Pre-Clinical Development of Viral-Vectored Transmission-Blocking Malaria Vaccines

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ABSTRACT

Malaria transmission-blocking vaccine candidate antigens have been developed to induce antibodies using different delivery systems, mainly protein-in-adjuvant formulations, independently in various laboratories giving varied transmission-blocking activity (TBA). However, only one candidate antigen has been tested in clinical trials. In order to advance the most efficacious target(s) for possible clinical development, a rank order of the leading antigens based on TBA in a head-to-head comparison using a single delivery platform was made. Candidate antigens, AnAPN1, PfsHAP2, Pfs230-C, Pfs25, and Pfs48/45 (with or without N-glycosylation site substitution), were generated as recombinant viral-vectored vaccines using simian adenovirus and modified vaccinia Ankara and administered to mice in a heterologous prime-boost regimen. Vaccine-induced antibody responses were induced to all except PfsHAP2 were maintained up to ten and a half months post-boost. TBA was assessed at the peak response against *Plasmodium falciparum* NF54 laboratory strain and African field isolates by *ex vivo* membrane feeding assays in *Anopheles stephensi* and *A. gambiae* respectively. Antibodies to three antigens [Pfs230-C, Pfs25 and Pfs48/45$_{+NGln}$] had TBA against *P. falciparum* NF54, and those against Pfs230-C and Pfs25 consistently showed efficacy regardless of the parasite exposure in both mosquito species. Further analysis of antibody responses to these two candidate antigens showed concentration-dependent efficacy against *P. falciparum* field isolates. In a rabbit study, responses to Pfs230-C, Pfs25 and Pfs48/45$_{+NGln}$ also showed IgG concentration-dependent efficacy. To assess TBA against AnAPN1, antibody responses to three fragments were tested. TBA was observed only against N-terminal 135 amino acid fragment. Pfs230-C and Pfs25 were generated as fusion vaccines using either a self-cleaving or glycine-proline linker sequence. Comparable antibody responses were induced between the two fusion strategies that had synergistic effects at inhibiting *P. falciparum* NF54 development in *A. stephensi*.

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ABBREVIATIONS

3D7	*Plasmodium falciparum* clone of parasite isolate NF54
αα	amino acid
AnAPN1	*Anopheles* alanyl aminopeptidase N 1
ASC	Antibody-secreting cell
AU	Antibody unit
BGH_Poly A	Bovine Growth Hormone Polyadenylation sequence
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CEF	Chicken embryonic fibroblasts
CH	Cycloheximide
ChAd63	Chimpanzee Adenovirus serotype 63
ChAd63-MVA	Heterologous prime-boost 8week regime of ChAd63 followed by MVA (individually expressing the same transgene)
CMV_LP	Cytomegalovirus long promoter
CRD	Cysteine-rich domain
DFA	Direct feeding assay
DMFA	Direct membrane feeding assay
DSF	Direct skin feeding
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot assay
F2A	Foot and mouse disease 2A self-cleaving peptide
FCS	Foetal calf serum
FMDV	Foot and mouth disease virus
GP	Glycine-proline
GPI	Glycophosphatidylinositol
HEK293	Human embryonic kidney 293 cells
HEK293E	HEK293 Epstein Barr virus nuclear antigen 1 modified
IC50	Half maximal inhibitory concentration: concentration of antibody required to inhibit 50% of oocyst development
i.m.	Intramuscular route of administration
i.p.	Intraperitoneal route of administration
ID PCR	Polymerase chain reaction to identify the presence of an antigen
IgG	Immunoglobulin G
IL-12p40	Interleukin 12 p40 subunit
IU	Infectious units
IVOA	*In vitro* ookinete development assay

LIPS	Luciferase immunoprecipitation system
MVA	Modified vaccinia virus Ankara
NCBI	National Centre for Biotechnology Information
NF54	*Plasmodium falciparum* patient isolate
NGln	N-linked glycosylation
-NGln	N-glycosylation sites mutated
+NGln	N-glycosylation sites intact
OVA	Ovalbumin
Pbs	*Plasmodium berghei* sexual-stage antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
Pfs	*Plasmodium falciparum* sexual-stage antigen
PFU	Plaque forming units
PH	Phenylhydrazine
PPP	Three shot protein-in-adjuvant vaccine regimen
Pvs	*Plasmodium vivax* sexual-stage antigen
rAnAPN1	Recombinant N terminal AnAPN1 protein vaccine
RBCs	Red blood cells
REFSEQ	Reference sequence
SMFA	Standard membrane feeding assay
SP	Signal peptide
TBA	Transmission-blocking activity
TBE	Transmission-blocking efficacy: inhibition of both infection intensity and prevalence
TBI	Transmission-blocking immunity
TBV	Transmission-blocking vaccine
TMD	Transmembrane domain
tPA	tissue Plasminogen Activator

1. Introduction

1.1. Hypothesis

Transmission-blocking vaccine candidate antigens based on *Plasmodium falciparum* have been characterised and developed independently in different laboratories across the globe in a number of expression systems. In this study, I aim to comparatively assess the functional ability of antibodies raised to five leading candidate antigens using a single expression and vaccine delivery platform. Recombinant adenovirus and poxvirus viral-vectored vaccines will be generated and used to induce antibodies that will be assessed for their ability to inhibit the development of *Plasmodium falciparum* in compatible mosquito vectors. This assessment will be utilised to narrow down the best candidate(s) antigens which will be further assessed, in combinations, to optimise blockade of parasite development.

1.2. Malaria

Malaria is a deadly infectious disease that causes high numbers of morbidity and mortality, especially in children under 5years and pregnant women, and contributes to reduced work capacity in adults [1]. It is estimated that over 3billion individuals are under the threat of disease with the largest population at risk living in sub-Saharan Africa [1, 2]. The disease is transmitted from one vertebrate host to another through a mosquito bite. This disease is caused by the apicomplexan parasite *Plasmodium*, where *P. falciparum* and *P. vivax* are the major human pathogens contributing significantly to the global burden of disease with the former accounting for the severe form of disease and mortality. Other human malarias are caused by the species *malariae, ovale* and the most recently recognised zoonotic *knowlesi* mainly reported in Southeast Asia [3-7]. Malaria is preventable and treatable with growing evidence of reduction in disease incidence [8-10] even though this appears not to be a uniform scenario especially in sub-Saharan Africa [11-13]. Therefore, there is need for

interventions that will enable the uniform decline of disease burden in endemic areas. One such intervention is in the form of vaccines, as this due process has been shown to be effective against a number of infectious diseases (reviewed in [14-16]). To date, however, there is no vaccine available for malaria, but there are increasing efforts to develop efficacious vaccines. More so, development of effective malaria vaccines remains an important but elusive goal. Hence vaccine strategies need to be developed that would curtail infection, disease and transmission resulting in reduced mortality, control, elimination and/or eradication.

1.2.1. Infection, disease and transmission

The life cycle of *Plasmodium* can be described in three stages; pre-erythrocytic, erythrocytic and sporogonic and involves two hosts; vertebrate secondary host and invertebrate mosquito definitive host. These stages are distinct and involve the parasites establishment first, in the vertebrate host after a mosquito bite resulting in infection and invasion; then successful replication and propagation in red blood cells (RBCs) leading to disease onset; and finally differentiation and transmission to susceptible mosquitoes.

1.2.1.1. Infective stages

Infection begins when an infected female mosquito, mainly of the anopheline species, takes a blood meal and injects motile sporozoites into the skin or blood stream. These sporozoites then migrate to the liver where the invade hepatocytes. These liver-stages then mature into distinct forms referred to as schizonts. This part of the cycle is referred to as the pre-erythrocytic stage as it involves replication and development of asexual parasites outside RBCs. Once mature, schizonts rupture releasing merozoites into the blood stream which invade RBCs.

1.2.1.2. Disease-causing stages

The invasion of RBCs results in disease onset where clinical symptoms are evident. These symptoms are as a result of several rounds of asexual replication and multiplication involving rupture and invasion of RBCs. During this process, infective merozoites invade RBCs, which develop into rings, immature, early or late [mature] trophozoites, and then become schizonts within which merozoites develop, causing the infected RBC to rupture releasing a new set of infective merozoites that invade uninfected RBCs. This blood-stage cycle of replication is repeated every 44-48 hours and forms part of the erythrocytic stage that is responsible for clinical disease. This stage if left uncontrolled by either host immunity and/or drug cure can result in death.

1.2.1.3. Transmission stages

During rounds of asexual replication, some rings differentiate into sexual erythrocytic stages, gametocytes. Gametocytes, male and female (micro and macro respectively), develop in RBCs into five morphologically distinct stages in *P. falciparum* before differentiation into micro- and macro-gametes after being taken up in the blood meal of a mosquito. The midgut microenvironment propagates the release of the gametes from RBCs which fertilise via zygote transition, develop into motile ookinetes and then into oocysts that rapture and release infective sporozoites. Microgametes exflagellate resulting in the formation of 'exflagellation centres' and in a 'swim-like' manner seek out macrogametes thus marking the beginning of sexual reproduction. However, for transmission to be successful, ookinetes traverse the midgut epithelium, develop into oocysts that undergo sporogonic replication, maturation, rupture and release sporozoites that migrate to mosquito salivary glands. Upon taking a blood meal, these sporozoites are injected and an infection is established in the vertebrate host, re-establishing the cycle.

1.2.2. The call for malaria elimination

Over the last decade, a number of strategies for the control and prevention of malaria have been implemented. These efforts have been strengthened by the call to eliminate/eradicate malaria [17]. These strategies largely involve mosquito control and drug treatment. Even though these measures have and can result in meaningful gains [8, 9, 18-22], eradication hasn't been achieved. On the other hand, a third strategy, which has refocused the research agenda, is development of vaccines to control malaria [23, 24].

1.2.2.1. Mosquito control

Historically, malaria has been successfully eliminated in parts of the world by interventions targeted at mosquito control. These interventions included use of insecticides and draining of breeding sites. However, these measures especially use of insecticides weren't implemented on a large scale due to environmental concerns. Thus to date, most mosquito control interventions largely involve environmentally friendly insecticides. These are mainly deployed in the form of indoor insecticide residual spraying (IRS), larviciding and long-lasting insecticide-treated nets (LLINs) [10, 18, 25-29]. On the other hand, use of such tools has resulted in the development of insecticide resistance in a number of mosquito species [26, 30-34]. These strategies, however, don't control existing asexual and gametocyte infections in vertebrate host populations [35]. Thus, in order for these interventions to be completely effective, there is need to develop novel tools and sustain implementation by wide coverage and usage [34, 36]. Hence biological non-insecticide approaches have been explored which include use of sterile male mosquitoes [37, 38] and symbionts such as Wolbachia [39, 40] to control mosquitoes. These approaches, however, pose a challenge for large scale deployment.

1.2.2.2. Drug treatment

Antimalarial drugs are effective in the control of infection and treatment of disease resulting in the implementation of intermittent preventive treatment (IPT) programs [41, 42]. These have been deployed either as prophylaxis or in mass drug administration campaigns [42]. However, due to the development of resistance to first-line drugs such as chloroquine, drug combination regimens have been employed. The most widely used combination therapy includes artemisinin-based combination therapies (ACTs) [21]. Nonetheless, these therapies are also failing due to drug resistance in Southeast Asia [43, 44] and in parts of sub-Saharan Africa there are reports of reduced clearance against *P. falciparum* infections over time [45]. On the other hand, they are unable to clear gametocytes with evidence of mosquito infectivity post-treatment [46-48]. Thus the use of drugs as a control measure is limited due to acquisition of drug resistance and only a handful of drugs have gametocytocidal effects. For instance, primaquine has gametocytocidal effects and has been shown to reduce the infectivity of gametocytes to mosquitoes [49-51]. Even though primaquine has these effects [50], activity is mainly targeted against mature stages [52] and the drug has contraindications in individuals with glucose-6-phosphate dehydrogenase deficiency [53-56] and methaemoglobin reductase deficiency [57, 58]. These contraindications aren't restricted to individuals with such deficiencies [56].

Nevertheless, a more recent approach has been to test-and-treat on a massive scale using drugs that have adverse effects against mosquitoes [59]. Kobylinski et al. (2011) used ivermectin, a drug used to treat onchocerciasis and lymphatic filariasis, for mass drug administration [59]. This drug has been shown to have transmission-blocking activity (TBA) against the parasite causing filariasis [60]. However the use of this approach against malaria might have implications for drug resistance development for onchocerciasis and lymphatic

filariasis. Therefore, even though combinations of IRS, LLINs and/or ACTs for malaria control have reduced disease burden [10, 19, 61, 62], there is still need for concerted efforts to circumvent risks of using such approaches either for mass coverage or administration. Thus alternative approaches and/or strategies are required that can supplement these efforts for malaria control.

1.2.2.3. Vaccines

Vaccines have been an important tool in controlling infectious disease and eradicating smallpox. Progress has been made with the development of antibody-based vaccines, which have proven useful in preventing and augmenting the fight against most childhood infectious diseases [14]. Thus are a favourable approach at tackling and combating disease. In 2006, the Malaria Vaccine Technology Roadmap group envisaged that a vaccine should be developed and licensed against *P. falciparum* by (a) 2015 with >50% efficacy and (b) 2025 with 80% efficacy against severe and clinical disease respectively [63]. The duration of protective efficacy for these milestones are one and four years respectively. The most advanced vaccine likely to be licensed by 2015 in its current formulation has <50% efficacy against both severe and clinical disease [64, 65]. Since this meeting and following the call for elimination, there are renewed efforts in the development of vaccines that also aim to eliminate/eradicate malaria [24, 66].

A more recent update on the Roadmap has revealed that in addition to (a), by 2030, vaccines against *P. falciparum* and *P. vivax* should be developed and licensed which (i) have 75% efficacy against clinical disease to risk-populations; and (ii) can be mass administered to reduce transmission thus eliminating malaria [67]. Thus ideally, vaccines that target pre-erythrocytic and transmission stages would be licensed. An advantage of vaccines that target transmission [transmission-blocking vaccines (TBVs)] is the potential of being incorporated

into a multistage component vaccine or augmenting, on their own, current control strategies. Thus malaria vaccines are considered an important tool for the control and elimination of malaria and remain a priority in the research agenda [23, 68, 69] with a number of targets currently in various stages of pre-clinical and clinical development [70].

1.3. Malaria Immunity: insights for vaccine correlates of protection

The evidence for existence and mechanisms of immunity to malaria mainly come from immuno-epidemiological studies on natural infections [71, 72] and animal models [73-75]. Natural acquired immunity to infection develops slowly and over time [71, 76, 77] with continued parasite carriage and no evidence of sterile immunity [78]. Therefore it is imperative to design and develop vaccines that would induce responses that either confer immunological memory or are directed at immunological signatures that augment and/or are associated with controlling natural infection [79]. To control infection, a plethora of responses are induced and required. Therefore, stage-specific responses to malaria will be discussed in relation to possible mechanisms required for protection and immune correlates of vaccine-induced efficacy. Emphasis will be made on immune mechanisms thought to be involved in natural immunity and targeted by most vaccine approaches.

1.3.1. Pre-erythrocytic immunity

Immune responses to *P. falciparum* begin, when a mosquito injects sporozoites, with involvement of antibodies directed against the major surface antigen on sporozoites (reviewed in [79-81]). However, the migration from the skin to the liver by sporozoites occurs quickly for antibodies to have an effect at clearing infection [82]. On the other hand, there is evidence that the antibodies raised block the invasion of hepatocytes [83, 84] and can be associated with vaccine-induced protection [85-89]. However, it appears that antibodies

induced by current anti-sporozoite vaccines, including the leading subunit vaccine, aren't enough to mediate sterile protection in all vaccinees [90]. However, there is strong evidence that sterile protection can be achieved using whole sporozoite vaccination, that induce cell-mediated responses targeting infected hepatocytes with interferon gamma (IFN-γ) secreting CD8+ T-cells being the dominant response required [91-95]. Interestingly, subunit vaccine approaches targeting antigens expressed in the liver-stage have also been shown to induce CD8+ T-cell responses that mediate protection in a number of regimens [80]. The IFN-γ–CD8+ T-cell responses have also been shown to be protective against natural infection [96].

On the other hand, CD4+ T-cells have also been induced by both subunit and whole sporozoite vaccination in both pre-clinical and clinical studies [94, 97-100] and have been shown to be important for mediating liver-stage immunity and correlate with protection from infection and disease in natural infections [101]. A recent approach of infection using whole sporozoite mosquito challenge under chloroquine cover showed sterile protection associated with a plethora of responses including IFN-γ secreting CD4+ T-cells [102-104]. The importance of CD8+ and CD4+ T-cell responses has been validated by the identification of human leukocyte antigen class I and II haplotype involvement in protection against disease [105]. Overall, antibody and T-cell-mediated responses in both natural infections and vaccination have achieved either suboptimal or sterile protection, however, it is yet to be determined what the *in vivo* correlates of protection against pre-erythrocytic infection are [79].

1.3.2. Blood-stage immunity

Early studies on natural acquired immunity identified the importance of antibodies in mediating protection against blood-stage disease [106-109]. This has been further confirmed by immuno-epidemiological studies which have identified targets of protective antibodies (reviewed in [71, 72, 110]) and animal studies which have aided identification of mechanisms involved in antibody-mediated immunity [111, 112], but they appear to be species-specific [113]. Antibody targets include surface antigens on infected RBCs [114] and merozoites [115] thus preventing invasion and infection of uninfected RBCs. *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) has been shown to be a target involved in the former [116] however this poses a challenge for immunity and vaccine development due to antigenic variation as a consequence of immunity [117-119]. However, it is apparent that merozoite antigens are plausible targets for both natural and vaccine-induced responses as invasion of RBCs is a crucial step for infection [115, 120-123]. However, due to antigen variability, polymorphism and allele-specificity, it has been difficult to induce vaccine protection against merozoites antigens, mainly due to the antibody titres required and their specificity [121].

On the other hand, cell-mediated immunity against merozoites and infected RBCs has been implicated in natural infections [124, 125], pre-clinical [126-131] and clinical [104, 132-134] studies. It is thought that CD4+ T-cells provide help for antibody responses [135] even though there is evidence that T-cells can mediate protection independently of antibodies [126, 127, 132, 136]. Studies by Pombo et al. (2002) showed that exposure to blood-stage parasites resulted in inducing a potent IFN-γ CD4+ and CD8+ T-cell response with no detectable antibodies in protected volunteers [132]. These findings supported an earlier study describing the inhibition of parasites *in vitro* by this phenotype of cells [137]. More recently, exposure to

parasites by experimental mosquito bite has been shown to induce blood-stage specific IFN-γ producing CD4+ T-cells that mediate protection [102-104]. However, most of the evidence for T-cell involvement stems from studies in mouse models with inconclusive data from natural infections though vaccines have been shown to induce especially CD4+ T-cells against blood-stage antigens. Evidently, a potent antibody response is required due to functional translation of these responses in *in vitro* measures of efficacy and correlation with protection [138, 139].

1.3.3. Transmission-blocking immunity

Insights on the acquisition of transmission-blocking immunity (TBI) have come from studies looking at natural and experimental infections, and vaccinations using sexual-stages in animal models (reviewed in [140-143]). From early studies of vaccinations, it was observed that the parasite and mosquito interaction could be exploited by both innate and adaptive components of the immune response [144-146]. The parasites' progression is susceptible to antibodies [147-149], complement [150-155], cytokines [156-160], and peripheral blood leukocytes [161-164] ingested with the blood meal and mosquito immune components [165]. TBI predominantly involves induction of antibodies by the vertebrate host that neutralise or opsonise their target in the mosquito independently [166-168] or aided by complement [169-173] without the need for phagocytosis [174].

There is extensive evidence of antibodies being raised against gametocyte antigens in endemic populations. A number of studies have identified that antibodies are present in endemic populations in mainly sub-Saharan Africa [175-179] and Asia [180, 181] against natural *P. falciparum* exposure. Bousema and colleagues have been able to identify the targets of antibody responses to major gametocyte surface antigens [178, 182]. These responses have been shown to have an impact on mosquito infectivity, with a number of

studies showing their ability to inhibit parasite infectivity [176, 177, 183]. Antibody responses with inhibitory activity have also been shown to correlate with antibody responses to gametocyte antigens [176, 177, 184]. However, there is also evidence of infection enhancement in a subset of individuals with naturally-acquired antibodies [180, 184-186]. This, on the other hand, has not been fully explored to understand the reason for evidence of naturally-induced antibody responses enhancing infection. However, it appears that the level of antibodies acquired against gametocyte antigens are important in mediating either inhibition or enhancement of infection with lower antibody responses acquired after natural infection in this subset of the population being responsible for enhancement [180, 184, 187]. Acquisition and detection of antibodies in natural infections is dependent on age, endemicity, exposure and seasonality [178, 181, 182, 188-190]. The ability of antibodies to inhibit parasite infectivity is also dependent on these factors [179]. It has further been shown that antibodies correlate with transmission-blocking activity (TBA) and in turn efficacy [191].

Apart from antibodies, the role of cell-mediated immunity has been identified in natural and vaccine-induced responses [192-194]. Harte et al. (1985) showed that CD4+ T-cells had TBA in the absence of antibodies with an additive effect when antibodies were co-administered [194]. Good et al. (1987) later showed that CD4+ T-cell responses could be induced against gametes from non-immune unexposed individuals [195]. There is strong evidence from both natural infections and vaccine studies, that the immune correlate of efficacy is the antibody response which could be augmented by CD4+ T-cell help.

