting IFNγ, TNFα, or IL2 in each immunization group using three biological replicates ± standard error. The background from unstimulated controls was subtracted in all cases. The pie charts represent the magnitude and percentage of $CD4^+$ and $CD8^+$ T cells secreting cytokines in each immunization group.
doi:10.1371/journal.pone.0025938.g005

proteins, based on an NP backbone, which is one of the more well-conserved influenza virus proteins. Also utilized in our vaccine design were the N-terminal of NA and the C-terminal of HA, which are the well-conserved regions of H5N1 influenza virus subtypes. These recombinants were part of a DNA-prime/poxvirus-boost vaccination strategy in which a mammalian expression vector expressing the same sequences that were inserted into the vaccinia virus genome was used for the DNA priming stage. While our vaccination strategy elicited an immune response marked by $CD4^+$ T cells expressing IFNγ, $CD8^+$ T cells expressing IFNγ and TNFα, and resulted in decreased viral replication in the lungs of influenza-virus infected mice, these mice were not protected against mortality.

There has been some disparity regarding the ability of influenza virus NP in vaccine constructs to protect mice from mortality. A recent report describes a human vaccine trial using a vaccinia virus-based vaccine encoding the NP and M1 proteins against influenza virus [24]. This vaccine protocol elicited increased IFNγ-secreting $CD8^+$ T cells in response to NP and M1. On the other hand, Lawson, et al., observed no protection following vaccination with vaccinia virus constructs encoding NP, even though they observed a reduction in lung viral load [20]. Ohba, et al. described a DNA-based vaccine based on the N-terminal of NP and observed protection upon challenge with influenza virus [18]. Saha, et al. also observed an improvement in vaccination using NP when used in conjunction with the VP22 gene of herpes simplex virus [17]. Finally, Altstein, et al. developed a vaccine using recombinant NP that included a proteolysis signal which provided some protection, especially when challenged with low doses of influenza virus [19]. Therefore, it was our goal to improve upon vaccine designs based on NP using a strategy in which we included human T cell epitopes for other influenza virus proteins within the NP backbone. A strategy based on multiple epitopes was successfully used to vaccinate against Japanese encephalitis virus in mice [27], as well as against hepatitis B virus and SIV [28,29]. While we did not specifically test if every influenza virus epitope was correctly processed and presented, we know that the NP peptide used for the ELISPOT and ICS experiments was correctly presented. Following rationale design principles, we expect that the other epitopes would be presented [30]. However, future experiments with overlapping peptides for the entire influenza proteins to fully characterize T cell epitopes, perhaps using a transgenic humanized MHC class I mouse, should be performed. In addition to the multi-epitope NPmix recombinant vaccinia virus, we also constructed a recombinant virus encoding the conserved regions from HA and NA of H5N1 viruses to examine if the inclusion of more T cell epitopes would provide cross-clade protection when challenged with H1N1 viruses.

Although we observed that T cells were able to be stimulated to produce cytokines with influenza virus antigens, we did not observe a protection from lethality of the virus. A number of other