1.4. Pre-erythrocytic vaccines

Vaccine strategies using whole parasites against pre-erythrocytic stages have induced protective responses in animal models [91, 196]. Attenuated *P. falciparum* sporozoites, whether by genetic manipulation or irradiation, have since showed protection in unexposed

individuals [197-199]. Even though this approach seems successful in inducing protective responses, the deployment is currently not feasible due to the method of administration (which involves mosquito bite) and the requirement for cryopreservation. In efforts to circumvent the need for administration via mosquito bite, Hoffman and colleagues have examined possible routes of administration using the irradiated whole sporozoite vaccine (PfSPZ) with protection observed by intravenous inoculation [200-202]; albeit more than four inoculations are required for sterile protection [201]. On the other hand, it appears that administration of non-attenuated whole sporozoites achieves sterile protection using fewer mosquito bites under chloroquine cover [103]. In spite of this, the deployment of these approaches is still impractical and thus other alternative approaches are favoured as vaccine strategies.

The most advanced malaria vaccine is a protein-in-adjuvant subunit formulation based on *P. falciparum* 3D7 circumsporozoite protein (CSP), a major surface protein on sporozoites and liver-stage schizonts [203]. This vaccine incorporates NANP repeat region (**R**) B-cell epitopes and C-terminal T-cell epitope region (**T**) of CSP conjugated to Hepatitis B surface S antigen (HBsAg) (**S**) and expressed with unconjugated S antigen (**S**); referred to as RTS,S [204]. This, however, hasn't yielded promising results as outlined above despite being tested in a number of populations, using different regimen and adjuvant formulations [64, 65, 86, 88-90, 205-207]. Long term follow-up has shown a reduction in vaccine efficacy however plans are imminent for licensure [67, 208]. On the other hand, RTS,S isn't the only vaccine candidate based on CSP. Other approaches have involved varying lengths of NANP repeats, T-cell epitope region, and full-length antigen all delivered using various regimen and formulations resulting in vaccine-induced antibody and/or T-cell responses that mediated

protection [207, 209-218]. Alternatively, using RTS,S as a boost for viral-vectored CSP enhanced responses but there is no published evidence of efficacy [219].

An alternative approach to pre-erythrocytic subunit vaccines is the induction of cell-mediated responses against liver-stage parasites. The most advanced liver-stage subunit vaccine is based on thrombospondin-related adhesion protein (TRAP) which includes a multi-epitope string of CD8+ T-cell epitopes [including NANP repeat from CSP] fused to TRAP; referred to as ME-TRAP [220]. This has been tested in non-immune adults as well as populations in endemic regions as a viral-vectored prime-boost vaccine, considered safe and immunogenic resulting in the induction of antigen-specific CD8+ T-cell responses which conferred either sterile, partial, or no protection depending on the regimen and population vaccinated [221-229]. This candidate antigen remains in clinical development to-date and has been assessed in combination with a blood-stage antigen [138].

Even though CSP and TRAP are the most advanced vaccine candidate antigens targeting the sporozoite and liver-stage respectively, there are a number of other targets that have been characterised and are in various stages of development. For instance, antigens that are expressed on sporozoites but may have an effect across more than one stage have been identified and characterised as potential vaccines. CelTOS, cell-traversal protein for ookinetes and sporozoites, has been identified as being essential for ookinete motility and sporozoite infectivity [230]. Angov and colleagues have demonstrated that this conserved antigen induces both antibody and cell-mediated responses that confers cross-species protection upon sporozoite challenge in animal models [231, 232]. Plans are underway to test CelTOS in clinical trials. Another antigen is the liver-stage antigen-1 (LSA-1) which has been shown to correlate with protection in epidemiological studies [233] but as a vaccine failed to induce protective immunity [234]. Alternatively, pre-erythrocytic antigens have been designed as

multi-antigen combinations, assessed in various regimen and formulations with the aim of enhancing antibody and cell-mediated responses and protection [235-238].

1.5. Blood-stage vaccines

Vaccine strategies against blood-stage malaria include a number of candidate antigens that have been assessed either individually or in combination. Initially, the most advanced approach for a malaria vaccine was a peptide-based vaccine, Spf66, that consisted of three blood-stage antigens fused together by CSP NANP repeats [239]. This vaccine initially showed promise and was studied extensively in field trials in three different continents [240-243]. However, analysis of the overall effect on efficacy revealed that there was no effect on infection, disease or death against human *Plasmodium* species [243]. Nonetheless, the disappointing results of Spf66 didn't deter blood-stage vaccine development. A different approach was developed, protein-in-adjuvant formulation, consisting of two merozoite surface proteins (MSP1 and MSP2) and ring-infected erythrocyte surface antigen (RESA) collectively referred to as Combination B. This vaccine has been assessed in a number of trials [244] resulting in reduction of parasite density in an allele-specific manner [245].

Apical membrane antigen-1 (AMA1) and MSP1 candidate antigens are the most advanced blood-stage targets to-date despite the existence of strain polymorphisms [246-249]. Draper and colleagues have been able to express both AMA1 and MSP1 vaccines, in viral-vectors, to circumvent antigenic diversity resulting in induction of antibodies with activity against diverse strains [247, 248]. Bruder et al. (2010) have also been able to induce antibodies capable of potent inhibition of blood-stage parasite growth against both AMA1 and MSP1 using viral-vectors [250]. Both candidates have been clinically assessed mainly as protein-in-adjuvant formulations [251-263] or viral-vectored vaccines [138, 264, 265]. However, there has been no evidence of sterile protection using either approaches in both non-immune [138,

256, 257] and populations from endemic regions [253, 255, 258] despite *in vitro* inhibition and *in vivo* reduction of parasite growth [256, 257]. In efforts to improve efficacy, AMA1 and MSP1 have been assessed in combination [138, 266, 267] or with CSP [268, 269]. In these studies, *in vitro* inhibition of parasite growth if evident, was attributed to AMA1 antibody responses [267]. In trials assessing efficacy, there was delay to parasitaemia or sterile protection in but a small number of vaccinees [138, 268, 269]. Chuang et al. (2013), demonstrated that sterile protection, following vaccination with AMA1 and CSP, was as a result of cell-mediated responses against AMA1 [269]. Considering that AMA1 is expressed on sporozoites and merozoites, efficacy could have been as a result of infection-blocking immunity as demonstrated in a *P. yoelii* model [270].

Other blood-stage vaccines tested to-date in clinical trials includes a fusion protein-in-adjuvant formulation of MSP3 and glutamate rich protein (GLURP) [271-273], which has induced antibodies with activity against a diverse range of parasites [274]. Other potential target antigens have been described and evaluated [275-277]. Reticulocyte-binding protein homologue 5 (Rh5) is one such promising target which has been shown to inhibit *in vitro* growth of not just vaccine homologous parasite strains but heterologous parasites [275, 276]. Plans are underway to progress a viral-vectored Rh5 vaccine to Phase I/IIa clinical trials.

1.6. Transmission-blocking vaccines

This approach for vaccine development isn't unique to malaria and has been employed in other vector-borne diseases. Leishmune® vaccine, based on fucose-mannose-ligand of *Leishmania donovani*, is the only licensed vaccine with TBA against canine visceral leishmaniasis [278, 279], with herd immunity conferred to dogs having subsequent effect against human visceral leishmaniasis [280]. Saraiva et al. (2006) demonstrated that Leishmune® vaccine-induced antibodies were able to inhibit *L. donovani* infection in

susceptible sandfly vectors [279]. This inhibition was also detected 12months after vaccination and wasn't only against the vaccine strain but also *L. chagasi* parasites. Furthermore, Tonui and colleagues showed that targeting parasite surface antigens against cutaneous leishmaniasis caused by *L. major*, led to inhibition of parasite infectivity to sandflies [281, 282].

Since the late 1970s, when first evidence of malaria TBA mediated by vaccination was demonstrated [144, 145], there has been little progress in the development of TBVs compared to other stage-specific parasite targets [283]. This is evidenced by only two phase I clinical trials published to-date against TBV candidate antigens [284, 285]. However, following the call for elimination, there has been a renewed interest in research towards development of TBVs [286, 287]. Targeting both the parasite and mosquito forms the rational basis for development of malaria TBVs by reducing and/or inhibiting parasite infectivity to mosquitoes and in turn to the vertebrate host. TBV development over the last three decades has largely focused on surface proteins expressed on the parasite. Various antigens have been identified, tested for potential to induce TBA and can be subdivided into two categories: parasite-based and mosquito-based antigens. Considering that this study focuses on TBVs, candidate antigens will be described in detail in relation to their role in parasite transmissibility and their potential as TBV candidate antigens.

1.6.1.1. Parasite-based TBV candidate antigens

The parasite exists predominantly in an extracellular phase during its development in mosquitoes and hence surface antigens are potential targets for vaccine development. Surface proteins expressed on gametocytes, gametes and ookinetes have been tested as potential candidate antigens. These antigens can be divided into pre-fertilisation targets such as P230, P48/45 and PHAP2 [288-295] and post-fertilisation targets P25 and P28 [296-299]. In

comparison to blood-stage antigens, there is evidence of limited strain polymorphisms to especially pre-fertilisation antigens possibly due to immune pressure [291, 300-310].

1.6.1.1.1. P230 and P48/45

P230 and P48/45 are expressed on gametocytes and gametes with expression seen after gametogenesis and fertilisation; and have been shown to play an important role in fertilisation by aiding membrane-to-membrane fusion of micro- to macro-gametes [293, 294]. These proteins have cysteine-rich domains (CRDs) with characteristic conserved cysteine positions [291, 311]. Antibodies against *P. falciparum* targets are present in populations from endemic regions which have TBA [175, 176, 178, 179, 182, 312]. These are putatively raised by rupture and/or phagocytosis of infected gametocyte RBCs. Occurrence of antibodies to these antigens after natural infection has led to their development as TBVs with the likelihood of boosting either vaccine-induced or natural responses. However, it appears that recent exposure is required for this to be effective [179, 182]. On the other hand, polymorphisms might impact their development as TBVs [291, 301, 303, 305].

Analysis of P230 structure and sequence, revealed two distinct regions, variable and conserved, with a number of orthologs identified for *P. falciparum* 230 (Pfs230) [288, 291, 313, 314]. There's a variable region situated on an N-terminus repeat sequence stretch of Pfs230 that is cleaved off during RBC emergence [290, 291, 315, 316]. Pfs230 is expressed only on micro-gametocytes and gametes and been observed on stage V gametocytes in the absence of gametogenesis [290]. Upon emergence, it undergoes proteolysis with the resulting cleaved product being involved in formation of exflagellation centres [316-318]. It has been demonstrated that it forms a complex with Pfs48/45 on the gamete surface [319] and antibodies to both pre- and post-cleavage forms are present in sera from individuals living in endemic regions [312, 316, 320]. However, a distinct region of the antigen has been the focus

of Pfs230-based TBV efforts to-date due to the induction of antibodies with potent TBA [321].

P48/45 is expressed on both micro- and macro-gametocytes and gametes and has been shown, in gene disruption studies, to be involved in the fertility of microgametes [292-294]. van Dijk and colleagues have demonstrated that P48/45 is involved in fertilisation of micro- and macrogametes by aiding membrane fusion and zygote formation [293, 294], possibly mediated by formation of complexes with P230 resulting in stable membrane-to-membrane interactions. Like Pfs230, orthologs have been identified in other *Plasmodium* species, which exhibit similar phenotypic functions [293, 294] with evidence of polymorphisms in *P. falciparum* 48/45 (Pfs48/45) [302-305]. Pfs48/45 has been observed to be susceptible to antibodies resulting in inhibition of zygote formation [176, 187, 322] with antibodies induced after natural exposure exhibiting TBA [175, 176, 178, 182]. This has potentiated the development of Pfs48/45 as a promising TBV target.

1.6.1.1.2. HAP2

Plasmodium male gamete sterility genes are also potential targets as these have conserved roles in nature and are essential for fertilisation, leading to successful development of any organism. HAP2, exclusively expressed on micro-gametocytes and gametes, putatively mediates fertilisation of micro- and macro-gametes. Evidence of this comes from gene knockout studies which have demonstrated that micro- and macro-gametes come together but are unable to fertilise [295]. Experiments in *P. berghei* have indicated that antigen-specific antibodies inhibit development of ookinetes and oocysts, an effect that could be mediated during transition of zygotes to ookinetes or on the actual fertilisation process [323]. More recently, *P. falciparum* HAP2 (PfsHAP2), in a protein-in-adjuvant regimen, was used to

induce antibodies with TBA [324]. Thus is a suitable candidate antigen for vaccine development.

1.6.1.1.3. P25/P28

These antigens unlike six-cysteine pre-fertilisation antigens have characteristic epidermal growth factor-like (EGF) domains which vary in number between P25 and P28, with the former having more than the latter [296, 308]. P25 and P28 have similar structures, pattern of expression, are conserved across *Plasmodium* species [325-330]; play similar albeit redundant roles in ookinete invasion [331-336]; and have limited sequence polymorphisms [306-310]. It is postulated that these EGF-like domains maybe involved in aiding binding of ookinetes to ligands on mosquito midgut epithelia. *P. falciparum* 25, Pfs25, is a 25kDa protein expressed on the surface of zygotes and ookinetes after initiation of gametogenesis and fertilisation and is the only candidate antigen that has been tested in clinical trials. This antigen has been demonstrated to induce antibodies with TBA in both pre-clinical [337-340] and clinical studies [285] which correlate with TBA [191].

1.6.1.2. Mosquito-based TBV candidate antigen

Sequencing of the *A. gambiae* genome has led to identification of molecules that are involved in interacting with pathogens and in mediating immune responses to these infections [341]. Ligands and/or receptors used by *Plasmodium* to traverse mosquito midguts for instance are potential targets [342-344]. This strategy, if successful, has potential to block more than one species of *Plasmodium* and could be translated to other mosquito-borne diseases if ligands are used by other pathogens and thus mosquito-based antigens are attractive TBV candidates.

1.6.1.2.1. AnAPN1

Several studies have described midgut-specific proteins as potential targets as mosquito-based TBVs [148, 149, 345, 346]. There is evidence that ookinetes interact with and attach to glycans on midgut glycoproteins and use them as ligands to traverse midgut epithelia [347]. *Anopheles* alanyl aminopeptidase N1 (AnAPN1) is one such midgut-specific glycoprotein that is localised on the apical epithelial surface and present in at least four anopheline species [348]. This glycoprotein has been postulated to be involved in the ookinete invasion of the midgut epithelium. Antibodies raised against the N-terminus (henceforth referred to as rAnAPN1) in a protein-in-adjuvant regimen, partially block development of *P. falciparum* and *P. berghei* in *A. gambiae* and *A. stephensi* [348, 349]. Thus the pan-TBA of antibodies raised make AnAPN1 an attractive TBV candidate.

1.7. Assessment of vaccine efficacy

In clinical trials, malaria vaccine efficacy is assessed in a number of ways depending on the study population. For instance, in non-exposed populations efficacy is assessed by exposure to infected mosquitoes, direct sporozoite inoculation or infected erythrocytes [350, 351]. Experimental *P. falciparum* malaria infections in the form of controlled human malaria infections (CHMI) have been extensively used for assessing especially pre-erythrocytic and blood-stage vaccines [210, 211, 240]. This has been made possible by the ability to grow gametocytes *in vitro* and successively infect mosquitoes leading to infective falciparum sporozoites [352-354]. CHMI involves infection of vaccinees and controls with mainly five infectious mosquito bites and monitored for parasitaemia by microscopy and PCR [198, 354, 355]. This approach has led to the advancement of vaccine candidates including RTS,S and ME-TRAP into field trials. In these field trials, vaccine efficacy is assessed by natural exposure to malaria and absence of clinical episodes in vaccinees compared to controls. This

has enabled assessment of RTS,S and ME-TRAP in various populations in endemic countries with episodes of clinical malaria as the primary endpoint [64, 65, 208, 225, 227].

On the other hand, blood-stage vaccine efficacy has been assessed using sporozoite and infected RBCs CHMI in non-exposed populations [138, 257, 356-358]. Even though infection using infected RBCs is a favoured approach for assessing blood-stage vaccine efficacy, due to the ability to directly measure impact of vaccine-induced responses (reviewed in [257]). Duncan et al. (2011) have employed this experimental infection challenge with no evidence of efficacy with an AMA1-based vaccine [257]. Additionally, for vaccine candidates that have been assessed in field trials, efficacy has been assessed based on natural exposure with clinical malaria as the primary endpoint [245, 253, 255, 258].

Other ways of assessing vaccine efficacy include *in vitro* assays that are used as surrogate markers of protection. This is particularly used for assessing blood-stage vaccines where *in vitro* inhibition of parasite growth is an indicator of the functionality of vaccine-induced responses measured by growth inhibition assay (reviewed in [359]). This assay has been shown to correlate with both *in vivo* parasite multiplication rates and antibody titres [257]. This has been useful in assessing whether vaccine-induced antibody responses are able to inhibit parasite growth against both vaccine homologous and heterologous parasite strains and employed in both pre-clinical and clinical studies [252, 256, 261, 266, 267, 274, 275].

1.7.1. Measurement of TBV efficacy

Assessment of TBV efficacy is unique in that efficacy is assessed based on ability to reduce and/or inhibit parasite infectivity to mosquitoes. Biological function of vaccine-induced antibodies is assessed mainly in mosquito feeding assays, which ascertain inhibition of parasite development. This has mainly been achieved using laboratory-adapted parasite lines

in compatible mosquito species. However, there are a number of assays such as the ookinete development assay that have been utilised, albeit the mosquito feeding assays are the main assays used to estimate efficacy.

1.7.1.1. Laboratory-based assays

To-date, a handful of laboratory-based assays has been developed to ascertain potential efficacy of candidate antigens [360, 361]. Most of these assays have been developed mainly in rodent *Plasmodium* species especially *P. berghei* [361, 362]. The use of transgenic parasites with reporters such as green fluorescent protein (GFP) have reduced assay laboriousness and increased throughput [360, 363-365]. However, this is mainly applicable to lab-based assays. Ability to culture *P. falciparum* malaria sexual-stages has further enabled utility of lab-adapted parasite strains to be used in these assays [352, 366-368]. Formation of motile microgametes (exflagellation) *in vitro* can be exploited to investigate potential impact of antibodies raised against sexual-stage antigens [323]. This has also been used to investigate impact of potential TB drugs in *P. falciparum* [365]. However, the exflagellation assay can only be used to investigate the potential impact of antibodies raised against antigens involved in microgametogenesis and/or fertilisation. Even though the assay is limited; nonetheless, identification of antigen function underpins the biological significance of this assay [323, 361].

On the other hand, ability to investigate the influence of antibodies to antigens expressed on gametocytes, gametes and ookinetes can be done using two lab-based *in vitro* and *in vivo* assays [360, 361]. *In vitro* ookinete development assay (IVOA) allows assessment of efficacy against a range of targets with the readout being reduction and/or inhibition of the number of ookinetes that develop from gametocytes [323, 361, 363]. However, successful application of this assay has been limited to *P. berghei* [323, 365] due to the inability to cultivate enough

numbers of *P. falciparum* gametocytes converting to ookinetes despite culture improvement efforts [367, 368]. In spite of this, this assay has been valuable at assessing efficacy against PbsHAP2 where 80% inhibition of ookinete development was observed [323].

In vivo standard membrane feeding assay (SMFA), though laborious, is considered the preferred assay of choice ('gold standard') with the readout being oocyst detection in the mosquito midgut [175, 369]. This assay can be performed for both *P. falciparum* and *P. berghei* species assessing efficacy of both vaccine and drug targets [323, 361, 362, 365]. Additionally, direct skin feeding of mosquitoes, direct feeding assay (DFA), under lab conditions mainly using *P. berghei* infected mice, is performed to evaluate efficacy of transmission-blocking targets [361, 362, 370-372]. This has been successful especially using transgenic parasite lines expressing *P. falciparum* or *P. vivax* antigens [370-372].

Ability of vaccine-induced antibodies to inhibit infectivity is assessed by feeding lab-reared mosquitoes with a mixture of pRBCs from *in vitro* grown gametocyte cultures with antigen-specific sera or IgG relative to a control. The mosquitoes feed on infected blood through an artificial membrane that mimics skin, in a feeder system, resulting in infected mosquitoes, which are individually dissected for oocyst detection [373, 374]. Lab-adapted *P. falciparum* isolate NF54 and its clone 3D7 are the most widely used parasite strains for gametocyte production and infectivity of compatible lab-reared *A. stephensi* in SMFA [361, 373, 375]. Historically, sera or monoclonal antibody preparations have been used [at varying dilutions and concentrations] assessing efficacy of a number of targets in relation to control [361, 369, 376-378].

1.7.1.2. Field-based assays

Even though lab-based assays, especially SMFA are able to provide estimates of efficacy, this is only measurable against lab-adapted parasites strains in lab-reared mosquitoes. Thus

field-based assays provide an additional advantage of evaluating vaccine-induced antibodies against human circulating parasite strains in field-collected mosquitoes [360, 379]. Human DFAs have been mainly used to assess infectivity of individuals to mosquitoes [380-383]. However, if ethical considerations can be overcome, DFAs are advantagous for evaluating TBV efficacy in clinical trials due to the reliability of infectivity [380-383].

On the other hand, direct membrane feeding assays (DMFAs) overcome the ethical considerations imposed and allow evaluation of TBVs against naturally circulating parasites [360, 379]. This is achieved by drawing blood from gametocyte-positive individuals and assessing the infectivity of the parasites in the presence of vaccine-induced antibodies. DMFAs are used to evaluate efficacy of *P. vivax* TBV candidates due to the inability to culture the parasite [381, 384]. To successfully measure the impact of vaccine-induced responses, autologous plasma needs to be replaced due to possible interference of natural responses in estimating vaccine efficacy [381, 383]. Thus DMFA is a suitable assay for assessment of TBV efficacy allowing evaluation of interventions against diverse parasites in field mosquitoes. This assay has been employed to-date, in *P. falciparum* infections, mainly to assess natural responses and infectivity [in comparison to DFA] and should have wider applications in determining advancement of potential candidate antigens. Regardless of the source of parasite or method of mosquito feeding, feeding assays are the primary assay for measuring either vaccine efficacy or transmission-reducing capability.

1.7.1.3. Estimation of efficacy

The primary readout in mosquito feeding assays is reduction or inhibition in the number and/or prevalence of oocysts in mosquito midguts. The number of oocysts (henceforth referred to as infection intensity) that develop has historically been used as the main readout of TBA. The prevalence (infection prevalence) is equally important as this indicates the

numbers of mosquitoes that haven't been infected as a result of the intervention [377]. Majority of studies report reduction or inhibition of intensity [191, 285, 348, 349, 385] or inhibition of prevalence [377, 386] as efficacy endpoints. However, it has been demonstrated that the distribution of oocysts varies within and between feeds following a negative binomial distribution regardless of parasite exposure [369, 377, 378, 386]. Medley et al. (1993), in an effort to understand and overcome this variability, proposed that inhibition of prevalence was a better efficacy endpoint to measure [377]. In this analysis and that later performed by Billingsley et al. (1994), it was apparent that there was a correlation between intensity and prevalence [both for lab and field-based observations], which could be summarised with an equation [377, 386]. They observed that large infection burdens (means in excess of 250 oocysts) were required to result in oocyst distributions that weren't skewed. Medley and colleagues argued that the number of mosquitoes dissected should be >50/group. On the other hand, other studies have argued that control means of at least 35 oocysts are required to enable reliable interpretation of observed efficacy based on infection intensity [369].

However, even though these stipulations enable efficacy endpoints based on infection intensity and prevalence to be accurately estimated with certainty. It is not feasible to obtain infections in the control group with such oocyst intensities due to the biological nature of the assays; or accurately dissect large numbers of mosquito midguts per intervention ensuring precision in oocyst detection. Therefore, in an attempt to re-examine the relationship between these two efficacy endpoints, Churcher et al. (2012) have argued that reporting both inhibition of intensity and prevalence be adopted to allow effective interpretation of efficacy [378]. The statistical analysis adopted in this study utilised a generalised linear mixed model (GLMM). This analysis was performed on both lab and field-based observations resulting in the estimation of the degree of certainty for both intensity and prevalence [378]. Ideally,

sample size calculations to determine the number of mosquitoes to be dissected for each experiment and replicate assays should be undertaken. However, van der Kolk, et al. (2005) have concluded that due to inter- and intra-assay variability, a comparative approach to assess TBVs within experiments should be adopted as opposed to assessing interventions separately and/or individually [369].

1.8. Viral-vectored vaccine platform

Current vaccine delivery platforms for licensed vaccines utilise either live-attenuated and inactivated whole micro-organisms or for subunit vaccines, protein-in-adjuvant approaches with the majority aimed to induce antibody responses [16]. However, there still remains a spectrum of diseases that need licensed vaccines with delivery platforms that circumvent limitations imposed by the existing platforms. Traditionally, protein-in-adjuvant platforms have proved useful for subunit approaches, however, this requires safe but immunogenic adjuvants. On the other hand, recombinant viral-vectors provide a useful delivery platform expressing foreign transgenes; where adenoviruses (Ad) have had a wide application in cancer gene-based therapeutic treatments and other viral-vectors such modified vaccinia virus Ankara (MVA) used as vaccine testing platforms for a number of other diseases [387, 388]. Viral-vectored vaccine platforms have been altered to render them replication-defective and hence don’t cause disseminated infections in the target host [389].

Viral-vectors act as an immune-potentiating carriers enabling the host to mount an immune response against both the carrier and foreign antigen being expressed [390]. These have been used in a number of regimes, primarily for the induction of antigen-specific T-cells and more recently antibodies for malaria liver- and blood-stage vaccines. These responses have been shown to be efficacious upon challenge against the antigen parasite strain in pre-clinical [133, 214, 275, 391, 392] and clinical studies [223, 229, 264, 265, 393]. Furthermore, viral-vectors

have also been used to develop and test subunit vaccines against HIV, tuberculosis, hepatitis C virus, influenza to name but a few (reviewed in [394, 395]). This extensive use of viral-vectors provides a useful platform to allow testing and screening of candidate antigens with the aim of advancing the most efficacious antigens into clinical development as they have a proven safe clinical record.

1.8.1. Adenoviruses

Adenoviruses belong to a group of double stranded DNA viruses with genome sizes ranging from 34-43kbps [396]. They are responsible for mainly respiratory infections in susceptible hosts but this is dependent on the serotype using different mechanisms to infect [396, 397]. The host tropism of these viruses can be exploited, circumventing infections by attenuating them thus rendering them replication-defective, resulting in recombinant adenoviruses that have been generated and widely employed as vaccine delivery platforms [396]. Adenoviruses are of different serotypes, where human Ad serotype 5 virus (HAdV5), has been used widely in pre-clinical and clinical studies as a viral-vector subunit vaccine delivering HIV and malaria antigens [396, 398-401]. HAdV5 is able to induce both cell-mediated and antibody responses that are potent at mediating protection [391, 398, 402]. However, the use of this viral-vector suffers from existence of neutralising antibodies from pre-existing infections [403, 404]. These responses have been shown to impact transgene responses and efficacy [399] though this appears to be influenced by the route of administration [405]. Thus use of HAdV5 to express malaria antigens in pre-clinical studies hasn't translated into the clinic due to the existence of neutralising antibodies from pre-existing infections in individuals living in endemic areas [406, 407]. Recent efforts to circumvent pre-existing neutralising antibodies to HAdV5 malaria-based vaccines, have involved modification of the hypervariable region

responsible for inducing these antibodies and resulted in inducing transgene-specific protective responses [408].

To further circumvent the existence of neutralising antibodies to HAdV5, simian adenoviruses, such as chimpanzee adenoviruses (ChAd), have been developed, characterised and tested as potential vaccine platforms [397, 409-412]. These have been demonstrated to have lower pre-existing levels of neutralising antibodies compared to HAdV5 in humans [406, 407, 411-413]. Though neutralising antibodies to these viral-vectors have been described to be higher in individuals from malaria endemic areas in sub-Saharan Africa, this could possibly be due to cross-species reactivity [413]. Furthermore, it appears that transgene-specific responses aren't affected by the presence of pre-existing responses at least in mice [405]. Moreover, simian viral-vectors have been shown to have a safe clinical record inducing potent antibody and cell-mediated response against a number of targets [138, 228, 264, 265, 394, 411, 414]. O'Hara et al. (2012) were the first to demonstrate the use of ChAd serotype 63 (ChAd63) expressing ME-TRAP resulting in the induction of potent cell-mediated responses after boosting with MVA that were maintained for up to 30months [228]. ChAd63 has further been used to induce not only T-cell responses but antibodies against blood-stage malaria antigens [138, 264, 265]. In pre-clinical studies, induction of antibody responses has been demonstrated against Pfs25, where responses induced after priming with ChAd63 were similar to those induced using HAdV5 [372].

1.8.2. Poxviruses

The use of poxviruses as vaccines stems from the first in-human testing of the concept of vaccination [16]. Edward Jenner used cowpox against smallpox and eventually this disease was eradicated with the use of a vaccinia virus-based vaccine [14]. Highly attenuated strains of vaccinia virus, MVA and New York vaccinia virus (NYVAC) has since been developed as

recombinant viral-vectors. These viral-vectors are replication-deficient and have capacity to express large foreign transgenes [415-417]. Both these platforms have been used to express malaria, HIV, tuberculosis, and influenza antigens [217, 225, 418-425]. MVA expressing a tuberculosis antigen 85A has recently been evaluated in a large field trial. This study demonstrated that the vaccine was safe and induced cell-mediated responses in infants though no efficacy was observed [425]. MVA has been demonstrated to induce mainly cellular immune responses [415] but has also been effective at inducing antibody responses administered as a booster [275, 372, 426].

In addition to MVA and NYVAC, other poxviruses have been attenuated and developed as vaccine platforms. Fowlpox (FP) and canary poxvirus (ALVAC) have both been used to express antigens in various regimen. FP has been used in combination with both DNA and MVA to express and induce responses against ME-TRAP in field trials [225-227, 427]. On the other hand, ALVAC was the first platform used to test HIV vaccine efficacy [428]. The RV144 trial demonstrated that antibody responses induced by antigens expressed in ALVAC resulted in the reduction in risk to HIV infection [429, 430].

1.8.3. Prime-boost vaccination

Vaccine development has currently led to the use of prime-boost approaches, which are in the form of homologous and heterologous strategies [15, 431, 432]. These strategies take into account priming the response with an immunogenic preparation and then boosting with, at a later time point, the same immunogen using varying delivery platforms [432, 433]. This increases and makes a better vaccine-induced response. Homologous strategy employs the use of the same viral-vector as opposed to the heterologous strategy that uses two different viral-vectors [432]. Heterologous strategy induces greater immune responses that potentiate vaccine-induced efficacy [214, 434, 435].

Viral-vectors have been shown to induce transgene-specific responses in various regimen with the most potent being the heterologous prime-boost incorporating an Ad prime followed eight weeks later by a boost with MVA [214, 407, 426, 436]. This approach has been used to induce T-cells against malaria antigens with heterologous boosting enhancing and resulting in efficacy after challenge [437]. This has also been beneficial for antigens that primarily induce antibodies [214, 426]. Heterologous prime-boost strategies are essential for optimal vaccine-induced responses [438]. Prime-boost regimen based on HAdV5 and MVA have been shown to induce potent antibody responses that have inhibitory effects against the parasite [275, 426]. Though a number of viral-vectored vaccine platforms have been developed to-date, this study focuses on the use of the Ad-MVA viral-vectored approach. Therefore, ChAd63-MVA heterologous prime-boost regimen would be used to test and screen pre-clinical TBV candidate antigens.

1.9. Aims and overview

The overall objective of this project was to generate recombinant ChAd63 and MVA viral-vectors expressing leading TBV candidate antigens and comparatively assess immunogenicity and importantly efficacy against *P. falciparum*. Concomitant assessment of each of the antigens would be achieved thus narrowing down to the best candidate(s) which would be further assessed, in combinations, to optimise blockade of parasite development. Therefore, this thesis will focus on pre-clinical development of TBVs using a delivery system that is human compatible with the aim of accelerating the most efficacious candidate antigen(s) into clinical development.

1.9.1. Aims

a) To generate recombinant viral-vectored vaccines expressing leading TBV candidate antigens.

b) To determine immunogenicity of leading candidate antigens expressed in recombinant viral-vectors and a rank order based on efficacy by comparatively assessing: (i) ability of antibodies to inhibit development of *P. falciparum* lab strain in *A. stephensi*; and (ii) whether efficacy observed in (i) can be replicated against development of *P. falciparum* field isolates in *A. gambiae.*

c) To assess combinations of top ranked candidate antigens based on (b) and down-select candidates to be prioritised for clinical development.

d) To investigate whether efficacy against *P. falciparum* determined in (c) can be replicated in a *P. berghei* animal model.

e) To optimise design of AnAPN1 and thus improve efficacy against *P. falciparum*.

1.9.2. Chapter overview

The work described in this study reports the use of a single delivery platform, recombinant viral-vectors, to comparatively assess and determine the most efficacious candidate antigen(s) for clinical development.

Chapter 2 gives a detailed account of all the materials and methods used to generate the results.

Chapter 3 outlines the procedures used to design and generate recombinant ChAd63 and MVA viral-vectored vaccines for leading candidate antigens used in the comparative

analysis. The procedures described were used to generate all the other viral-vectored vaccines in the study.

Chapter 4 describes the comparative assessment of the ability to inhibit parasite infectivity to mosquitoes by ChAd63-MVA vaccine-induced antibody responses to leading TBV candidate antigens. Efficacy outcomes were tested against two parasite sources, *P. falciparum* lab strain and field isolates, in two mosquito vectors, *A. stephensi* and *A. gambiae* to enable advancement of the most efficacious target antigens.

Chapter 5 outlines procedures to further narrow down the most efficacious candidate antigens by assessing the possibility of combination strategies. Combinations of the two most efficacious candidate antigens were further tested for antibody induction and ability to inhibit *P. falciparum* infectivity to *A. stephensi* as fusion vaccines.

Chapter 6 describes the use of the top three efficacious candidate antigens in *P. falciparum* and whether transmission-blocking immunity and efficacy can be predicted and replicated using a *P. berghei* rodent model. This also illustrates the use of an *in vitro* assay as a surrogate to predict efficacy outcomes *in vivo* standard membrane feeding assays.

Chapter 7 describes the optimisation of the mosquito-based antigen, AnAPN1 by designing, constructing and generating two additional fragments and comparatively testing the ability of vaccine-induced antibodies to inhibit *P. falciparum* development in *A. stephensi*.

Chapter 8 provides a summary of the overall findings, the possible impact of the results and suggestions for possible follow-up studies.

2. Materials and Methods

2.1. Materials

Details of all commercially available reagents are listed in the **Appendix** (Reagents, **Table 1**). The buffers and solutions used in this study are also documented in the **Appendix** (Buffers and solutions).

2.2. Molecular Biology and Cloning

2.2.1. Design, Cloning, and Generation of Viral-Vectors

2.2.1.1. Design of Vaccine Candidates

Protein sequences were accessed through the National Centre for Biotechnology Information (NCBI) protein database (unless specified) and bioinformatics determined for the presence of signal peptides (SP) [439]; glycophosphatidylinositol (GPI) anchors [440]; transmembrane domains (TMDs) [441]; and N-glycosylation sites [442] (**Table 2.2**). Endogenous SP sequences were replaced by the human tissue plasminogen activator (tPA) signal sequence [443]. GPI anchor sequences were omitted whilst N-glycosylation sites were either substituted or left intact (only for variations of the P48/45 constructs). A Kozak consensus sequence (5'-CCACC-3') and an ATG site were incorporated upstream of the tPA leader sequence to allow for optimal initiation of translation of the insert antigen whilst a stop codon was inserted at the end [444]. Sequences were codon optimised by GeneArt (Life Technologies, Germany) for either human or murine usage. This process has been shown to improve DNA vaccine immunogenicity against Adenine+Thymine (AT) rich *Plasmodium* genes [445]. For cloning purposes, the following restriction sites were inserted: Acc65I site upstream of the Kozak sequence, BglII site between the antigen and tPA sequences, and EcoRI, XhoI and NotI respectively downstream of the antigen sequence. Presence of these restriction sites within the antigen sequences was modified.

Table 2.1. Bioinformatics of antigen inserts

Antigen	Accession No.	Full αα length	αα in construct	Features
AnAPN1 (PEST)	XP_318000.4	1020	Full length 20 – 996 Domain I 20 – 269 N terminus 20 – 194	SP (1-19αα); GPI anchor (997-1020αα); No TMD; No N-glycans.
Pfs230 (3D7)	AAA29725.1	3135	443 – 1132	SP (1-20αα); No GPI No TMD; 23 N-glycans
Pfs48/45 (3D7)	CAA80119.2	448	28 – 427	SP (1-27αα); GPI anchor (428-448αα); No TMD; 7 N-glycans.
PfsHAP2 (3D7)	XP_001347424.1	889	34 – 688	SP (1-33 αα); GPI anchor (872-899 αα); 2 TMDs; 8 N-glycan sites.
Pbs230* (ANKA)	PBANKA_030610	2756	233 – 790	SP (1-22 αα); No GPI no TMD; 7 N-glycans.
Pbs25 (ANKA)	BAA19925.1	217	24 – 193	SP (1-23αα); GPI anchor (194-217αα); No TMDs; 2 N-glycans.
Pbs48/45 (ANKA)	AAG59815.1	455	32 – 431	SP (1-31αα); GPI anchor (432-455αα); No TMDs; 2 N-glycans.

*Accessed via the PlasmoDB Genomics Resource; AnAPN1, anopheline alanyl aminopeptidase N 1; Pfs, *Plasmodium falciparum* sexual-stage antigen; Pbs, *Plasmodium berghei* sexual-stage antigen; Strain shown in parenthesis under antigen abbreviation; αα: amino acid; SP: signal peptide; GPI: glycosylphosphatidylinositol; TMD: Transmembrane domain.

2.2.1.2. Restriction Endonuclease Digests

Restriction enzyme digests were set up in 20µl reactions for 2hours at 37°C as follows:

5µg	DNA
2µl	Restriction Enzyme Buffer
2µl	10X BSA (if required)
1µl	Restriction Enzyme (10,000 Units)
up to 20µl	dH_20

For double enzyme digests, 0.5µl of each enzyme was added with the exception of digests with AgeI and DrdI where 1.5µl and 1µl were added respectively and incubated for 3hours.

2.2.1.3. Agarose Gel Electrophoresis

For standard agarose gel electrophoresis, DNA reaction products were run on 0.8, 1 or 2% agarose gels in 1x Tris-acetate-EDTA buffer. Agarose was melted for 2minutes after which 10µl of 10,000X SYBR® Safe gel stain was added and allowed to solidify for 45minutes. 6x DNA loading dye was added to the products and loaded onto the gel as well as Smart ladder DNA marker and run in 1x TAE buffer for 1hour at 100V. For low-melting agarose gel electrophoresis, 1% low melting agarose gels were prepared in 1x TAE buffer and allowed to solidify after adding 10µl of 10,000x SYBR® Safe gel stain at 4°C for 2hours. DNA samples containing loading dye were loaded as well as DNA Smart ladder and run for 2hours 30minutes at 70V with the gel tank on ice. Gels were imaged under blue light using a transilluminator.

2.2.1.4. Agarose Gel Extraction and Purification of DNA

DNA was purified from excised gels using a Qiagen Minelute gel extraction kit according to the manufacturer's protocol. For phenol gel extraction and purification, phenol mix (25ml

Phenol, 15ml dH20, 400ml 1M Tris-HCl supplemented with 5M NaCl pH8.0) was placed at 37°C for 1hour. Tubes with excised low-melting agarose were placed in a 65°C water bath for 10minutes and then 37°C for 2minutes. 0.5x volume of the phenol mix was added to each tube and vigorously shaken for 45seconds and then spun down at 13,000rpm for 5minutes. The aqueous layer was transferred to a clean tube and 500µl of the Phenol mix was added. After spinning for 5minutes at 13,000rpm, the aqueous layer was removed and transferred to a clean tube and 1/50th volume of 5M sodium acetate was added and the tubes shaken vigorously for 45seconds and the spinning process repeated. The aqueous layer was transferred to a clean tube and 2x volume of 100% ice-cold ethanol added to precipitate the DNA at -20°C. After a 3day precipitation period, the tubes were span for 15minutes at 13,000rpm and the pellet washed with 500µl of 70% ethanol. The pellet was then air-dried for ~5minutes and resuspended in 10µl of dH_20. DNA concentration was quantified using a NanoDrop spectrophotometer.

2.2.1.5. DNA Ligation Reactions

Insert DNA was ligated into vectors in 10µl total reaction volume at 16°C overnight. Control ligation reactions were set up using water. The reaction mix was made up as follows:

6µl	Insert DNA/ dH_20
2µl	Vector DNA
1µl	T4 DNA Ligase
1µl	1X T4 DNA Ligase reaction buffer

2.2.1.6. Transformation and Growth of Bacterial Cells

Transformation reactions were performed using Max Efficiency®DH5α™-TIR competent cells. The cells were thawed on ice until the last ice had melted. 5µl of the ligation reaction mix was added to the cells and left to incubate on ice for 30minutes. Cells were then heat-

shocked for 30seconds in a 42°C water bath, immediately placed on ice for 5minutes and incubated at 37°C for 1hour at 225rpm (250µl of super optimal broth with catabolite repression (SOC) medium was added to plasmids under kanamycin selection). Luria Bertani (LB) agar plates containing either 1000x Ampicillin or 2000X Kanamycin were used to grow the transformed cells at 37°C overnight. Single colonies were picked the following day and grown in starter cultures of 3ml LB media containing either of the antibiotics by incubating overnight at 37°C at 225rpm. Subsequently, 1ml of the cells were grown up in 100ml LB cultures overnight at 37°C with vigorous shaking at 225rpm.

2.2.1.7. Plasmid DNA Purification from Transformed Bacterial Cells

Plasmid DNA was purified from bacterial cells using a Qiagen miniprep kit for starter cultures and Qiagen plasmid midi kit for 100ml cultures according to the manufacturer's protocol. DNA concentration was determined using a NanoDrop spectrophotometer.

2.2.1.8. Restriction Cloning into Chimpanzee Adenovirus 63 (ChAd63)

Antigen inserts were cloned into pENTR™4LPTOS shuttle vector (see **Figure 3.2**), with antigen expression driven by the Cytomegalovirus (CMV) long promoter (CMV_LP containing a regulatory element, enhancer and intron A), between the two attL sites. Acc65I and NotI double restriction digests were set up for the entry vector and the insert DNA. The resulting products were screened by gel electrophoresis and band-specific DNA extracted and purified from excised gels for ligation reactions. After ligation, competent cells were transformed with the entry vector, grown on kanamycin agar plates after which colonies were picked and grown in 3ml LB starter cultures. DNA was extracted and purified from the bacterial cultures using the Qiagen miniprep kit and restriction digest screened by agarose gel electrophoresis for the presence of the insert in the entry vector clones. 1ml of the correct cultures was used to inoculate a 100ml LB culture after which DNA was extracted using the

Qiagen plasmid midiprep kit and quantified. The entry vector clone was recombined into the destination vector, ChAd63-DEST, by Gateway® cloning according to the manufacturer's instructions. The following reaction mix was made up for each entry clone:

150ng	Insert DNA
150ng	ChAd63-DEST DNA
up to 8µl	TE Buffer pH8.0

LR Clonase II was thawed on ice for 2minutes, added to the reaction tubes, mixed well and left to incubate at RT overnight. The reaction was terminated by adding 1µl of Proteinase K for 10minutes at 37°C. The DNA was then used to transform competent cells which were grown under ampicillin resistance overnight. Six individual colonies were screened for the presence of the insert after being grown in starter cultures after which the correct colony was bulked up in 100ml cultures, DNA extracted using the Qiagen plasmid midi kit, quantified and then linearised using PmeI as follows:

6µg	DNA
4ml	Pme I
10µl	10X BSA
10µl	Buffer 4
up to 100µl	dH_20

10µl was used to check linearisation by running on an agarose gel.