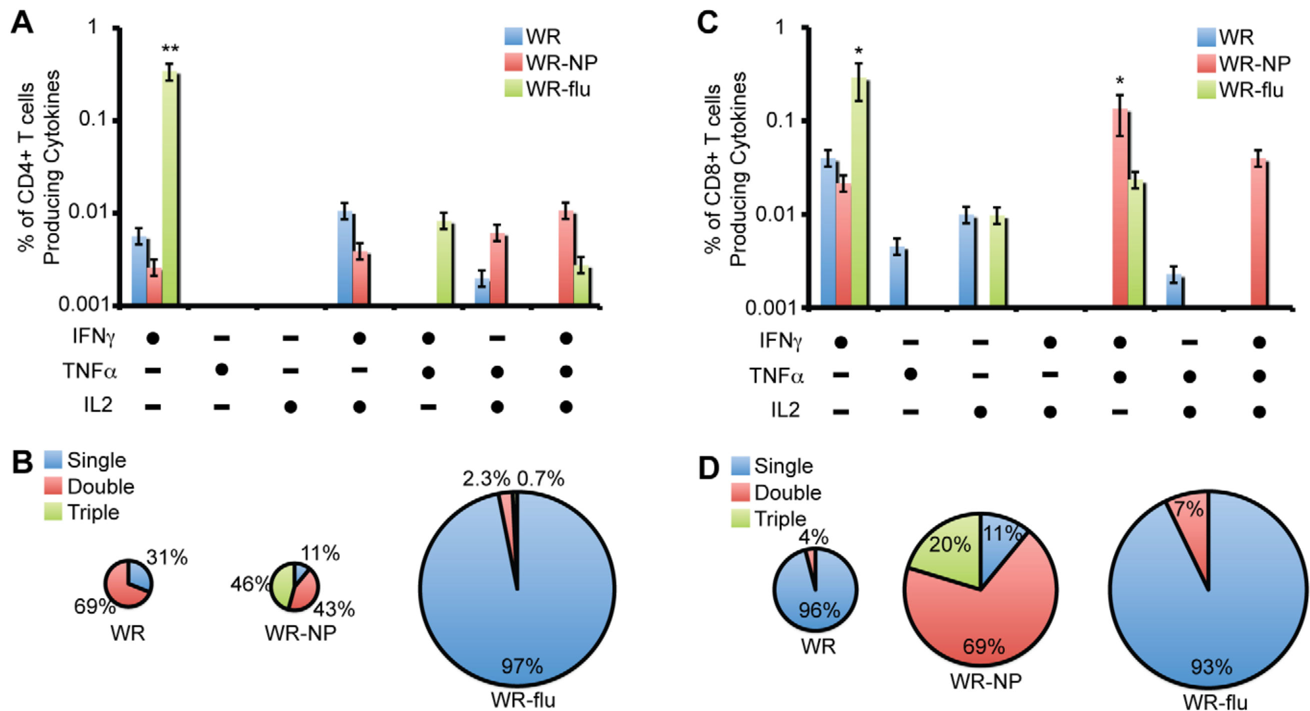

Figure 6. Polyfunctionality of influenza virus-specific CD4$^+$ and CD8$^+$ T cells. (A, C) Functional composition of CD4$^+$ (A) or CD8$^+$ (C) T cells responses against influenza virus NP peptide based on the secretion of IFNγ, IL-2, or TNFα. All the possible combinations of the responses are shown on the x-axis, whereas the percentages of the functionally distinct cell populations are shown on the y-axis. Bars correspond to the fraction of different functionally distinct T-cell populations within total CD4$^+$ or CD8$^+$ populations. Responses are grouped and color-coded on the basis of the number functions. (*, $P<10^{-5}$; **, $P<10^{-25}$) (B, D) The pie chart summarizes the data and each slice of the pie correspond to the fraction of CD4$^+$ T cells with a given number of functions within the total CD4$^+$ (B) or CD8$^+$ (D) T cell populations. The size of the pie chart represents the magnitude of the specific influenza virus immune response induced.
doi:10.1371/journal.pone.0025938.g006