2.2.1.9. Restriction Cloning into Modified Vaccinia Ankara

The antigen constructs were cloned into an MVA-GFP2 shuttle vector (see **Figure 3.3**) with antigen expression being driven by the P7.5 promoter between the thymidine kinase sites (TKL and TKR) by restriction digest cloning using Acc65I and XhoI. Double digests using these enzymes were set up for both the insert plasmid and shuttle vector after which the cut DNA was run on an agarose gel and subsequently gel purified. The insert and vector DNA

were ligated overnight after which competent cells were transformed and grown on ampicillin plates. Six single colonies for each were grown up into starter cultures overnight, used to extract DNA, restriction digest screened for the presence of the insert using Acc65I and XhoI and then grown up in larger cultures overnight. DNA was extracted, quantified and linearised using AatII for efficient recombination with the parental virus as follows:

5µg	DNA
5µl	Buffer 4
3µl	Aat II
up to 50µl	dH_20

10µl was used to check linearisation by running on an agarose gel.

2.2.1.10. In-Fusion Cloning

Insert DNA was cloned into a destination vector using In-Fusion cloning kit. In-Fusion cloning reactions were performed at a 2:1 insert:vector ratio according to the manufacturer's recommendation as follows:

2µl	5X In-Fusion Reaction buffer
1µl	In-Fusion enzyme
150ng	Linearised vector
50-200ng	Purified PCR insert
up to 10µl	dH_20

Control reactions were set up using linearised pUC19 as the vector with/without the insert. Reaction tubes were left to incubate at 37°C for 15minutes followed by 15minutes at 50°C and the final volume brought up to 50µl by mixing well with TE buffer (pH8). Competent cells were transformed and grown on kanamycin (for the inserts) and ampicillin (for pUC19) plates overnight at 37°C.

2.2.2. Polymerase Chain Reactions (PCRs)

The primers used for the PCR reactions were designed using either PrimerSelect™ (v10, Lasergene® software, DNASTAR, USA) or Primer3 software [446] synthesised by Sigma Aldrich, UK. The reactions were run using a Bio-rad or G-storm thermal cycler and the resulting products visualised on agarose gels. Insert-specific primers were designed and used in identity (ID) PCR reactions whilst primers flanking the insert were used in flank-to-flank PCRs. Purity PCRs were performed for recombinant MVA generation. DNA was extracted from viral material using DNA Releasy reagent according to the manufacturer's instructions. PCR reactions were performed using KAPA Long Range, hot start polymerase ready mix in 25µl reactions as follows:

12.5µl	2X Master Mix
1.25µl	Primer 1 (10µM)
1.25µl	Primer 2 (10µM)
2µl	Template DNA
8µl	dH_20

The reaction conditions for identity and purity PCRs were set to run for 35cycles, 95°C for 3minutes, 95°C for 40seconds, annealing Tm of 60°C for 15seconds, and extension at 68°C for 1minute/1000bp product expected and 72°C for 3minutes. For the Flank PCR reactions, the extension conditions were modified to 68°C for 3minutes and 72°C for 5minutes.

2.2.3. DNA Sequencing

All DNA sequencing reactions were carried out by Source Bioscience Limited. Sample reactions were carried out at 5µl per reaction with the following requirements specified as follows: plasmid DNA at 100ng/µl, PCR products at 1ng/µl per 100bp and primers at

3.2pmole/μl. Primers were designed using PrimerSelect™ and the sequencing results analysed using SeqMan Pro™ (v10, Lasergene® software, DNASTAR, USA).

2.2.4. Cloning and Generation of Luciferase antigen constructs

The luciferase immunoprecipitation assay system (LIPS) employs the use of Renilla luciferase for the detection of antigen-specific antibodies by luminescence using a Renilla with 8 point mutations that is active when secreted (rLuc8) described by Burbelo et al [447]. In order to detect antigen-specific luciferase expression the antigen needs to be in tandem with rLuc8, and thus to obtain the Renilla-antigen fusion proteins, plasmid DNA containing the insert was Phusion PCR amplified. Insert-specific primers were designed to be 15bp complementary to the rLuc8vector excluding the tPA leader sequence. The rLuc8 vector was linearised using BamHI and HindIII. The PCR and restriction products were then run on an agarose gel, DNA purified from excised gels. The insert DNA by In-Fusion cloned into the rLuc8 vector. The resulting product was used to transform competent cells. The resulting colonies were PCR screened after which the colony with the correct product size was grown from which DNA was purified, quantified and sequenced.

2.2.5. Generation of Recombinant His-tagged Proteins

HEK293 suspension cells have been shown to produce high yields of proteins with the cell line modified to express *Epstein Barr* virus nuclear antigen 1 (EBNA1, HEK293E) producing [448] even greater yields. This system allows for the episomal replication of transfected plasmids containing the EBNA1 replication origin. This transient transfection of HEK293 cells grown in suspension has been used to produce *Plasmodium* proteins that have been otherwise difficult to produce in other expression systems [275, 449, 450]. This expression

system was used to produce recombinant *Plasmodium berghei* sexual-stage antigens in this study as His-tagged proteins.

Codon optimised antigen inserts were cloned from pENTR™4LPTOS shuttle plasmid generated from restriction cloning into a pENTR™4LPTOS containing a biotin acceptor peptide (BAP) in tandem with six histidine (his) residues. Protein expression was driven by the CMV promoter with a tPA leader sequence. The inserts were cloned into the vector by In-Fusion cloning with insert-specific primers. The vector was linearised using Acc65I and BamHI. The presence of the insert within the vector was confirmed by colony screen PCR and subsequently sequenced after bulking up of the DNA.

HEK293E cells were grown in suspension using Erlenmeyer culture flasks in Freestyle™ 293 Expression medium (F293E medium) at 37°C in a humidified incubator at 8% CO_2 at 125rpm. Twenty-four hours before transfection, the cells were seeded at a density of $2.5x10^5$ cells/ml in transfection medium (F293E medium, G418 (1:1000) and 1% FCS)) to allow a density of $5x10^5$ cells/ml for DNA transfection. For transfection, F293E medium was warmed up to RT and mixed with 66μg of DNA for every 100ml of culture. Plasmid DNA expressing biotin ligase (BirA) and GFP were premixed with the DNA at 5% each to enable biotinylation of the protein and determination of transfection efficiency respectively. Polyethylenimine (PEI) solution was added to the medium/DNA mix (100μl per 100ml culture), mixed and left to complex for 10minutes. Transfection medium was removed from the culture and the medium/DNA/PEI mix added drop wise whilst stirring after which the culture was immediately returned to the incubator to allow protein expression. After 3days, transfection efficiency was determined by GFP expression using a stage illuminator. Supernatant was harvested the following day by spinning at 750xg for 10minutes and filter sterilised using a 0.22μM filter. The resulting filtrate was dialysed using a Slide-A-Lyzer

dialysis cassette in PBS according to the manufacturers' instructions. The protein concentration was determined by NanoDrop spectrophotometer and stored at 4°C until use.

2.2.6. IgG Purification

Total IgG was purified using Protein G columns as follows. The samples, binding buffer (BB), elution buffer (EB), and Protein G columns were brought to RT. The columns were equilibrated with BB after which the samples (1:1 mixture with BB) were added and eluted with EB in 300µl of 1M Tris pH9.0. To concentrate, the eluted fraction was transferred to Amicon centrifugal filter device (30,000MWCO), mixed by inversion with PBS to a final volume of 15mL and centrifuged at 3095xg for 45minutes at 4°C until the samples were concentrated. The flow-through was discarded and PBS added up to 15mL, mixed and the centrifugation process repeated. This procedure was repeated to concentrate the sample, which was then filtered through a 0.22µm filter. The sterilised IgG was and quantified using a NanoDrop spectrophotometer and the final concentration adjusted to 2, 2.5 3.5, 4, or 20mg/ml.

2.3. Molecular Virology

2.3.1. Transfection and Generation of Recombinant ChAd63 Virus

The Jenner Institute Viral-Vector Core Facility generated all the recombinant adenoviruses using linearised DNA (**2.2.1.8.**). HEK293 cells expressing a tetracycline repressor protein (HEK293 T-REx™) that suppress the insert protein from being expressed during gene expression thus enabling high-level induction were used to stably transfect according to established Jenner Institute protocols. The cells were seeded in D10B medium in T25 flasks at 90% cell density and incubated at 37°C, 5% CO_2. Transfection mix comprising of OptiMEM, Lipofectamine™ 2000 and DNA restriction enzyme mix were prepared, mixed

and incubated at RT for 5minutes and used to transfect the cells after D10B media replacement for 4hours at 37°C, 5% CO_2. Subsequently the media was replaced with D10B media without Pen/Strep. After 48hours, the cells were passaged into T75 flasks to maintain cell viability and promote virus rescue until cytopathic effects (CPE) were observed. After CPE, the adenovirus-infected cells were harvested and centrifuged (1500xg, 5minutes). The supernatant was discarded, the pellet resuspended in lysis buffer (5ml 1M Tris-HCl pH7.8, 0.5ml 1M $MgCl_2$ in 500ml dH_20), freeze-thawed 3x to release the virus and stored at -20°C. This material was then used to prepare a premaster seed virus stock by infecting HEK293T-REx™ cells in a 6-well plate. The cells were infected in a twofold serial dilution and after 48hours; the well with the most uniform CPE was harvested. ID and Flank-to-Flank PCR reactions were performed on 20µl of the sample to confirm the presence of the insert (**2.2.2.3.**) and the rest stored until for bulk preparation. The premaster seed was thereafter used to inoculate a hyperflask to obtain recombinant virus for purification after observed CPE.

2.3.2. Purification & Quantification of Recombinant ChAd63 Virus

Recombinant ChAd63 virus was purified on a caesium chloride (CsCl) gradient by density centrifugation to established protocols. The harvested infected cells from the hyperflask were pelleted (1500xg, 5minutes) and resuspended in lysis buffer, freeze-thawed twice and treated with benzonase (250U/ml of cell lysate) on a rotary mixer for 1hour at RT and centrifuged (1500xg, 5minutes). CsCl gradient solutions were prepared by underlaying 3.5ml of 1.25g/ml CsCl solution with 3.5ml 1.35g/ml CsCl solution in 14ml Beckman Ultraclear tubes. The cell lysate supernatant was added to the prepared CsCl gradients and centrifuged for 2hours (110,000xg at 4°C) using a Beckman ultracentrifuge. The virus band was removed and added to 6ml of 1.35g/ml CsCl and centrifuged (160,000xg for 16-18hours). The lower

band was collected and dialysed in storage buffer (10mM Tris, 7.5%w/v sucrose pH7.8) for 90minutes changing the buffer every 30minutes. The recombinant virus was removed from the cassette and added as 50µl aliquots into cold labelled tubes in order to preserve the integrity of the recombinant viruses and stored at -80°C. An aliquot of the preparation was also sterility tested before storing at -80°C. The titre of the virus in infectious units (IU) was determined by a dilution immunoassay and the virus yield by UV spectrophotometry according to established protocols.

2.3.3. Transfection & Purification of Recombinant MVA Virus

The Jenner Institute Viral-Vector Core Facility generated the recombinant virus by homologous recombination into the MVA genome (parental virus, MVA expressing red fluorescent protein (RFP)) using the linearised MVA-insert shuttle vector (**2.2.1.9.**) to allow for homologous recombination between the thymidine kinase locus (TKL). Chicken embryo fibroblasts (CEFs) or Doug Foster strain cells 1 (DF1s) seeded in D10 media overnight (37°C, 5% CO_2) to reach 90% confluence in 6-well plates, were infected with MVA-RFP at multiplicity of infection (MOI) of 2.5 and left to incubate for 90minutes (37°C, 5% CO_2). Transfection complex of 1µg of linearised DNA (**2.2.1.9.**) and effectene transfection reagent (according to the manufacturer's instructions) was made and left to incubate for 10minutes. The D10 media in cells was replaced with D2 medium and the transfection complex mix added and left to incubate for 48hours (37°C, 5% CO_2). Recombination and transfection efficiency was visualised by GFP expression after which cells were harvested and stored at -20°C.

The harvested recombinants were sorted by MoFlo using a MoFlo™ XDP cell sorter for expression of GFP according to established Jenner Institute protocols. MoFlo sorting was

conducted after plaque picking from infected cells to purify the GFP-expressing recombinants from MVA-RFP. After the plaques were free of parental virus by fluorescence, ID, flank-to-flank and purity PCRs were performed (**2.2.2.3.**) to confirm the presence of the insert and absence of the parental virus. Upon confirmation, the plaque lysate was bulked up by infecting CEFs or DF1s in T150 flasks to obtain the master seed virus. The pure recombinant virus was purified on a 36%w/v sucrose cushion according to established protocols. ID, flank-to-flank and purity PCRs were performed (**2.2.2.3.**) on the purified virus and stored in aliquots of 200µl until use at -80°C. An aliquot of the preparation was also sterility tested before storing at -80°C. The viral titre was determined in 6-well plates using either CEFs or DF1s using X-Galactosidase liquid staining solution by dilution immunoassay to determine the plaque forming units (PFU).

2.4. Immunology

2.4.1. Animals

For mouse studies, female 6-8 week old BALB/c (H-2^d) mice were used supplied by Harlan (UK) and housed in specific pathogen-free environments. All procedures were performed in accordance with Animal Scientific Procedures Act, UK under the Project Licence (PPL 30/2889) and Personal Licence (PIL 30/8942) and approved by the University of Oxford Animal Care and Ethical Approval Committee. For rabbit studies, New Zealand white rabbits were used and procedures performed by BioGenes (Germany).

2.4.2. Vaccinations

2.4.2.1. Anaesthetic

Mice were vaccinated with the aid of inhalational anaesthesia using a mixture of isofluorane (3.5%) and oxygen (2l/min). This combination was also used as terminal anaesthesia for cardiac bleeds.

2.4.2.2. Vaccines

All ChAd63 vaccines were prepared in sterile endotoxin-free PBS with Ca and Mg. All other vaccines were prepared in sterile, endotoxin PBS without Ca and Mg. For protein vaccination, Ovalbumin (Grade VII) was obtained from Sigma, UK. Pfs25 was produced in *Pichia pastoris* as previously published [339] and was a kind gift from Dr. Y. Wu, National Institute of Allergy and Infectious Diseases. Pfs230-C was produced using the wheat-germ cell-free expression system described previously [451] and a kind gift from Prof. T. Tsuboi, Ehime University.

2.4.2.3. Dose and route

All animals were vaccinated via the intramuscular (i.m) route into both quadriceps. For mouse experiments, $1x10^8$ infectious units (IU) of ChAd63 were administered and $1x10^7$ plaque forming units (PFU) of MVA. For rabbit studies, $5x10^8$IU and $5x10^7$PFU of ChAd63 and MVA were administered respectively. All protein doses were at 1.5μg administered with Abisco®-100 (12μg/dose) in PBS per mouse.

2.4.3. Preparation of Anopheles midgut lysates

120-150 midguts from *A. gambiae*, G3 and Yaoundè, and *A. stephensi*, SDA500 were dissected in cold PBS. The midguts were then transferred to a solution containing 750μl of

M-Per mammalian extraction reagent, 2% ASB-C8ϕ (Merck4Biosciences, UK) and protease inhibitors (1:100 dilution, Sigma, UK) supplemented with 150mM NaCl as described [346]. The midguts were homogenised on ice, span down at 14,000g for 10minutes at 4°C and supernatant transferred to clean tubes. The protein concentration was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions.

2.4.4. Preparation of splenocytes

Mice were subjected to terminal inhalational anaesthesia and spleens obtained by dissection after cervical dislocation. Individual spleens were crushed in PBS and passed through a 70μm cell strainer. The cells were centrifuged at 1350rpm for 5minutes, supernatant discarded and pellet resuspended in ACK lysis buffer for 5minutes. The cells were then immediately mixed by inversion with 25mL PBS and the centrifugation repeated. The supernatant was discarded and the cells resuspended in 5mL D10 medium. The splenocytes were counted using a CASY counter (Schärfe Systems, Germany).

2.4.5. Immunohistochemistry Assay

Frozen cryopreserved tissue sections were warmed up to RT and blocked for 15minutes using Novocastra protein block (Leica, UK). Serum (naive, anti- AnAPN1$_{fl}$, -AnAPN1-Domain I and –AnAPN1-Nterm (pooled and individual samples), and vector control) was diluted 1:50 in dilution buffer (10% FCS in RPMI 1640 supplemented with 2% sodium azide), added to the tissues after discarding the blocking solution and left to incubate for 1hour at RT in a humidified chamber. Monoclonal Abs (mAbs) against CD13, cytokeratin (stains epithelial cells), CD68 and dilution buffer were used as controls. Primary antibody (Ab) was washed off in PBS twice and secondary Ab (goat anti-mouse Ig-HRP, DAKO, UK) diluted 1:100 in dilution buffer was added and left to incubate for 30minutes. The tissues were further washed

twice in PBS and DAB Chromogen substrate buffer (DAKO, UK) added for 6minutes. Slides were then immersed in haematoxylin and eosin stain and washed in tap water. The slides were mounted in oil and allowed to dry overnight before viewing under the microscope.

2.4.6. Enzyme Linked Immunosorbent Assays (ELISAs)

2.4.6.1. Endpoint ELISAs

Midgut lysate (5µg/ml) or recombinant protein (2µg/ml) was used to coat maxisorp or streptavidin 96-well plates 100µl/well in PBS overnight. The coating solution was discarded; plates washed 6x in PBS/T and blocked with 300µl/well of either Casein, 1%BSA in PBS/T or 5% skimmed milk in PBS/T for 1hour. Test sera were diluted in PBS/T and tested either in threefold dilutions down the plate or as single dilutions in duplicates. These were allowed to incubate for 2hours at RT after which the plates were washed 6x in PBS/T and 100µl/well of secondary antibody (goat anti-mouse IgG for mouse samples, goat anti-rabbit IgG for rabbit samples, or goat anti-human IgG for human samples) added. Development was done by adding 100µl of pNPP substrate diluted in diethanolamine buffer and plates were read at OD_{405}. Endpoint for each antigen was determined as the x-axis intercept of the dilution curve at an absorbance value greater than the OD_{405} value for naïve serum plus 3x standard deviation.

2.4.6.2. Standardised ELISAs

Standardised ELISAs were set up according to Miura et al. [452]. Day70 sera with high titres (measured by endpoint ELISA **2.4.6.1.**) were pooled to make a standard reference for which the antibody units (AUs) were determined. To determine the ELISA units of the reference pool, the pool was diluted 3x in independent twofold dilutions starting at 1:1,000 up to 1:512,000. Recombinant protein pre-coated plates (**2.4.6.1.**) were used to which 100µl/well of

the diluted standard samples were added on the plates for 2hours and secondary antibody added for 1hour after washing. Signal was detected by adding 100µl/well of pNPP substrate buffer (1.4.5.1), developed for 20minutes and read at OD_{405}. The ELISA procedure was repeated on the second day with fresh dilutions of the reference pool. The standard reference pool dilutions were assigned arbitrary units starting at 20f or the 1:1,000 dilution and subsequent dilutions assigned units at a factor of 0.5. Four-parameter curve was generated using the values from independent experiments. AUs of the standard were calculated based on the values of the parameters of the curve. AUs of the two runs were accepted if the difference was less than 30% and the average of the two runs was calculated as the AUs of the reference pool. To determine the ELISA units of the test samples, the ELISA was performed (**2.4.6.1.**) with the standard reference pool curve being diluted at 1:1,000 up to 1:512,000 in two independent dilutions. An internal control was added using the standard reference serum diluted to the AUs that gave an OD value of 1. For Pfs25 ELISAs, the reference pool, a kind gift from Dr. Y. Wu, National Institute of Allergy and Infectious Diseases [453], was used to determine the AU of the test samples.

2.4.6.3. IgG Isotype ELISAs

Recombinant protein was used to coat plates as in the Endpoint ELISA above and the ELISA performed as in **2.4.5.1.** with the following exceptions: secondary antibody – biotinylated anti-mouse IgG1, IgG2a, IgG2b or IgG3 was added at 1µg/mL in PBS/T after which Extravidin, diluted 1/5000 in PBS/T, was added after an hour and left to incubate for 30minutes at RT. The plates were washed and subsequent development carried out as outlined in **2.4.6.1.**

2.4.6.4. Synthetic peptide-based ELISA

The ELISAs were performed at Johns Hopkins Malaria Research Institute by Jennifer Armistead as previously described [454]. Maxisorp plates were pre-coated with 100µl/well poly-L-Lysine (50µg/ml in 0.05 M sodium bicarbonate buffer, pH 9.6) per well and incubated for 1hour at RT. All incubations were subsequently performed at RT. Plates were then washed once in PBS and 100µl of 1% (v/v) Glutaraldehyde in PBS added to each well and left to incubate for 15minutes. The plates were washed and 100µl of synthetic peptides added to the appropriate wells and left to incubate overnight. The following day, the plates were washed twice in PBS and 200µl of 1M Glycine added to each well and left to incubate for 1hour. The wash was repeated and 300µl of 1:1 solution of 5% milk and 1% gelatin added and left to incubate for 1hour for further blocking. Afterwards the plates were washed once in PBS, 100µl of diluted antisera added the appropriate wells and left to incubate for 1hour. The plates were then washed 5x in PBS/T after which 100ml of secondary antibody (goat anti-mouse HRP-conjugated, diluted 1:500) added and left to incubate covered in foil for 1hour. The wash in PBS/T was repeated after which 100µl of peroxidase substrate was added and incubated covered in foil for 5minutes. To stop the reaction, 100ml of stop solution was added and the OD_{450} measured.