studies also had difficulties in showing that HA and NP could provide protection from lethality [31,32,33,34,35,36]. It may have been that our immunization scheme was sub-optimal, or most importantly, we used the mouse as a test animal. It should be highlighted that human T cell epitopes were used in the immunization regimen, and hence, these epitopes in the mouse might not be properly presented within MHC class I to stimulate T cells. This is why we had used only a known NP mouse epitope for stimulation of splenocytes for ELISPOT and ICS experiments. Also, it may be that the vaccinia virus boost suppressed the presentation of NP-generated T cell epitopes, as previously reported [37]. Since we challenged mice with $10\times LD_{50}$, this may have been too high of a dose to observe the protective effects of vaccination. While we did observe a decrease in viral replication in the lungs of mice vaccinated with WR-NP and WR-flu, the amount of virus in the lungs was still greater than 10^4 PFU/ml, which is high enough to cause lethality in mice [38]. It is likely that mouse lung pathology would be similar in all vaccination groups, and this would have contributed to mortality. Additionally, since our vaccine construct utilized a muti-epitope design, we may have observed increased immunogenicity upon stimulating splenocytes or T cells, if instead of the single NP peptide that we used pools of peptides spanning the influenza virus NP, M1, NS1, PB1, and PA proteins, similar to previously described studies [10,11].

Many approaches exist to improve the vaccine design presented in this study, one of them being the use of adjuvants. Rapamycin has been shown to have immunostimulatory effects by improving antigen presentation and aiding in cytokine production from macrophages and dendritic cells. Furthermore, it is able to improve on the generation of memory CD8$^+$ T cells following vaccination with a poxvirus vector [39,40]. Recent results have shown that STING plays an important role in the generation of IFNγ-secreting CD8$^+$ T cells [41]. Upon vaccination with a DNA vaccine, wild-type mice generated significantly increased amounts of IFNγ as compared to STING$^{-/-}$ mice in response to peptide stimulation. These results suggest that the development of an adjuvant to stimulate STING during vaccination would augment the immune response to antigen presentation. In addition to the use of adjuvants, many other future directions exist for the improvement of our vaccine design to move closer to a universal influenza virus vaccine. Our constructs that elicit T cell responses could be combined with a vaccine specifically design to elicit a humoral B cell-producing neutralizing antibody response. Secondly, we would have to test the vaccine for its efficacy in protection after challenge with other influenza virus subtypes, such as H5N1, H3N2, H9N2, and H7N7, all of which contain conserved NP genes. It would also be prudent to challenge with influenza B subtypes, since seasonal influenza virus vaccines contain an attenuated B subtype virus. Since challenge with wild-type H5N1 strain of influenza virus requires high biosafety levels, we could challenge with a recombinant strain of PR8 that expresses HA and NA from H5N1 viruses.

In summary, our study describes the design and development of recombinant DNA and vaccinia virus vaccine constructs for use in a DNA-prime/poxvirus-boost vaccine protocol as proof-of concept protocol for a universal vaccine candidate against influenza virus. Since these experiments were performed with the aim to show proof-of-principle, we used the replication-competent WR strain

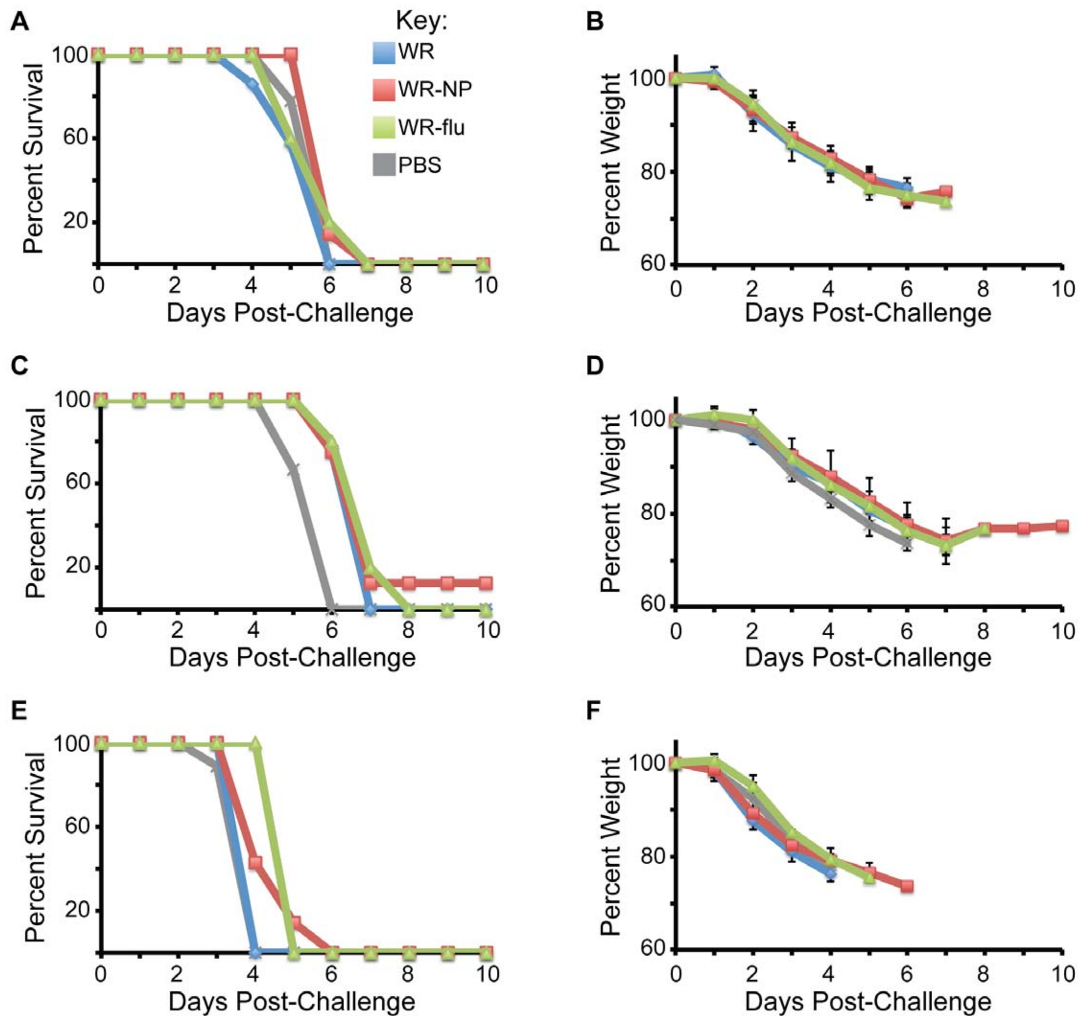

Figure 7. Vaccination delays mortality in influenza virus-challenged mice. Two weeks following the end of the vaccination protocol, 5–8 mice from each vaccination group and 9 mice from control PBS-inoculated animals were infected with $10\times LD_{50}$ of the A/WSN/33 (A, B), A/PR/8/34 (C, D), or A/California/07/09 (E, F) strains of influenza virus. Mice were sacrificed when body weight reached 75% of starting weight.
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of vaccinia virus; any future vaccine trials, especially in humans, non-replicative and safe, attenuated vaccinia virus strains, such as MVA or NYVAC [12] must be used. Our construct contains multiple T cell epitopes against influenza virus antigens, which were aimed to broaden and increase immune responses upon influenza virus challenge. While our construct elicited an immune response marked by increased $CD4^+$ and $CD8^+$ T cells expressing IFNγ or TNFα, and resulted in decreased viral replication in the lungs of influenza-virus infected mice, these mice were not protected against mortality. Taken together, our results suggest that a human mutli-epitope vaccine design in a DNA prime/poxvirus boost approach can stimulate the breath and quality of specific T cell immune responses to influenza virus antigens leading to reduction in viral load in the lungs. This reduction might play an important role to limit influenza virus replication in a natural infection and to develop host immune resistance. The protocol of immunization described here can be further improved through the use of a recombinant attenuated vaccinia virus strain, like MVA, and combination with adjuvants and vectors inducing neutralizing antibodies to influenza virus proteins. Hence, a vaccine construct that elicits broad T cell responses and limits virus replication to some extent, as described here, could be combined with a second vaccine that elicits a neutralizing antibody response to further restrict the virus load. Together, such a vaccine strategy could bring us closer to creating a universal vaccine against influenza virus infection.