2.4.7. Luciferase Immunoprecipitation System Assay (LIPS Assay)

Antigen-rLuc plasmid DNA (3µg), negative (non-rLuc expressing) and positive (rLuc expressing) control plasmids were mixed with 1µg of GFP-expressing plasmid in individual tubes to which Opti-MEM (250µl) was added whilst a lipofectamine mix was prepared by mixing Lipofectamine™ 2000 (10µl/tube) and Opti-MEM (250µl/tube) and left to incubate for 5minutes. The DNA and lipofectamine solutions were mixed and left to incubate for

20minutes. D10 media was carefully aspirated from HEK293 cells in a 6-well plate seeded at 90% confluence, the DNA-lipofectamine mix added to each well (made up to a final volume of 800µl for each well with Opti-MEM) and left to incubate at 37°C and 5% CO_2 overnight. Transfection efficiency was checked by quantification of GFP expression using the OPTIMA fluorescence microplate reader. The cells were then harvested on ice in lysis buffer after aspirating the media. The cells were scrapped off, transferred to pre-cooled tubes, sonicated for 15seconds and span down for 4minutes at 12,500rpm to obtain cell lysates. Luciferase expression (luminescence light units/ml, LU/ml) of the cell lysate was measured using a Varioskan Flash Multimode Reader where $\geq 2x10^8$LU/ml was set as the baseline luciferase expression for the assay to be performed.

Serum samples were diluted in Buffer A (1:100 for pre-boost and 1:1000 for post-boost samples) and 50µl/well added to an equal volume of $2x10^8$LU/ml in a 96-well plate and left to incubate at RT on a rotary shaker. 30% of Protein A/G UltraLink Resin was prepared in PBS of which 5µl was added to a 96-well Multiscreen HTS filter plate. The serum-rLuc8-antigen mixture was transferred to the filter plate and left to incubate for 1hour on a rotary shaker at RT. After incubation, the plates were washed 8x in Buffer A and then 2x in PBS. 50µl/well of PBS was added to prevent the plates drying out whilst Renilla luciferase assay buffer was brought up to RT to which 100x Renilla luciferase assay substrate was added. This was used to detect the luciferase expression/well and the reaction immediately stopped/well with 2M HCl to avoid 'leakage' of luciferase spilling into the subsequent wells.

2.4.8. Western Blot Analysis

HEK293 cells seeded onto 6-well plates were transfected with plasmid DNA expressing antigen of interest (**2.4.7.1.**) and incubated overnight at 37°C and 5% CO_2 from which

supernatant and cell lysate were harvested. Purified *P. falciparum* cultured gametocyte (kind gift from Dr. R. Dinglasan, Johns Hopkins University); *P. berghei* purified gametocytes and ookinetes; Anopheline midgut lysates; and supernatant and lysate from transfected cells were boiled at 95°C for 5minutes in non-reducing, and reducing Laemmli buffer and run on 4-20, 8, 10 or 12% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and blocked in 3% BSA/PBS overnight. Blocking solution was discarded and the blots were washed 2x with PBS/T before incubating with sera or IgG diluted in 3% BSA/PBS for 1 h. The blots were then washed and incubated for another hour with donkey anti-mouse or anti-rabbit IgG conjugated to HRP. After repeating the washing procedure, the protein bands were detected by chemiluminescence using ECL substrate and images captured using a LAS 4000 biomolecular imager. Colorplus Pre-stained Protein ladder was used to estimate the relative protein mobility.

2.4.9. Antibody secreting cell ELISPOT

Prepared splenocytes (**2.4.4.)** from vaccinated mice were used to assess the IgG specific antibody secreting cells (ASCs) as described previously [131]. MAIP ELISPOT plates were coated with 2μg/ml of recombinant protein and incubated overnight at 4°C. The plates were washed twice with PBS and blocked with D10 medium for 1hour at 37°C, 5% CO_2. Resuspended splenocytes were diluted to $5x10^5$cells and plated at a single dilution with more than 6replicates per sample. Plates were incubated for 4hours at 37°C, 5% CO_2 after which they were washed 6x with PBS and incubated overnight with biotinylated goat anti-mouse IgG (1:1000 in PBS) at 4°C. The plates were then washed 6x in PBS and Streptavidin alkaline phosphatase polymer (1:1000, 50μl/well in PBS) added and left to incubate for 1hour. The spots were developed after washing 6x in PBS with Bio-Rad alkaline phosphatase

conjugate substrate kit for 10minutes, washed in water and left to dry overnight. The plates were counted using AID plate reader software (AID, Cadama Medical).

2.5. Parasitology

2.5.1. Parasite strains

P. falciparum NF54 strain and *P. berghei* ANKA strain clone 2.34 were used. For experiments with fluorescent parasites, *P. berghei* (clone 15cy1A) expressing GFP (PbCTRPp.GFP) previously described [455] was used, a kind gift from Dr C. Ramakrishan, Imperial College London. *P. berghei* Pf25DR expressing Pfs25 based on ANKA 2.34 strain previously generated [372] was a kind gift from Dr. A. Blagborough, Imperial College London.

2.5.2. Parasitised RBC Challenge

Female 6-10 week old Tuck Ordinary (TO) mice (Harlan, UK) were used as donor mice for challenge with parasitised RBCs (pRBCs) as previously described [361, 362]. Frozen aliquots of *P. berghei* parasite-infected blood (100µl, ~$5x10^7$ parasites) were thawed and immediately injected via the intraperitoneal (i.p.) five days prior to challenge. Parasitaemia was checked by thin blood smear. Giemsa staining was performed by air-drying the smears, fixing in methanol for 1minute and placed in Giemsa solution for 30minutes. Slides were then examined at 100x oil immersion using a light microscope. The percentage parasitaemia was determined by taking the average of counts from 3 fields from which an estimate of the number of parasitised RBCs per ml was determined. On the fifth day, mice were checked for parasitaemia and cardiac bled under terminal anaesthesia. Anaesthetic (2% Rompun solution (20mg/ml), Ketaset (100mg/ml), and PBS mixed at a ratio of 1:2:3 respectively) was

administered i.m. and the blood drawn through a 26-gauge needle and syringe containing heparin (300U/ml of Heparin sodium salt in PBS).

2.5.3. Gametocyte and Ookinete Production and Purification

P. berghei gametocyte and ookinete culture and purification was conducted according to published protocols [362]. TO mice were injected i.p. with phenylhydrazine (PH, 6mg/ml in PBS) to induce reticulocytosis. Three days later, mice were infected with *P. berghei* (**2.5.2.**). Three days post-infection, mice were checked for parasitaemia (**2.5.2.**) and exflagellation. To check exflagellation, a drop of exflagellation medium was added to a drop of blood and after 10minutes viewed at 40x using a light microscope. Mice with good exflagellation (>10 exflagellation centres per 4 fields of view) were cardiac punctured under anaesthesia (**2.5.2.**). Parasitised blood was added to RPMI-1640 (pH 7.4) (ratio of 40% RBCs to 60% medium) and kept at 37°C to prevent gametocyte activation. White blood cells were purified from the blood using plasmodipur filters by passing through the filter onto CF-11 columns followed by 10mls ookinete medium (with FCS) per ml of mouse blood. For ookinete production, 1.3mls of the flow-through was added to 32mls of ookinete medium with FCS per flask and left to incubate for 24hours at 19°C.

For purification of gametocytes, the procedure was carried out as previously described [323, 456]. For the purification of ookinetes, the 24hour cultures were mixed and a 20µl sample diluted in PBS (1:1) used to count ookinetes using a haemocytometer. The cultures were then transferred to 50ml tubes, placed on ice and spun at 500xg for 5minutes at 4°C. The supernatant was discarded, pellet resuspended and 50ml of cold 0.17M NH_4Cl added to lyse RBCs on ice for 20minutes. The mixture was pelleted at 500xg for 5minutes at 4°C after which the cells were resuspended in 2ml of ookinete medium. This was then layered onto 5ml 17.3w/v Nycodenz solution (made up in PBS), spun at 500xg for 30minutes at 4°C (slow

acceleration, no brake), the interphase removed and topped up with 12ml of ookinete medium. The cells were pelleted at 300xg, 10minutes at 4°C (low deceleration), supernatant discarded, pellet resuspended in 12ml of ookinete medium and spun at 200xg. The pellet was transferred to a 15ml tube and made up to 10ml with ookinete medium. The ookinetes were counted as above, spun at 180xg for 10minutes 4°C (low deceleration), transferred to 1.5ml tubes and further spun at 500xg for 1minute. The supernatant was discarded and snap frozen on dry ice and stored at -20°C until use.

2.5.4. Immunofluorescence Assays (IFA)

Slides were coated with poly-L-Lysine (0.5%) for 2minutes and then washed off and left to dry overnight. *P. falciparum* gametocyte culture was spun down and the supernatant decanted after which the pellet was resuspended and added to tubes containing 20µl of ookinete media without FCS at 0, 5, 10, 15 and 20 minutes time intervals. To fix the cells, 180µl of 4% paraformaldehyde (PFA) was added before the addition of the parasite material for each time point. For ookinete reactivity, the culture was spun down, the pellet resuspended and the fixative added. The mixed samples were then pipetted or smeared onto labelled slides and left to set overnight at 4°C. The slides were washed twice in TBS and then permeabilised (0.2% TritonX-100 in PBS) for 5minutes, washed 5x and blocked for 45minutes using 10% goat serum, 1% BSA in TBS (BSA/TBS). Sera was diluted in PBS/BSA at 1:50 and 1:100 and after washing off the blocking solution, the diluted sera was added and left to incubate for 1hour in a humidified chamber. The slides were then washed 4x in TBS, secondary antibody (goat anti-mouse Alexa-488) diluted at 1:200 in BSA/PBS) added and incubated for another hour. The slides were then washed 3x and mounted in vectashield-DAP1 and left to set overnight at 4°C. Immunofluorescence staining was detected using a Leica DMI3000 B microscope at 100x oil-immersion.

2.5.5. In vitro Ookinete development Assay (IVOA)

The *in vitro* development of *P. berghei* ookinetes (using PbCTRPp.GFP) was assessed using the ookinete assay as previously described [361, 457]. GFP expression was used as a read out and parasites from three separate infected mice were considered as triplicate independent experiments every time the assay was performed. Ookinete medium was prepared by adding FCS and Pen/Strep. Sera or purified IgG was diluted in prepared ookinete medium from test and vector control vaccinated animals. Cycloheximide (CH, 10mM), in triplicate per plate and wells with ookinete medium only served as controls. 100µl/well of diluted samples in duplicate was added to 96-well tissue culture plates.

Parasitised blood from each PH-treated mouse (**2.5.4.**) was diluted in ookinete medium (1:20) and 100µl/well immediately dispensed using an automated dispenser into the prepared 96-well plates. The plates were covered in foil and incubated at 19°C for 22hours. After the incubation period, the plate was read using an OPTIMA fluorescence microplate reader. CH treated wells were considered as baseline, the mean fluorescence of which was background subtracted and the results represented as percentage of the mean of the medium only and represented as inhibition of ookinete production [361, 457].

2.5.6. Feeding Assays

Feeding assays were performed in different locations using 5-7day old females of different mosquito species and parasite sources. The following mosquitoes were used for the respective parasite source; *A. stephensi* SDA500 strain [458] or *A. gambiae* G3 and Yaoundé strains for *P. berghei* infections (Jenner Institute and/or Imperial College); *A stephensi* Nijmegen strain for *P. falciparum* NF54 (NIH membrane feeding assay evaluation centre); and *A gambiae s.s* (M form) lab-adapted [459] for *P. falciparum* field isolates (IRSS-IRD,

Burkina Faso). The read-out for efficacy was the number of oocysts counted from dissected mosquitoes recorded as infection intensity and the number of infected mosquitoes recorded as infection prevalence. The percent inhibition of infection intensity and prevalence were determined using the following formulas respectively [361, 377, 378]:

$$100 - \left(\frac{\text{mean oocyst test}}{\text{mean oocyst control}}\right) \times 100\%$$

$$100 - \left(\frac{\%\ \text{infection prevalence test}}{\%\ \text{infection prevalence control}}\right) \times 100\%$$

In this study, a number of measures were adopted to allow effective comparative assessment based on efficacy. Firstly, all membrane feeding assays were conducted against the same parasite exposure in any given experiment. Thus allowing comparisons of TBVs against the same control intensity/parasite exposure. Secondly, efficacy was to be reported as inhibition of both infection intensity and prevalence. All results tabulated include mean intensity in the controls, number of mosquitoes dissected for each intervention including controls, and confidence interval estimates for both infection intensity and prevalence. Thus with respect to this study, transmission-blocking efficacy (TBE) was re-defined as the ability of an intervention to inhibit both infection intensity and prevalence. Thirdly, all the data were analysed using the methodology suggested by Churcher and colleagues [378] allowing effective interpretation of the results. Additionally, in order to determine whether the observed TBA [as a result of either mixing IgG or combining antigens in a single vaccine] was additive, sub-additive and/or synergistic. The following definitions were used for these parameters.

Additive: $a_{m+n} = a_m + a_n$

Sub-additive: $a_{m+n} \leq a_m + a_n$

Synergistic: $a_{m+n} \geq a_m + a_n$

Where, a_{m+n} represents the observed efficacy of the combination; a_m and a_n are the observed efficacies of the individual respective vaccines.

2.5.6.1. Direct Feeding Assay (DFA)

Direct feeding assays were performed according to previously published protocols [361, 362]. Mice were vaccinated and 2weeks post-boost were either PH-treated, infected (**2.5.4.**) and directly fed onto 24hour starved *A. stephensi* SD500 or *A. gambiae* Yaoundé mosquitoes prepared in a pint-size cup the day before the feeding experiment. After 24hours of feeding, unfed mosquitoes were removed by brief CO_2 exposure whilst the infected were maintained on a 12hour day-night cycle at 19-22°C, 50-80% relative humidity on fructose solution. Midguts were either dissected on 11-13days post-feeding from which the numbers of oocysts were counted and the number of infected mosquitoes recorded.

2.5.6.2. Standard Membrane Feeding Assay (SMFA)

2.5.6.2.1. *P. berghei* Infections

Female 4-7day old mosquitoes were placed in pints and starved for 24hours. On the day of feeding, test or control sera or purified IgG was diluted in PBS (100µl total volume) and kept at 37°C. Blood from PH-treated infected mice was obtained (**2.5.4.**) and immediately added to the serum or IgG, and transferred to the prepared parafilm membrane in glass feeder. The mosquitoes were allowed to feed for 30minutes in the dark. After 24hours, unfed mosquitoes were removed by brief CO_2 inhalation whilst the infected were maintained on a 12hour day-night cycle at 19-22°C, 50-80% relative humidity on fructose solution. Days 10-12 post-feeding, midguts were dissected, oocysts counted and the number of infected mosquitoes recorded blinded.

2.5.6.2.2. *P. falciparum* Infections

In vitro grown *P. falciparum* NF54 mature gametocyte cultures were mixed with RBCs and normal human serum with or without complement and kept at 37°C just prior to feeding to prevent exflagellation. This was then transferred to tubes containing diluted serum or purified IgG, mixed and then fed to 4-6day old starved female via a parafilm membrane in a glass feeder for 15-45minutes. The infected mosquitoes were then maintained at 26°C and 75% relative humidity on 10% Karo syrup for 7-8days after which midguts from 20 mosquitoes were dissected, oocysts counted and the number of infected mosquitoes recorded.

2.5.6.3. Direct Membrane Feeding Assay (DMFA)

5-11 year old children in Bobo-Dioulasso, Burkina Faso, were screened for the presence of asexual and sexual-stage *P. falciparum* parasites by thick blood smears using 10% Giemsa staining. Parasites, asexual and sexual, were quantified as an average of 8000 white blood cells per μl of blood. Informed consent from parents or guardians was obtained for children positive for gametocytes with microscopic gametocytemia of more than 20gametocytes/μl (Protocol 003-2009/CE-CM, Centre Muraz institutional ethical committee). The exclusion criteria for the gametocyte positive children was (a) presentation of severe clinical symptoms; and (b) presence of gametocytes from mixed parasite infections. Up to 10mLs of blood was drawn in heparinized tubes, from which 350μl was immediately aliquoted and span in a 37°C preheated centrifuge to remove plasma. The blood was washed once with an equal volume of RPMI 1640 and mixed with 180μl of test or control IgG. The mixture was then immediately fed to previously starved 5-7 day old female lab-adapted *A. gambiae sensu stricto* M form mosquitoes as previously published [459, 460] and described in **2.5.6.2.**

2.6. Statistical Analysis

All statistical analysis was performed using GraphPad PRISM 6.01 (GraphPad Software, San Diego, CA, USA) unless specified. The normality distribution of the data was determined by a D'Agostino-Pearson omnibus test. For non-parametric analyses the following were performed; Wilcoxon matched-pairs signed rank or Mann-Whitney tests to compare between two matched and unmatched groups respectively; Friedman or Kruskal-Wallis test with Dunn's correction for multiple comparison to test three or more matched and unmatched groups respectively. For parametric analyses, paired or unpaired *t*-test was performed comparing two groups whilst a One-way or Repeated-measures ANOVA with Holm-Sidak's correction for multiple comparison was used to compare three or more matched and unmatched groups respectively. All the antibody data was transformed (Log_{10}).

To determine correlation, Spearman's rank or Pearson's test correlation was used for non-parametric and parametric datasets. A Fisher's exact test was used to determine associations between proportions.

For statistical analysis of feeding experiments, the individual oocyst counts and prevalence of infection observed per mosquito were analysed using a generalised linear mixed model (GLMM) according to previously published data simulations [378]. A zero-inflated negative binomial error structure was used to describe oocyst intensity whilst a binomial error structure was used to determine oocyst presence or absence. Models were compared using a likelihood ratio test and confidence intervals generated using bootstrapping methodology. An overall analysis of the relationship between the oocyst intensity and prevalence was performed using a negative binomial distribution modified from published assumptions [377, 378]. This analysis was performed using R software GLMM statistical analysis package by Dr. T. Churcher.

Significance was considered at $p<0.05$. Asterisks (*) represent statistical significance where *$p<0.05$, **$p<0.01$ and *** $p<0.0001$.

3. Generation of Recombinant Viral-vectored Vaccines

3.1. Introduction

Development of TBVs, as protein vaccines, has been hampered by the expression of full-length protein with the induced antibodies having varying TBA depending on the antigen expression system and adjuvants used to elicit the response. However, the use of viral-vectors provides a robust platform to allow full-length antigen expression. Pfs25 and Pvs25 have been expressed in HAdV5, ChAd63 and MVA, where, in mice, these vectors have been able to induce transmission-blocking antibodies [340, 372]. This has provided a precedent and good evidence that transmission-blocking antigens can be expressed in viral-vectors and can be used to induce antibodies with functional activity.

For the effective delivery of any given transgene, the presence of regulatory elements in the expression vector are vital for optimal transcription and translation [461-463]. Xu et al. (2001) were able to show that use of a strong promoter is the first essential choice with viral promoters such as the Cytomegalovirus (CMV) immediate-early promoter being ideal for high-level transgene expression [462-464]. In addition, presence of a polyadenylation (polyA) signal such as the bovine growth hormone (BGH) sequence allows for stability and export of the transcript [462, 465]. Furthermore, the shuttle vector should include: cloning sites, origin of replication (ORI) e.g. *E. coli* ORI (ColE1), secretory signal sequences [248, 443, 466, 467], Kozak sequence [444], and codon optimisation of transgene sequence [445]. These together enable the effective expression of a transgene and were thus incorporated in the generation of viral-vectors used in this study.

This chapter explores whether a repertoire of TBV candidate antigens can be expressed in the ChAd63-MVA viral-vectored system. The design of the leading TBV candidate antigens, AnAPN1, Pfs230-C, Pfs48/45 and PfsHAP2, and their development as recombinant ChAd63

and MVA viral-vectored vaccines is described. The procedures and approaches described in this chapter were used to design and generate all the recombinant viral-vectored vaccines used in this study.

3.1.1. Generation of Recombinant Adenoviruses

Replication-deficient recombinant adenoviruses have been generated and adapted for use as vaccine vectors by deletion of genes required for viral replication and T-cell-mediated cytotoxic responses; as well as genes enabling addition of transgenes (E1 and E3 regions respectively) [402, 410, 412, 468, 469]. The removal of these regions enables accommodation of transgenes up to 8kbp of DNA [470] without compromising the stability and growth of the virus [463]. Due to the deletion of the E1 region, recombinant viruses require E1-supplementating cell lines, such as HEK293 and PER.C6, to supply the region *in trans* [471, 472]. This has mainly been achieved for human serotypes especially HuAdV5 whereas for ChAd63 and other simian serotypes, further modification to enable propagation in packaging cell lines has led to the substitution of the E4 region with that of HuAdV5 [411, 412, 473].

Replication-deficient ChAd63 viruses can be generated by recombination between homologous flanking sequences within the E1 region from molecular clones using Gateway® cloning technology. This system works on the premise of the site-specific recombination properties of bacteriophage lambda (λ) [474]. This systems allows for the integration of λ into the *E. coli* chromosome allowing recombination between DNA specific sites (*att* sites) mediated by recombinant proteins such as LR [474, 475]. The reaction with Gateway LR enables recombination between the *att*L sites in the entry molecular clone and the *att*R sites in the destination vector resulting in an *att*B expression clone containing the heterologous transferred DNA of interest and an *att*P by-product [475]. The resulting bacterial sequences

are cleaved by restriction enzyme digestion allowing the exposure of recombination sites, and upon transfection into the packaging cell line, pure recombinants are formed.

In order to obtain ChAd63 viruses expressing the individual candidate antigen(s), the antigen of interest was first inserted into the multiple cloning site in the entry clone (pENTR™) by restriction cloning. The resulting entry clone was then used to perform the LR recombination reaction with the pChAd63 destination vector to generate an expression clone (pChAd63-Insert) containing the candidate antigen of interest. After restriction digest, the pChAd63-Insert expression clone was used to transfect HEK293 cells from which antigen specific recombinant ChAd63 viruses were generated. To confirm the identity and purity of each generated recombinant virus: (1) identity PCRs were performed to check for the presence of the antigen of interest using antigen-specific primers (Identity PCR); and (2) purity PCRs were performed to confirm that the antigen of interest and the respective relevant regulatory elements were present in the recombinant(s) using insertion-site specific primers (Flank-to-flank PCR).