Materials and Methods

Cells, viruses, and infections

BSC40 monkey kidney epithelial cells and Madin-Darby canine kidney (MDCK) cells (ATCC) were grown as monolayers in supplemented high glucose Dulbecco's modified Eagle's medium (hgDMEM) supplemented to contain 2 mM L-glutamine, 0.1 mM nonessential amino acids, Fungizone Amphotencin B (0.5 μg/ml), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml) and 10% newborn calf serum (NCS) or fetal calf serum (FCS) (Sigma), respectively. Wild-type vaccinia virus (strain WR) and recombinant WR viruses expressing influenza virus proteins were grown and plaque-purified on monkey BSC-40 cells, purified by two 45% (w/v) sucrose cushions, and titrated on BSC40 cells by plaque assay.

Near-confluent monolayers of cells were mock-infected or infected with vaccinia virus diluted in supplemented hgDMEM

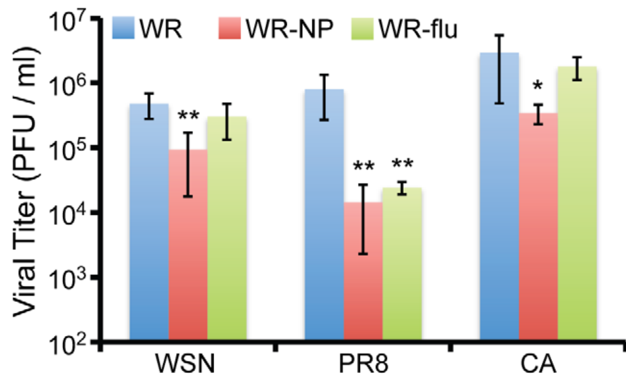

Figure 8. Vaccination reduces the levels of infectious virus in the lungs of influenza virus-challenged mice. Two weeks following the end of the vaccination protocol, mice from each vaccination group were infected with $10 \times LD_{50}$ of the A/WSN/33 (WSN), A/PR/8/34 (PR8), or A/California/07/09 (CA) strains of influenza virus. Mice were sacrificed at 5 d p.i. (except for some mice challenged with CA that died at 3–4 d p.i), and diaphragmatic lung lobes were isolated and homogenized. Levels of infectious virus were determined in triplicate by plaque assay on MDCK cells. The results represent the mean activity of 5–8 independent samples per group ± standard deviation. *P* values from a two-tailed t test assuming nonequal variance are indicated (*, $P<0.05$; **, $P<0.01$).
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to the indicated multiplicity of infection (MOI). After 1 h of adsorption at 37°C, virus and medium was removed. Fresh supplemented hgDMEM containing 2% NCS was added to the cells and infections were allowed to proceed at 37°C until the indicated time post-infection.

Mouse inoculations

At day 1, six- to eight-week old mice BALB/c mice (Harlan) were anesthetized with isoflurane and injected intramuscularly with PBS alone or 100 µg of empty pCIneo vector or containing NPmix, NA-HA, or both (n = 19–27). At day 15, mice were anesthetized and infected intraperitoneally with 10^7 PFU of WR, WR-NP, or WR-flu. At day 26, four animals were sacrificed for adaptive immune response analysis. At day 29, the remaining mice were anesthetized and challenged intranasally with $10 \times LD_{50}$ of the A/WSN/33, A/PR/8/34, or A/California/07/09 strains of influenza virus (n = 5–8 for each group). Animals were weighed each day for ten days and sacrificed when they lost at least 25% of their starting body weight. Blood and lung tissue was collected from each mouse at the time of sacrifice. All experiments were performed in a specially separated negative-pressure HEPA (high-efficiency particulate air)-filtered biosafety level 2 laboratory. All animals were handled in strict accordance with good animal practice as defined by the relevant national, international, and/or local animal welfare bodies, and with the Royal Decree (RD 1201/2005). All animal work was approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of the Centro Nacional de Biotecnología (CNB-CSIC). Permit number: 10015.

Generation and verification of recombinant vaccinia viruses

Genes for expression of the recombinant influenza virus proteins, NPmix and NA-HA (Figure 1B) were designed by us and sent to GeneArt® for synthesis and insertion into the pBlueScript vector. During the optimization process for the sequences, the following cis-acting sequence motifs were avoided: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; ARE, INS, CRS sequence elements; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites, branch points; and AscI, FseI, NotI, PmlI and SalI sites. The influenza virus genes were codon optimized and contain either an N-terminal FLAG tag (NPmix) or a C-terminal His tag (NA-HA). The genes were individually cloned into pCIneo for mammalian expression, and also cloned into the TK locus of vaccinia virus using the transfer vector pCyA. In this vector NPmix was inserted alone, or in front of NA-HA, both of which were driven by the vaccinia virus early/late promoter (pE/L). Homologous recombination in the wild-type vaccinia virus strain WR was performed as previously described [42]. Recombinant virus containing NPmix was called "WR-NP," and virus containing both NPmix and NA-HA was called "WR-flu". Expression of the influenza genes was driven by a synthetic early/late virus promoter (Figure 1A).

Protein analyses and plaque assays

Following vaccinia virus infection or transfection with the pCIneo expression vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions, cells were lysed at the indicated times p.i. in disruption buffer (0.5% Triton X-100, 50 mM KCl, 50 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 1× Complete protease inhibitor (Roche), 25 mM β-glycerophosphate, 1 mM Na_3VO_4). Total protein content was determined for clarified cell lysates by using the BCA protein assay kit (Pierce). Lysates were separated by SDS-PAGE with the same amount of total protein being loaded into each lane and then transferred onto nitrocellulose membranes. Immunoblots were blocked for 1 h in PBS containing 0.5% Tween 20 and 5% nonfat dry milk, washed in PBS containing 0.05% Tween 20, and incubated at 4°C overnight with a mouse monoclonal actin antibody (MP Biochemicals), a rabbit polyclonal NP antibody (a kind gift from Adolfo García-Sastre), or a sheep polyclonal HA antibody (provided by the National Institute for Biological Standards and Control) in PBS containing 0.5% Tween 20 and 1% nonfat dry milk. Subsequently, membranes were washed and incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-sheep immunoglobulin G (Sigma), and bound antibodies were detected with Amersham ECL Western blotting detection reagent (GE Healthcare).

At the indicated times post-infection, vaccinia virus-infected cells and cell media supernatant were collected and assayed in triplicate for viral yield by standard plaque assay on BSC40 cells. For influenza virus-infected mice, diaphragmatic lung lobes from each animal were weighed, homogenized in PBS, and samples were then assayed in triplicate for viral yield by standard plaque assay on MDCK cells. Viral yields were calculated according to the formula: $\log \text{yield}_{t=x} = [\log_{10}(\text{PFU/ml})_{t=x}] / [\log_{10}(\text{PFU/ml})_{t=0}]$, where *t* is time and *x* is the time post-infection.

For immunostaining of vaccinia virus plaques, infected cells were fixed 24 h p.i. with 1:1 methanol:acetone, washed in PBS, then incubated for 2 h with primary antibodies for vaccinia virus (WR strain) or influenza virus NP diluted in PBS containing 3% FCS. Cells were then washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit diluted in PBS containing 3% FCS. The spots were developed in PBS containing 1 mg/ml of the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma) with 0.03% hydrogen peroxide and 0.03% nickel sulfate.