3.1.2. Generation of Recombinant MVA

Modified vaccinia virus Ankara (MVA) is a 178kbp double stranded DNA virus [476] with an insertion capacity for transgene expression [416, 417]. This poxvirus viral-vector is a highly attenuated strain that was made replication-deficient via several rounds of serial passage in chicken embryonic fibroblasts (CEFs) rendering it non-infective in mammalian cells [415]. In spite of this, transgene expression in non-permissive cells such as mammalian cells is unaffected and is achieved at substantial levels comparable to that expressed by wild-type virus [477]. The early and late vaccinia virus p7.5 promoter, that enables perpetual transgene expression, has been shown to induce influenza transgene-specific antibody

responses comparable to virus infection [478, 479] and was thus used to drive antigen expression.

Generation of recombinant MVA viruses compared to recombinant adenoviruses involves a single insertion event mediated by homologous recombination at a desired locus [480]. The insert of interest is restriction cloned into a shuttle vector containing a green fluorescent marker (GFP) and used to perform homologous recombination with the parental virus (containing a red fluorescent marker, RFP). These crossover recombination events occur at low frequencies when transfected into CEFs or baby hamster kidney (BHK-21) cells within the Thymidine kinase (TK) gene locus. The recombinants expressing the transgene of interest are distinguished from the non-recombinants by clonal selection and plaque purification by expression of either GFP or RFP respectively.

MVA recombinant viruses expressing the antigens of interest were generated by cloning into the MVA.GFP shuttle vector. The resulting clones were linearised and transfected into either CEFs or the CEF immortalised cell line, DF-1 cells [481], infected with MVA.RFP parental virus. The products of the homologous recombination at the TK locus were sorted by a fluorescence-activated cell sorter (FACS) enriching the GFP-positive recombinants from the parental RFP non-recombinants. The recombinants were purified by repeated plaque picking to ensure all traces of the non-recombinants were eliminated. In line with what was described for ChAd63, to confirm the identity and purity of each generated recombinant virus: (1) identity PCRs were performed to check for the presence of the antigen of interest using antigen-specific primers (Identity PCR); (2) purity PCRs were performed to confirm that the antigen of interest and the respective relevant regulatory elements were present in the recombinant(s) using insertion-site specific primers (Flank-to-flank PCR); and (3) additional

purity PCRs were performed to confirm that all traces of the parental virus were eliminated by the detection of RFP using RFP specific primers (Purity PCR).

3.2. Results

3.2.1. Antigen Design

Protein antigen sequences were accessed from the NCBI database and bioinformatics determined for *A. gambiae* PEST strain and *P. falciparum* 3D7 strain for vector- and parasite-based antigens (**Table 2.2**). All the endogenous SP sequences were substituted for the tissue plasminogen activator (tPA) signal peptide (**Figure 3.1**). This sequence has previously been shown to allow optimal transgene expression and immunogenicity [248, 443, 467]. In addition to the insertion of the tPA sequence upstream of the antigen sequence, each antigen was designed to have a Kozak sequence before the ATG start codon site to allow for optimal initiation of translation as previously described [444]. *Plasmodium* GPI anchors have been shown to interfere with post-translational protein expression in mammalian cells [482-484] and therefore the GPI anchor sequences and TMDs were omitted (**Figure 3.1**). A stop codon was simultaneously inserted downstream of the antigen sequence. The predicted N-glycosylation sites were either substituted (changing of asparagine to glutamine residues (Asn-X-Thr/Ser to Gln-X-Thr/Ser)) or left intact (only done for Pfs48/45). The sequences were optimised for human codon usage by GeneArt®, as it has been shown to improve antibody responses of *Plasmodium* transgenes by enhancing expression [445, 485]. Restriction cloning sites corresponding to those utilised for cloning into ChAd63 and MVA were incorporated upstream and downstream the antigen sequence (**Figure 3.1**). The design of Pfs25 has been described previously and is comparable to the design of the antigens used in this study [372].

3.2.1.1. AnAPN1

AnAPN1 as a potential TBV candidate antigen was first described by Dinglasan et al. (2007) [348], based on *A. gambiae* Keele strain, as a conserved antigen across anopheline mosquito species. Thus for this study, the construct was designed based on the available sequence of *A. gambiae* PEST strain (NCBI reference sequence (REFSEQ) XP_318000.4 (**Table 2.2**)). The 1020αα protein sequence consisted of a SP (1-19αα) and a GPI anchor sequence (997-1020αα), which were substituted and omitted respectively, with no predicted putative N-glycosylation sites and TMDs (**Figure 3.1A**). The 135αα sequence of AnAPN1 previously described didn't induce complete blockade of either *P. falciparum* or *P. berghei* infection in *A. gambiae* and *A. stephensi* [348]. Therefore, for this study it was postulated that the full-length sequence would be able to induce complete inhibition owing to the possible breadth of antibody responses generated from this construct. Thus the codon optimised vaccine sequence included 977αα (20-996αα, 2931bp) in tandem with an N-terminal tPA signal sequence and referred to as AnAPN1 full-length ($AnAPN1_{fl}$) (**Figure 3.1A**).

3.2.1.2. Pfs230-C

Pfs230 is expressed on male gametocytes, gametes and ookinetes as a 360KDa (3135αα) protein that is processed to produce a 310KDa segment when the parasite emerges from RBCs in the mosquito midgut [316, 486, 487]. This protein in its entirety poses a challenge for vaccine design due to its length. Thus initial characterisation of the protein resulted in the production of six distinct regions to determine their TB potential of which only antibodies raised to region C (443-1132αα) exhibited TBA [321]. Further analysis of the TB potential of this region revealed that the entire region was essential to have a significant impact on inhibiting infectivity to mosquitos [488]. Therefore, the vaccine construct for Pfs230 was designed to incorporate amino acids 443-1132 (**Figure 3.1B**) and based on *P. falciparum*

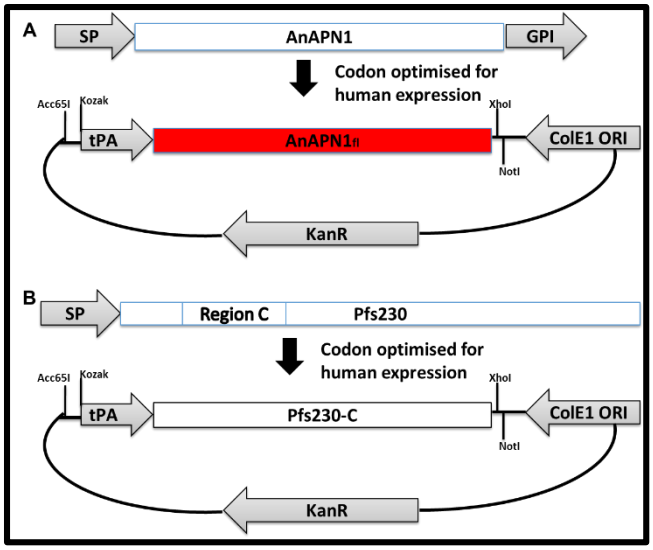
3D7 strain sequence (NCBI REFSEQ AAA29725.1 (**Table 2.2**)). The entire 3135αα protein has a SP (1-20αα), no predicted GPI anchor and twenty-three predicted N-glycosylation sites with only five present in region C. All five N-glycosylation sites within region C were substituted at positions 429, 1161, 1341, 1557 and 1911 after which an N-terminal tPA sequence was included, and codon optimised for human usage and referred to as Pfs230-C (**Figure 3.1B**).

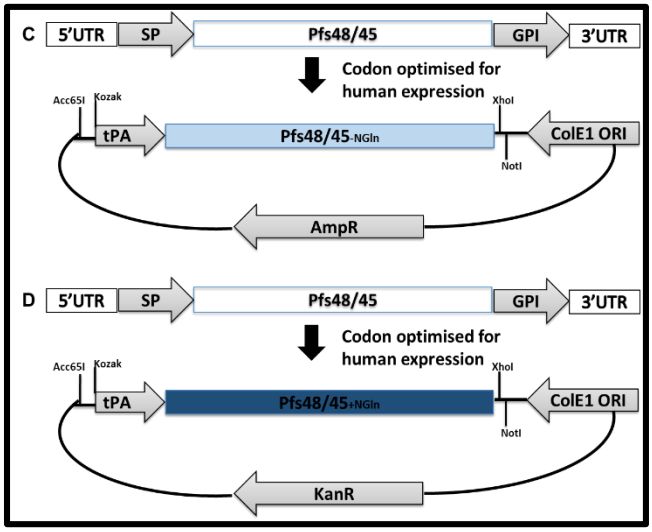
3.2.1.3. Pfs48/45

The gametocyte and gamete male specific protein Pfs48/45 is expressed as a doublet at 48kDa and 45kDa consisting of 448αα [292]. Characterisation of this protein by Kocken et al. (1993) revealed that it has a SP (1-27αα), GPI anchor (426-428αα) and seven potential N-glycosylation sites [292] which were all predicted by bioinformatics (**Figure 3.1C and D**). It has been suggested that *Plasmodium* doesn't N-glycosylate or possess the necessary machinery for formation of N-glycosylated proteins [489]. It has also been previously shown that expression of Pfs48/45 in mammalian expression systems has led to the production of a glycosylated product with no TB potential [490]. Thus to determine whether N-glycosylation site substitution had an impact on immunogenicity and efficacy, two versions of Pfs48/45 were designed. The vaccine constructs were based on *P. falciparum* 3D7 strain (NCBI REFSEQ CAA80119.2 (**Table 2.2**)), lacked endogenous SP and GPI anchor with either N-glycosylation sites substituted (referred to as Pfs48/45$_{\text{-NGln}}$, positions 50, 131, 190, 204, 254, 299 and 303 (**Figure 3.1C**)) or intact (referred to as Pfs48/45$_{\text{+NGln}}$, (**Figure 3.1D**)). The tPA signal peptide was added upstream of the antigen sequence for both constructs and the sequences codon optimised of human usage.

3.2.1.4. PfsHAP2

The TB potential of the conserved male sterility gene encoding HAP2 has been demonstrated in *P. berghei* ANKA strain with incomplete inhibitory activity generated from antibodies raised against a fragment (355-690αα out of 812αα) [323]. The vaccine construct designed was based on *P. falciparum* 3D7 strain full-length protein sequence (899αα, NCBI REFSEQ XP_001347424.1 (**Table 2.2**)) and referred to as PfsHAP2. A comparison of the PbsHAP2 and PfsHAP2 sequences revealed a 58% identity score and a positive coverage of 73% (NCBI protein basic local alignment search tool (BLAST)) with PfsHAP2 amino acid regions 41-736 and 748-836 being identical by Clustal W sequence alignment. PfsHAP2 had a predicted SP (1-33αα), GPI anchor (872-899αα), two TMDs (14-30αα and 689-711αα) and 8 possible N-glycosylation sites (**Figure 3.1E**). The endogenous SP, GPI anchor and TMDs were all removed with the vaccine construct consisting of an N-terminal tPA sequence, amino acids 34-688 with N-glycosylation sites substituted (positions 145, 297, 499, 481, 535, 541, 593, and 612). This sequence was codon optimised for human usage (**Figure 3.1E**).


A
SP
AnAPN1
GPI
Codon optimised for human expression
Acc65I
Kozak
tPA
AnAPN1fl
XhoI
NotI
ColE1 ORI
KanR
B
SP
Region C
Pfs230
Codon optimised for human expression
Acc65I
Kozak
tPA
Pfs230-C
XhoI
NotI
ColE1 ORI
KanR


C
5'UTR
SP
Pfs48/45
GPI
3'UTR
Codon optimised for human expression
Acc65I
Kozak
tPA
Pfs48/45-NGln
XhoI
NotI
ColE1 ORI
AmpR
D
5'UTR
SP
Pfs48/45
GPI
3'UTR
Codon optimised for human expression
Acc65I
Kozak
tPA
Pfs48/45+NGln
XhoI
NotI
ColE1 ORI
KanR

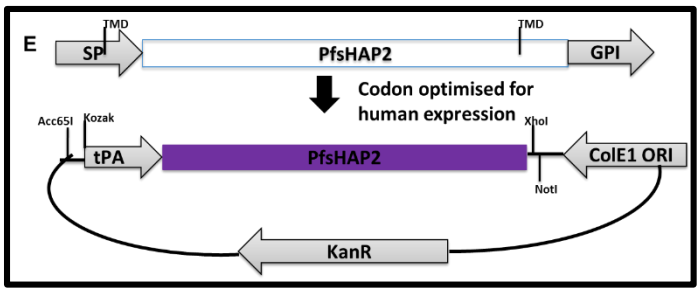

E
TMD
SP
PfsHAP2
TMD
GPI
Codon optimised for human expression
Acc65I
Kozak
tPA
PfsHAP2
XhoI
NotI
ColE1 ORI
KanR

Figure 3.1. Schematic of the design and construction of leading candidate antigens.

Antigen sequences were obtained from NCBI protein database and bioinformatics determined for the presence of signal peptides (SP); glycophosphatidylinositol (GPI) anchors; transmembrane domains (TMDs) and N-glycosylation (NGln) sites. Endogenous SPs, GPIs, and TMDs were omitted for (**A**) AnAPN1$_{fl}$; (**B**) Pfs230-C; (**C**) Pfs48/45$_{-NGln}$; (**D**) Pfs48/45$_{+NGln}$; and (**E**) PfsHAP2. N-glycosylation sites were substituted for all except Pfs48/45$_{+NGln}$ where they were left intact. The endogenous SPs were replaced with tissue plasminogen activator (tPA) signal peptide, a Kozak sequence and start codon placed upstream of the signal peptide and stop codon downstream of the antigen open reading frame, and respective restriction sites incorporated. The sequences were then codon optimised for human usage into respective plasmids expressing either kanamycin (KanR) or ampicillin (AmpR) resistance genes with an origin of replication (ColE1 ORI). For Pfs230-C (**B**), only the region corresponding to 443-1132αα was incorporated (Region C).

3.2.2. Generation of recombinant ChA63 vaccines

3.2.2.1. Restriction cloning of antigen inserts

Replication-deficient recombinant ChAd63 vaccines expressing each individual vaccine construct were generated by restriction cloning. The plasmids containing the respective codon optimised antigen inserts (tPA-Insert) (**Figure 3.1**) and the entry plasmid were restriction digested using Acc65I and NotI within the multiple cloning sites (**Figure 3.2A**). The entry plasmid contained the human cytomegalovirus (CMV) long promoter (with regulatory element, enhancer and an intron A sequence) (CMV-LP) that has been shown to enhance insert expression [491] and bovine growth hormone polyadenylation termination sequence (BGH polyA). In addition, this entry plasmid contained two tetracycline operator sequences (TOS) for regulated induction of the insert and hereafter referred to as pENTR™4-

LPTOS.CMV_LP.BGH_PolyA). The restriction derived entry plasmid was ligated with the tPA-Insert clone to generate pENTR™4-LPTOS.CMV_LP-tPA-Insert-BGH_PolyA clones that were screened for insert presence by restriction digest. The correct clone was then inserted into the pChAd63-destination plasmid by Gateway® cloning using LR Clonase (**Figure 3.2B**). The ChAd63 destination vector is based on the pUC19 bacterial plasmid and requires virus rescue to be performed by transfection of HEK293 cells after removal of the pUC19 genome by PmeI digest [411, 469, 471, 492].

Initial virus rescue of the individual pChAd63-tPA-Insert was performed using HEK293A cells. However, subsequent recombinant virus production utilised Tetracycline-Regulated Expression HEK293 cells (T-REx™ 293) that allow repression of the transgene and efficient virus growth and rescue [492]. These cells stably express the tetracycline repressor therefore upon transfection with a plasmid containing TOS, the expression of the insert of interest is repressed and only induced by selection using blasticidin. This reduces the possibility of any toxic effects the expressed protein might have on the cells and virus rescue. The resulting recombinant viruses were purified on a caesium chloride gradient and titred by immunoassay staining.

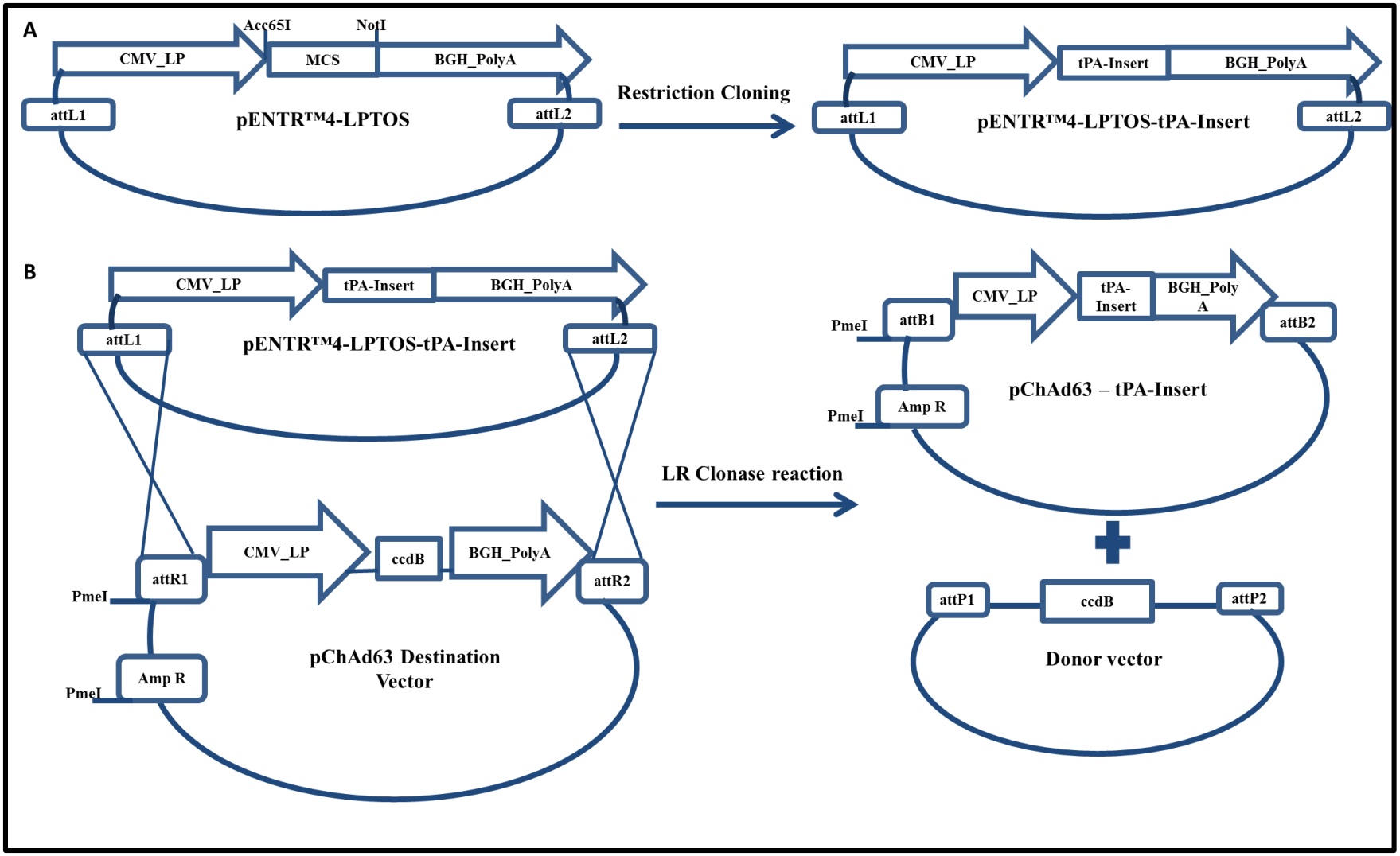

A
Acc65I
NotI
CMV_LP
MCS
BGH_PolyA
attL1
pENTR™4-LPTOS
attL2
Restriction Cloning
CMV_LP
tPA-Insert
BGH_PolyA
attL1
pENTR™4-LPTOS-tPA-Insert
attL2
B
CMV_LP
tPA-Insert
BGH_PolyA
attL1
pENTR™4-LPTOS-tPA-Insert
attL2
PmeI
attR1
CMV_LP
ccdB
BGH_PolyA
attR2
PmeI
Amp R
pChAd63 Destination Vector
LR Clonase reaction
PmeI
attB1
CMV_LP
tPA-Insert
BGH_Poly A
attB2
PmeI
Amp R
pChAd63 – tPA-Insert
attP1
ccdB
attP2
Donor vector

Figure 3.2. Schematic for the cloning and generation of recombinant ChAd63 viruses.

(A) Restriction cloning into the pENTR4-LPTOS entry vector. Respective codon optimised antigen insert (tPA-Insert) plasmids (**Figure 3.1**) were cloned into the entry plasmid vector pENTR™4-LPTOS.CMV_LP.BGH_PolyA by restriction digestion using Acc65I and NotI within the multiple cloning site (MCS). Ligation reactions were performed from the restriction digest generated sticky ends resulting in pENTR™4-LPTOS.CMV_LP-tPA-Insert-BGH_PolyA plasmid. (**B**) Generation of recombinant ChAd63. Resulting entry clones were used to shuttle the entry clone into the ChAd63-Destination vector (under ampicillin resistance (AmpR)) between attL1 and attL2, and attR1 and attR2 sites respectively by Gateway® LR clonase™ II enzyme reaction. The resulting expression clone, flanked by attB sites, was then linearised by PmeI digestion removing AmpR, and used to transfect HEK293 cells for subsequent recombinant virus rescue and purification. Antigen expression was driven by CMV long promoter (CMV_LP) with the BGH_PolyA termination sequence. CMV: Cytomegalovirus; BGH_PolyA: Bovine growth hormone polyadenylation sequence.

3.2.2.2. Identity and confirmation of recombinant ChAd63 viruses

To ensure that the individual recombinant ChAd63-tPA-Insert viruses generated contained the right antigen insert, DNA extracted from purified viruses was used to perform PCR with antigen-specific primers as well as primers flanking the open reading frame (ORF) (**Table 3.1**). PCR screen of the regions flanking the insert in the recombinant ChAd63-tPA-Insert viruses initially rescued from HEK293A cells showed mutants of varying sizes within the viral preparations that outgrew over time and weren't evident in pre-viral plasmids. These were observed for AnAPN1$_{fl}$ (left panel 1000bp, **Figure 3.4A**), Pfs230-C (left panel 2500bp, **Figure 3.5A**), Pfs48/45$_{-NGln}$ (left panel 1500 and 900bp, **Figure 3.6A**), and Pfs48/45$_{+NGln}$ (left panel 2000bp, **Figure 3.7A**) with the exception of PfsHAP2 (**Figure 3.8A**). Sequence analysis of the observed Pfs230-C mutant showed that part of the pUC genome backbone, that had been restriction digested prior to transfection, had been inserted within the ORF of Pfs230-C resulting in an antigen-specific C-terminal fragment of about ~500bp [492]. This was assumed for all the other three viruses with mutants at varying sizes.

To circumvent this problem, pChAd63 DNA for AnAPN1$_{fl}$, Pfs230-C, Pfs48/45$_{-NGln}$, and Pfs48/45$_{+NGln}$ were prepared from preserved glycerol stocks, PmeI digested, run on a gel, correct band excised, and phenol-chloroform purified to remove contaminating pUC19 backbone before transfection and virus rescue in T-REx™ 293 cells. This approach negated the observant of mutants in subsequent confirmation screening for insert presence by PCR (right panel, **Figures 3.4A – 3.7A**). Even though there were no mutants observed for the PfsHAP2 virus (left panel, **Figure 3.8A**), the pChAd63-PfsHAP2 was transfected and rescued in T-REx™ 293 cells to ensure that all the viruses were generated in the same manner. The presence of each insert was determined and confirmed by using insert-specific primers (**Table 3.1**) that were found to correspond to the expected product size as indicated in

the figures and corresponded to the pre-viral plasmid product sizes (middle panel **Figures 3.4A – 3.8A**).

3.2.3. Generation of recombinant MVA vaccines

3.2.3.1. Restriction cloning of antigen inserts

Recombinant MVA viruses expressing individual candidate antigens were generated by restriction cloning into a shuttle vector and subsequent recombination using CEFs infected with parental MVA virus. An MVA shuttle vector containing p7.5 promoter and GFP expressed by the fowlpox FP4b promoter [493] was used for restriction cloning (MVA.GFP, **Figure 3.3A**). The MVA shuttle and tPA-Insert (**Figure 3.1**) plasmids were restriction digested using Acc65I and XhoI and subsequently ligated to generate an MVA.GFP-tPA-Insert clone for each respective vaccine construct (**Figure 3.3A**). The clones were linearised and used to perform homologous recombination with parental MVA.RFP wild-type between the flanking TK loci in CEFs (**Figure 3.3B**). This double cross-over event resulted in recombinants, after replacement of RFP in the parental virus, expressing GFP and the transgene. However, these recombinants contained a percentage of contaminating RFP virus and thus were purified on the basis of GFP expression by FACS. Purified viral plaques expressing GFP were isolated and propagated in CEFs to generate recombinant MVA viral stocks that were titred by plaque assay.

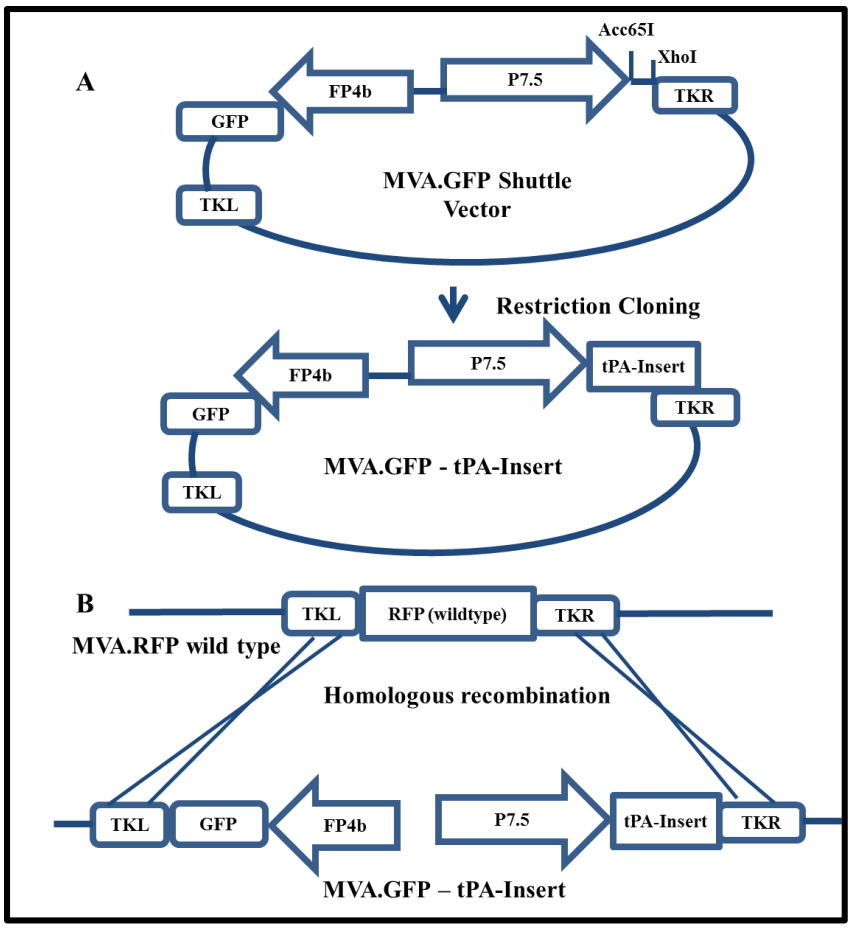


Figure 3.3. Schematic for the cloning and generation of recombinant MVA viruses.

(A) Restriction cloning into MVA.GFP shuttle vector. Codon optimised respective antigen insert (tPA-Insert) plasmids (**Figure 3.1**) and the MVA.GFP shuttle vector were restriction digested (Acc65I and XhoI). Ligation reactions were performed from the restriction digest generated sticky ends resulting in MVA-tPA-Insert clones that were linearised by AatII digestion. (**B**) Generation of recombinant MVA. Recombinant virus was generated by double cross-over homologous recombination with linear parental MVA-RFP virus. Homologous recombination occurred between the flanking thymidine kinase left (TKL) and right (TKR) loci in both the plasmid and parental virus resulting in recombinant virus expressing the gene of interest in tandem with GFP in the place of RFP. P7.5 promoter was used to drive tPA-Insert gene expression whilst the fowlpox FP4b promoter drove GFP expression. GFP: green fluorescent protein; RFP: red fluorescent protein.

3.2.3.2. Identity and confirmation of recombinant MVA viruses

To confirm the identity and presence of the antigen inserts in the purified viral preparations, viral DNA was purified and PCR performed using primers listed in **Table 3.1**. The identity of AnAPN1$_{fl}$ (left panel, **Figure 3.4B**), Pfs230-C (left panel, **Figure 3.5B**), Pfs48/45$_{-NGln}$ (left panel, **Figure 3.6B**), Pfs48/45$_{+NGln}$ (left panel, **Figure 3.7B**), and PfsHAP2 (left panel, **Figure 3.8B**) were confirmed and corresponded to the pre-viral plasmid DNA used to generate the viral stock. In addition, the entire vaccine construct for each of the antigens was present as indicated by PCR using primers flanking the tPA-Insert (flank-to-flank PCR, middle panel **Figures 3.4B – 3.8B**). Furthermore, PCR showed that the prepared viral stocks were pure and free of any contaminating RFP recombinants. This was evident by the lack of the 500bp product that is produced with primers for detecting RFP (plasmid containing RFP used as a control) (**Table 3.1**) as shown in the right panel of **Figures 3.4B**, **3.7B** and **3.8B**.

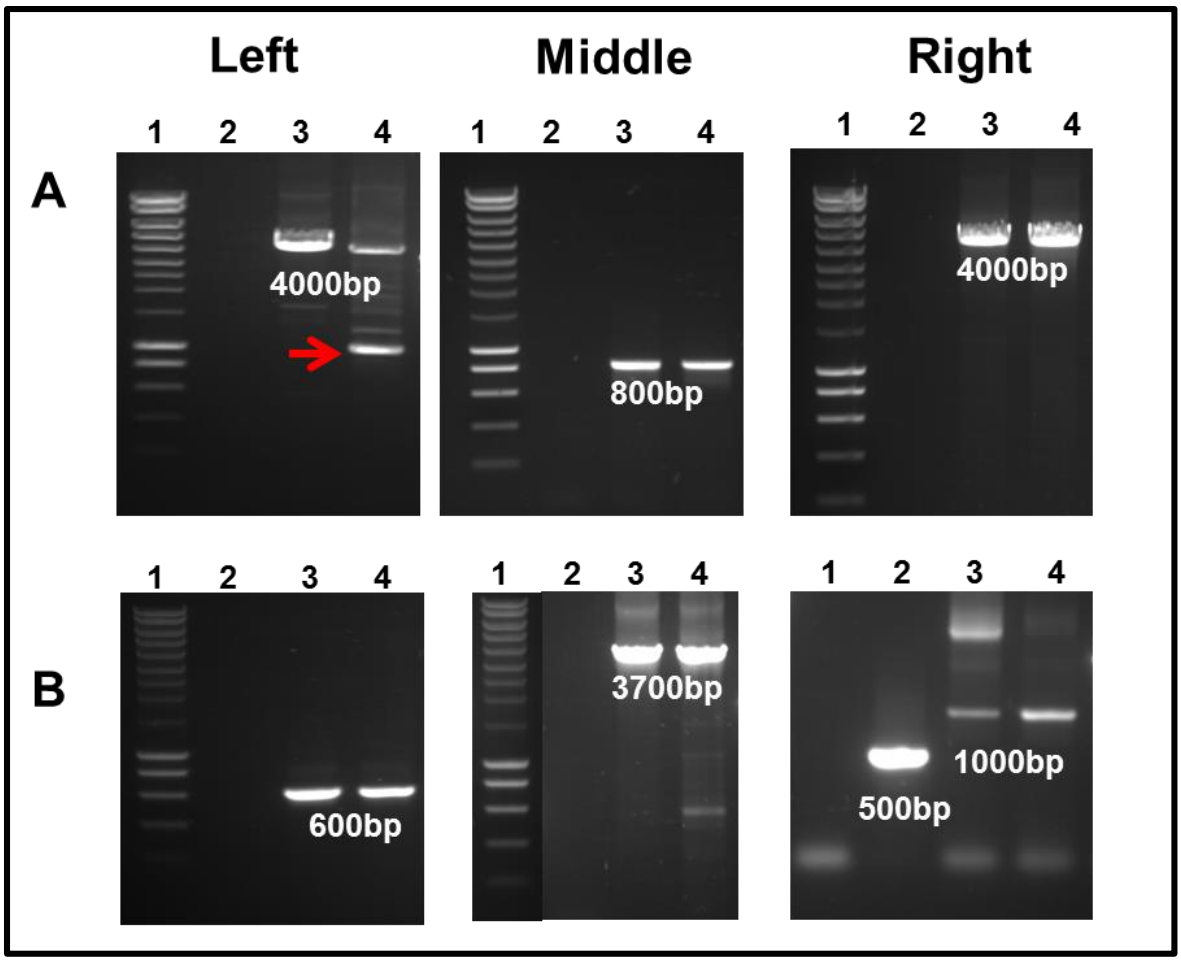


Figure 3.4. PCR confirmation of AnAPN1fl in recombinant viral-vectors.

PCR reactions were performed using purified viral DNA from rescued virus and products run on agarose gels. Pre-viral plasmid DNA and water were used as positive and negative controls respectively. (**A**) ChAd63 – Flank-to-flank (left) from HEK293As, identity (middle), and flank-to-flank (right) from T-REx™ 293. (**B**) MVA – identity (left), flank-to-flank (middle), and purity (right). Expected product sizes are shown with respect to corresponding bands whilst the red arrow shows the lower molecular weight band observed in HEK293A. Lanes: 1 – DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA with the exception of (**B**) right panel where lanes 1, 2, 3 and 4 are water, RFP plasmid, pre-viral plasmid and viral DNA respectively.

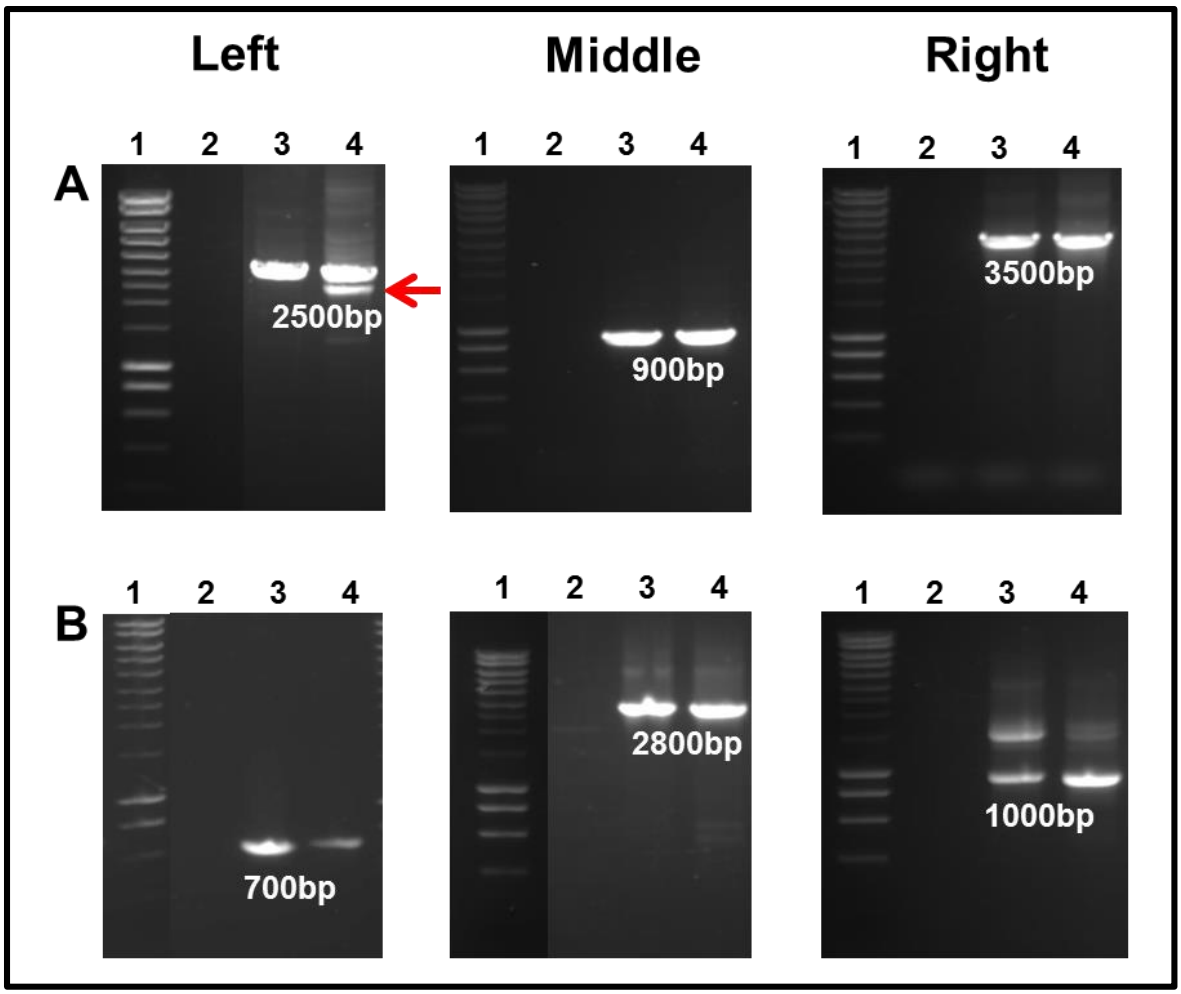


Figure 3.5. PCR confirmation of Pfs230-C in recombinant viral-vectors.

PCR reactions were performed using purified viral DNA from rescued virus and products were run on agarose gels. Pre-viral plasmid DNA and water were used as positive and negative controls respectively. (**A**) ChAd63 – Flank-to-flank (left) from HEK293As; identity (middle); and flank-to-flank (right) from T-REx™ 293. (**B**) MVA – identity (left); flank-to-flank (middle); and purity (right). Expected product sizes are shown with respect to corresponding bands whilst the red arrow shows the lower molecular weight band observed in HEK293A. Lanes: 1 – DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA.

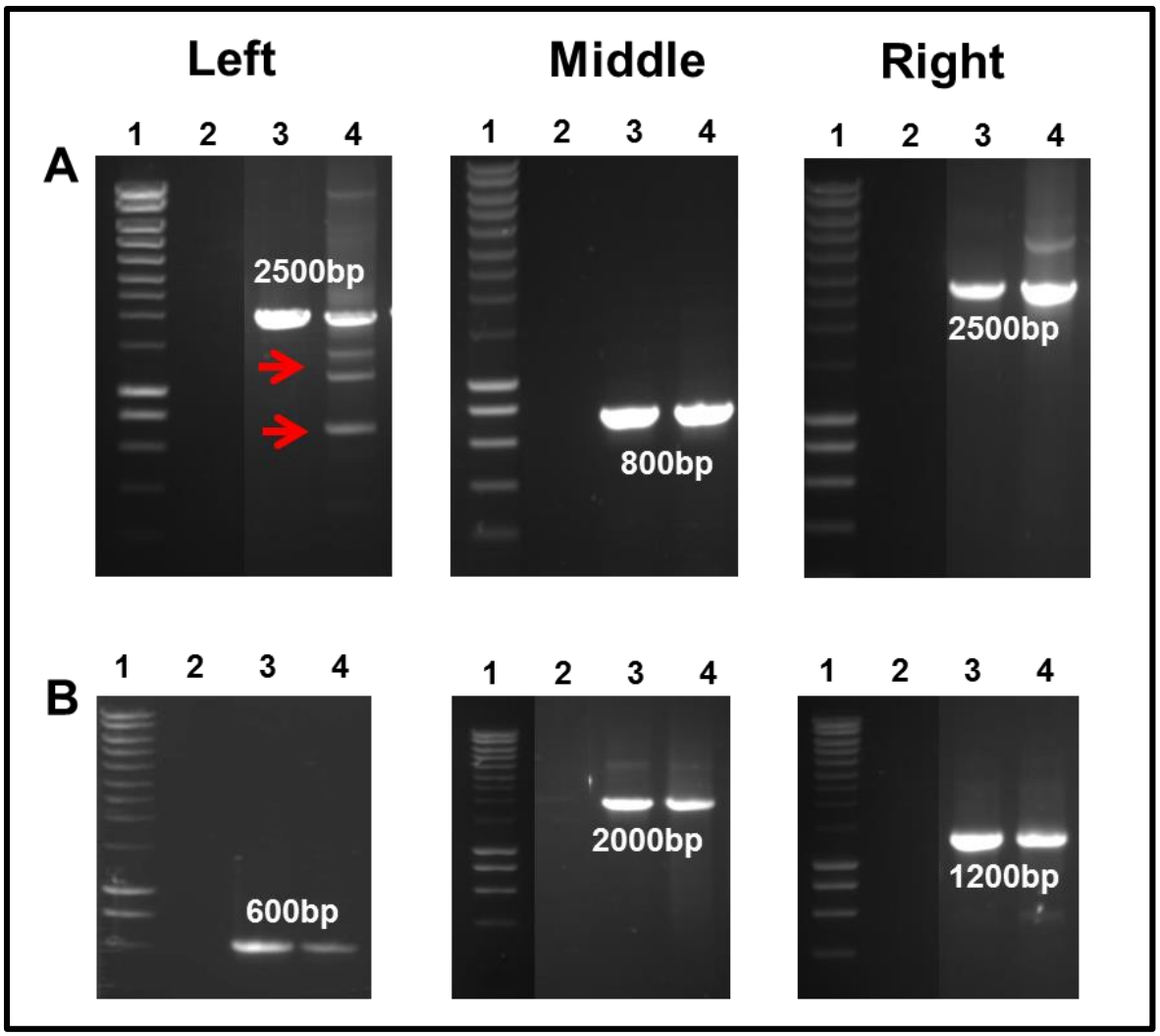


Figure 3.6. PCR confirmation of Pfs48/45$_{\text{-NGln}}$ in recombinant viral-vectors.

PCR reactions were performed using purified viral DNA from rescued virus and products were run on agarose gels. Pre-viral plasmid DNA and water were used as positive and negative controls respectively. (**A**) ChAd63 – Flank-to-flank (left) from HEK293As; identity (middle); and flank-to-flank (right) from T-REx™ 293. (**B**) MVA – identity (left); flank-to-flank (middle); and purity (right). Expected product sizes are shown with respect to corresponding bands whilst the red arrows show the lower molecular weight bands observed in HEK293A. Lanes: 1 – DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA.