Immunofluorescence

Following influenza virus infection of cells cultured on glass coverslips, cells were fixed in 2% paraformaldehyde in PBS, permeabilized in 0.1% Triton X in PBS, washed with 2.5% FCS and 10 mM glycine in PBS, and then blocked with 10% FCS in PBS. Cells were then incubated for 2 h with primary antibodies recognizing influenza virus NP, HA, or vaccinia virus 14 K (A27 gene) diluted in 10% FCS in PBS. Subsequently, cells were washed and incubated for 1 h with Alexa 488- or 586-conjugated anti-rabbit immunoglobulin G (IgG) (Invitrogen), or fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (Jackson Immunoresearch). Cells were washed and incubated for 20 min with DAPI (4′,6′-diamidino-2-phenylindole) (Sigma) and mounted onto glass slides using ProLong Antifade reagent (Invitrogen). Cells were imaged with the Leica TCS SP5 multispectral confocal microscope (Leica Microsystems) using photomultipliers for laser lines 405, 488, and 561 nm. LAS AF v.2.3.6 software was used for image acquisition.

IFNγ ELISPOT assay

The vaccine-specific cellular immune response in mice was determined using ELISPOT assay measuring the secretion of IFNγ by splenocytes after stimulation with a peptide specific for influenza virus NP (TYQRTRALV), as previously described [10,11]. Briefly, eleven days after boosting with vaccinia virus, mice were sacrificed and splenocytes depleted of red blood cells were isolated. 10^6 splenocytes were plated in triplicate in 96-well nitrocellulose-bottomed plates previously coated with 6 mg/ml of anti-mouse IFNγ mAb R4-6A2 (Pharmingen). Cells were stimulated with the influenza virus-specific peptide (2 μg/ml), a positive control peptide against the E3 protein of vaccinia virus (VGPSNSPTF, 5 μg/ml), or without peptide as a negative control. 48 h after stimulation, cells were washed and those secreting IFNγ were developed using a biotin-streptavidin sandwich system and counted using a stereomicroscope.

Intracellular Cytokine Staining (ICS) assay

Multiparameter flow cytometry was performed as previously described [10,11]. Briefly, 10^6 splenocytes were stimulated with the peptides described above in the presence of 1 μl/ml Brefeldin (BD Bioscience) for 6 hours in a 96-well plate. The cells were then washed, stained with the LIVE/DEAD Kit (Invitrogen), and Fc receptors were blocked using CD16/CD32 antibodies (BD Biosciences). The cells were then stained with the surface-specific mouse antibodies, CD4-Alexa700, CD3-FITC, and CD8-PerCP (BD Biosciences). Cells were permeabilized using the BD Cytofix/Cytoperm Kit and were stained for the intracellular cytokines, IFNγ-APC, IL2-PE and TNFα-PECy7. Sample acquisition was performed with an LSRII Flow Cytometer and FACSDiva software (BD Biosciences) and was further analyzed with FlowJo (Tree Star). All statistical analysis was performed as previously described [10,11].

Supporting Information

Figure S1 Alignment of nucleoprotein (NP) from various influenza virus subtypes. The NP protein of five influenza virus strains of different subtypes (H1N1, H2N3, H5N1, H9N2, and H7N7, from top to bottom) was aligned. The conserved region, as indicated by red underline, from amino acids 19–498, was used as the backbone for the NPmix multi-epitope protein.
(TIF)

Figure S2 Alignment of hemagglutinin (HA) and neuraminidase (NA) from different strains of the H5N1 influenza virus subtype. The NA protein (A) or HA protein (B) from seven or eight different H5N1 viruses were aligned. The conserved N-terminal of NA (amino acids 107–231) and conserved C-terminal of HA (amino acids 347–511), as indicated by red underline, were fused and used for the NA-HA construct along with NPmix in the WR-flu recombinant virus.
(TIF)

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Author Contributions

Conceived and designed the experiments: AG PH ME. Performed the experiments: AG SG AV. Analyzed the data: AG CS. Contributed reagents/materials/analysis tools: CG. Wrote the paper: AG ME.

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