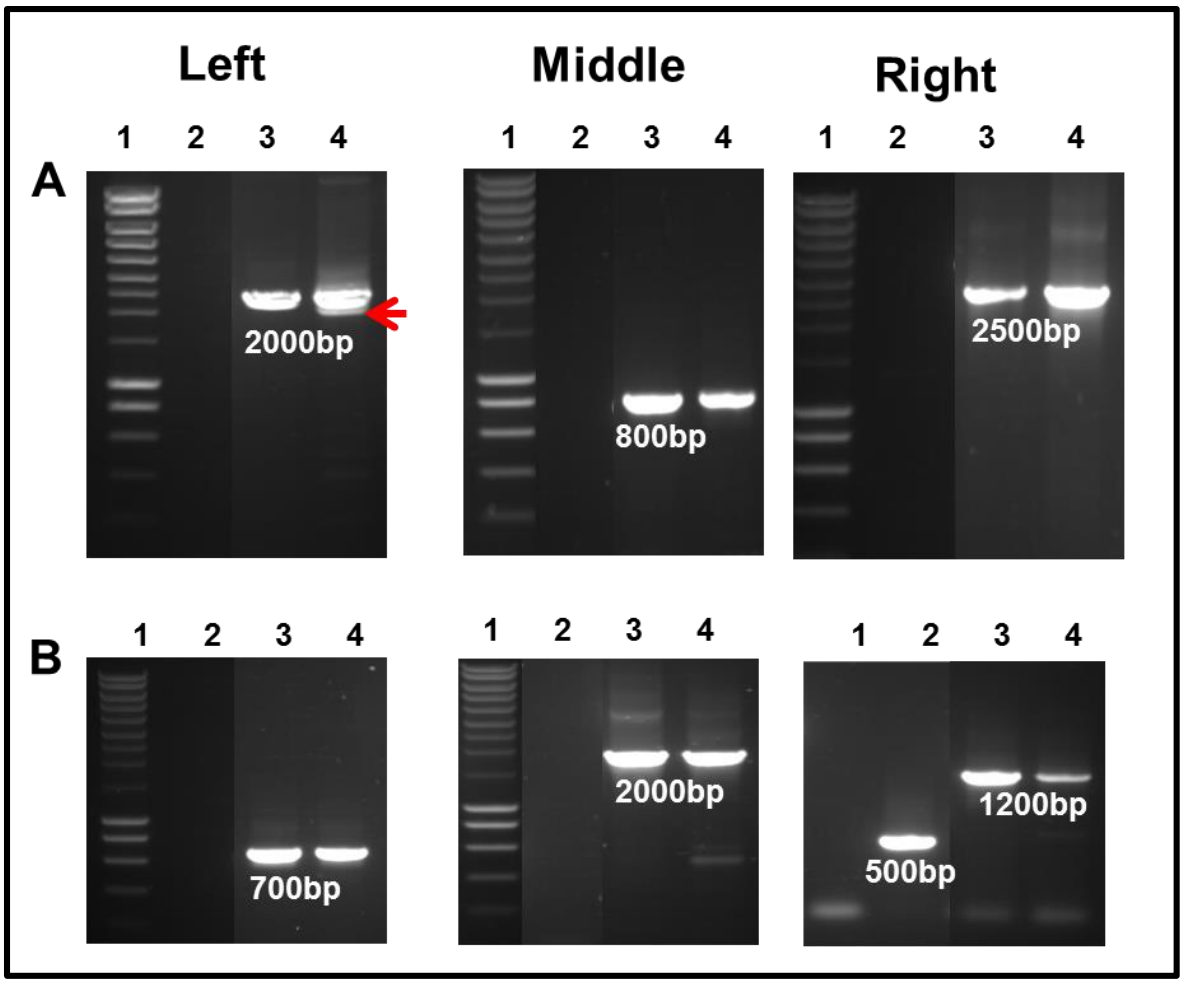


Figure 3.7. PCR confirmation of Pfs48/45$_{+NGln}$ in recombinant viral-vectors.

PCR reactions were performed using purified viral DNA from rescued virus and products were run on agarose gels. Pre-viral plasmid DNA and water were used as positive and negative controls respectively. (**A**) ChAd63 – Flank-to-flank (left) from HEK293As; identity (middle); and flank-to-flank (right) from T-REx™ 293. (**B**) MVA – identity (left); flank-to-flank (middle); and purity (right). Expected product sizes are shown with respect to corresponding bands whilst the red arrow shows the lower molecular weight band observed in HEK293A. Lanes: 1 – DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA with the exception of (**B**) right panel where lanes 1, 2, 3 and 4 are water, RFP plasmid, pre-viral plasmid and viral DNA respectively.

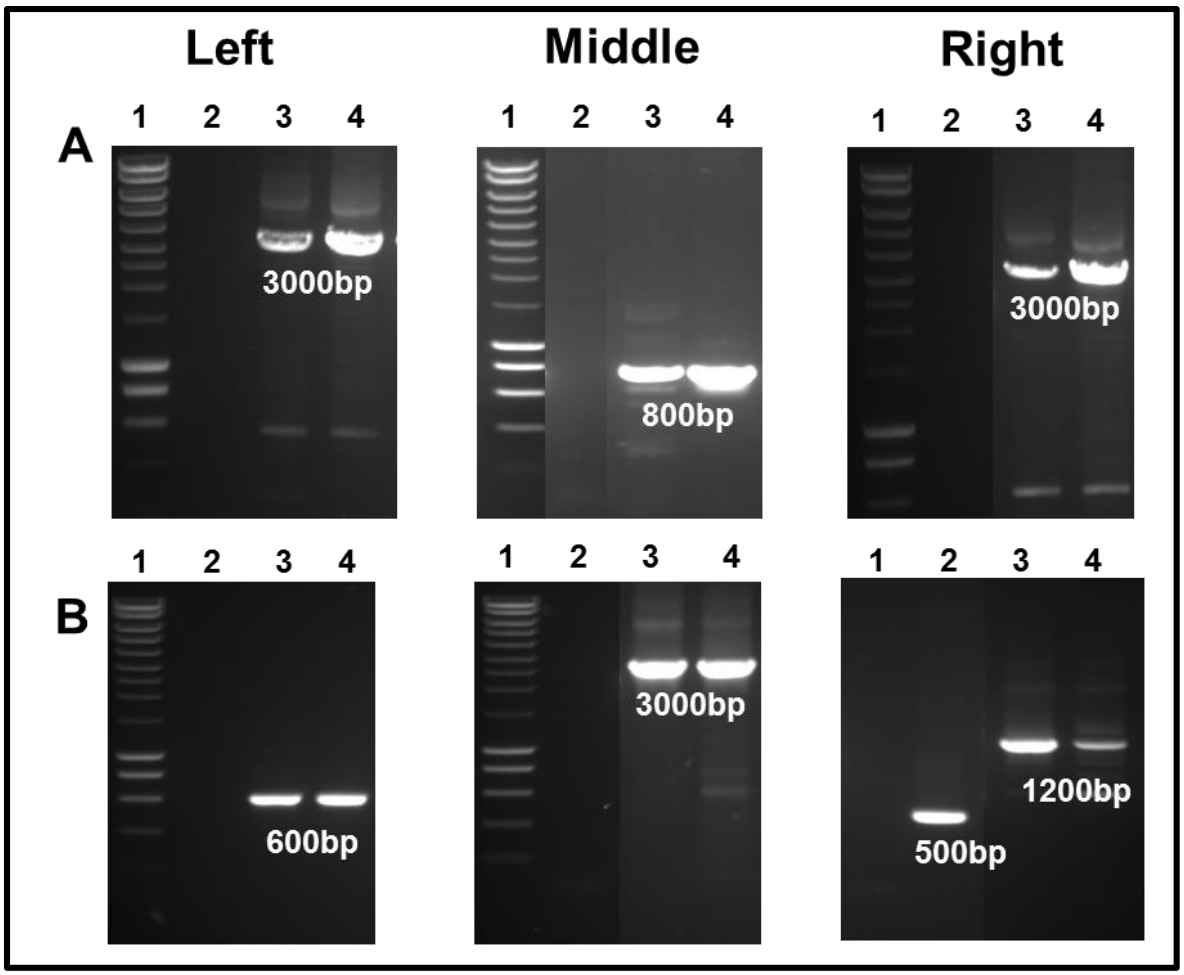


Figure 3.8. PCR confirmation of PfsHAP2 in recombinant viral-vectors.

PCR reactions were performed using purified viral DNA from rescued virus and products were run on agarose gels. Pre-viral plasmid DNA and water were used as positive and negative controls respectively. (**A**) ChAd63 – Flank-to-flank (left) from preparations made using HEK293As; identity (middle); and flank-to-flank (right) from preparations made using T-REx™ 293. (**B**) MVA – identity (left); flank-to-flank (middle); and purity (right). Expected product sizes are shown with respect to corresponding bands. Lanes: 1 – DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA with the exception of (**B**) right panel where lanes 1, 2, 3 and 4 are water, RFP plasmid, pre-viral plasmid and viral DNA respectively.

Table 3.1. List of primers used for PCR reactions.

Primer Pair	Sequence	PCR Purpose
J538	GAGTGGCACCTTCCAGGGTCAAGGAAGG	Flank-to-flank for
J680	GCGTGTACGGTGGGAGGTCTATATAAGC	ChAd63
J645	GCCGTCCACCGTGGATCCCATAACATTC	ID for ChAd63-
J683	GATGCTGAGCAACTTCCTGGCCGAGTTCG	$AnAPN1_{fl}$
J645	GCCGTCCACCGTGGATCCCATAACATTC	ID for ChAd63-
J688	CACAAGGACGTGAAGTACTTCGAGCAGAGC	Pfs230-C
J645	GCCGTCCACCGTGGATCCCATAACATTC	ID for ChAd63-
J685	AGCGAGGAACTGGAACCCAGCAACATCG	Pfs48/45*
J645	GCCGTCCACCGTGGATCCCATAACATTC	ID for ChAd63-
J684	GGAAAGCGAGGCCCAGATCAACAAATACG	PfsHAP2
J506	AGCGAGGAACTGGAACCCAGCAACATCG	Flank-to-flank for
J459	GCGACCTCATTTGCACTTTCTGGTTCG	MVA
J459	GCGACCTCATTTGCACTTTCTGGTTCG	Purity for MVA
J481	CTCCAAGCTGGACATCACCTCCCACAACGAG	
J459	GCGACCTCATTTGCACTTTCTGGTTCG	ID for MVA-
J683	GATGCTGAGCAACTTCCTGGCCGAGTTCG	$AnAPN1_{fl}$
J459	GCGACCTCATTTGCACTTTCTGGTTCG	ID for MVA-
J688	CACAAGGACGTGAAGTACTTCGAGCAGAGC	Pfs230-C
J459	GCGACCTCATTTGCACTTTCTGGTTCG	ID for MVA-
J685	AGCGAGGAACTGGAACCCAGCAACATCG	Pfs48/45*
J459	GCGACCTCATTTGCACTTTCTGGTTCG	ID for MVA-
J684	GGAAAGCGAGGCCCAGATCAACAAATACG	PfsHAP2
J1204	TGTGGGTAATGTTCTCGATGTC	Flank-to-flank for
J1205	CCATCGAGTGCGGCTACTAT	MVA sequencing
J1456	ATCCACGCTGTTTTGACCTC	Flank-to-flank for
J1210	CAGCGAGCTCTAGCATTTAGG	ChAd63 sequencing

*The same primer pairs and PCR conditions were used for both the Pfs48/45 variants, $Pfs48/45_{-NGln}$ and $Pfs48/45_{+NGln}$. ID: identity PCR.

3.3. Discussion

This chapter has outlined the work carried out to derive recombinant ChAd63 and MVA vaccines encoding the leading TBV candidate antigens AnAPN1 (being full-length and referred to as $AnAPN1_{fl}$), Pfs230 (region C and referred to as Pfs230-C), Pfs48/45 (N-glycosylated variants being $Pfs48/45_{-NGln}$ and $Pfs48/45_{+NGln}$) and PfsHAP2. The derivation of these viral-vectors using established protocols however didn't proceed without encountering some difficulties. In the generation of ChAd63 viral-vectors, an unexpected insertion of plasmid DNA in the place of the respective antigen was observed. This led to the propagation of mutant virus that was only detected during screening of purified viral stocks specifically observed for ChAd63-Pfs230-C. Even though the mutant was only fully investigated for this construct, it was perceived that the remaining vaccine constructs would have the same problem. However, this wasn't evident for PfsHAP2.

In light of this, a number of modifications were implemented. To circumvent insertion of plasmid DNA, the linearised pChAd63-tPA-Insert was gel excised and DNA phenol-chloroform extracted to get rid of the smaller pUC19 genome fragment. To promote efficient viral growth and rescue, the viruses were transfected using T-REx™ 293 cells. Viral propagation and rescue in T-REx™ 293 cells was considered in order to repress any possible effects that might occur due to transgene expression as evident during production of a variant version of CSP [492]. T-REx™ 293 cells have been shown to completely suppress the expression of transgenes during propagation which is possible due to insertion of two tetracycline operator sequences within the CMV promoter [494]. The occurrence of the mutants upon propagation in HEK293A cells isn't fully understood but one could speculate that transgene expression was possibly toxic to viral growth leading to the generation and selection of recombinant mutants that would allow for viral replication and growth in the

packaging cell line. Therefore, these modifications enabled subsequent propagation and production of recombinant ChAd63 viruses that were assumed error-free.

The identity and purity of viral stocks is pertinent especially in a study that seeks to comparatively assess different candidate antigens using a single delivery system. Thus, PCR screens to determine this were imperative and enabled the assessment of each of the candidate antigens expressed in corresponding viral-vectors. In addition, due to the derivation of the two Pfs48/45 variants, it was imperative to distinguish and determine that the products generated were true-to-type. Thus sequencing of the Pfs48/45$_{\text{-NGln}}$ variant for both ChAd63 and MVA recombinant viral-vectors showed that it was indeed true-to-type and different from its counterpart (**Figure 3.9**).

The methods and procedures described in this chapter were used to further generate all vaccine constructs and recombinant viral-vectors used in this study. **Table 3.2** outlines all the recombinant viral-vectors that have been generated in this body of work.

Summary of overall findings:

- Malaria transmission-blocking candidate antigens, of varying sizes, can be generated in ChAd63-MVA viral-vectors.
- Suppression of transgene expression is essential for the propagation and generation of error-free ChAd63 recombinant viruses.

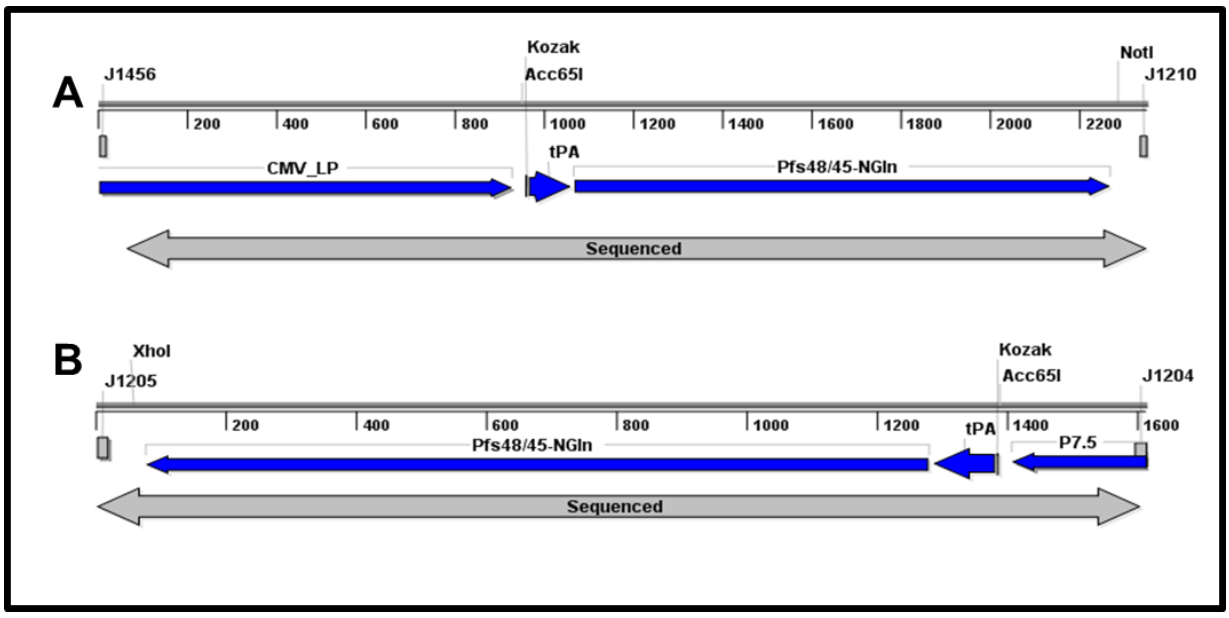


Figure 3.9. Confirmation of Pfs48/45$_{\text{-NGln}}$ by sequencing.

Flank-to-flank PCR was used to amplify purified viral DNA using respective primers flanking the antigen. PCR reaction products were run on agarose gels, excised and sent for sequencing. Sequence alignments were performed using SeqMan Pro™ against the sequence for (**A**) ChAd63-Pfs48/45$_{\text{-NGln}}$ and (**B**) MVA-Pfs48/45$_{\text{-NGln}}$.

Table 3.2. List of all the recombinant viral-vectored vaccines generated in the study.

Antigen(s)	Antigen source	Vaccine abbreviations
AnAPN1	*A. gambiae*	ChAd63 and MVA – $AnAPN1_{fl}$
	Derived from $AnAPN1_{fl}$	ChAd63 and MVA – AnAPN1 Dom I
	Derived from $AnAPN1_{fl}$	ChAd63 and MVA – AnAPN1 Nterm
Pfs230-C	*P. falciparum* 3D7	ChAd63 and MVA – Pfs230-C
Pfs230-C and Pfs25	*P. falciparum 3D7*	ChAd63 and MVA – Pfs25-2A-Pfs230-C
		ChAd63 and MVA – Pfs25-GP-Pfs230-C
Pfs48/45	*P. falciparum* 3D7	ChAd63 and MVA – $Pfs48/45_{-NGln}$
		ChAd63 and MVA – $Pfs48/45_{+NGln}$
PfsHAP2	*P. falciparum* 3D7	ChAd63 and MVA – PfsHAP2
Pbs230-C	*P. berghei* ANKA	ChAd63 and MVA – Pbs230-C
Pbs25	*P. berghei* ANKA	ChAd63 and MVA – Pbs25
Pbs48/45	*P. berghei* ANKA	ChAd63 and MVA – $Pbs48/45_{-NGln}$
		ChAd63 and MVA – $Pbs48/45_{+NGln}$

4. Comparative Assessment of Leading Transmission-blocking Candidate Antigens: Immunogenicity and Efficacy

4.1. Introduction

Pre-clinical initiatives investigating TBVs have led to the development of a number of *P. falciparum* candidate antigens of which Pfs230-C, Pfs25, and Pfs48/45 are the most thoroughly characterised. Different vaccine platforms utilising these antigens have been used to raise antibodies with demonstrable TBA [283]. Despite the lack of any reported head-to-head comparison, Pfs230-C, Pfs25, Pfs48/45 and AnAPN1 are currently considered leading TBV candidate antigens [495]. However, to-date, only Pfs25 has progressed onto human clinical testing with the others in the process of being developed independently as clinical products [285, 495]. These antigens have been expressed and tested independently in different laboratories, mainly as recombinant proteins produced in a variety of heterologous expression systems as well as delivered using various vaccine delivery technologies and assessed using a variety of endpoint estimates.

Cloning and expression of Pfs230 revealed a 360kDa protein that was expressed in *E. coli* and reacted with native gametocyte/gamete parasite extracts [290]. Due to the size of this protein, it was perceived that its potential development as a TBV candidate would be challenging and thus six fragments were expressed based on strain polymorphisms and potential B and T-cell epitopes [301, 321]. This initial expression of fragments in *E. coli* led to determination that the most potent TBA was mediated by antibodies raised against fragment C (Pfs230-C) [321, 488]. Pfs230-C has since then been expressed in cell-free wheat-germ expression system, DNA, and plant-based expression platform; where antibodies raised exhibit varying TBA [451, 496, 497] . In the case of Pfs25, it has been expressed in baculovirus, DNA, *Chlamydomonas* green algae, and ditto a plant-based platform and in

various yeast strains with antibodies induced also giving varied inhibitory activity [338, 339, 371, 498-503]. Concomitantly, Pfs48/45 has been expressed either as full-length protein or as fragments in baculovirus, DNA, *E. coli*, *Chlamydomonas* green algae, vaccinia virus, plant-based platform, and yeast [292, 385, 490, 504-506]. On the other hand, AnAPN1 and the *P. berghei* homologue of PfsHAP2 have been expressed in *E. coli* inducing TB antibodies with incomplete inhibitory effects on parasite infectivity [323, 348, 349].

Recombinant proteins have often been delivered in adjuvant using different regimen. Complete TBA has been reported with potent adjuvants such as Freund's (which isn't suitable for use in humans), whereas adjuvants licensed for human use have induced moderate to high TBA depending on the antigen. Adjuvants such as Montanide ISA-51 can be used in both pre-clinical and clinical studies enabling translational application of vaccine antigens [507, 508]. However, adverse reactions were reported with Montanide ISA-51 in the trial of Pfs25 [285]. Expression of full-length correctly folded native protein, especially of cysteine-rich antigens, has been hampered due to conformational-dependent epitopes that are implicated in mediating TBA [172, 294, 322, 385, 509]. For Pfs48/45, codon harmonisation of the native sequence has led to expression of full-length protein that induces antibodies giving ≥95% TBA in rodents and primates administered in Montanide ISA-51 [504].

It was thus imperative to conduct a study that comparatively assessed the ability of these antigens to induce antibodies with potential TBA using a single delivery platform. Therefore, a heterologous prime-boost regimen incorporating ChAd63 and MVA vaccines generated as described in **Chapter 3** were used in this study. The use of viral-vectors negates the need for use of adjuvants and both ChAd63 and MVA have proven clinical safety and immunogenicity profiles [228, 265, 510]. Thus assessment of a variety of antigens could lead to the prioritisation of candidate(s) for clinical testing. An 8 week time interval between

prime and boost has been shown to be optimal for the induction of antibody responses with responses peaking 2 weeks post-boost [391, 426]. Therefore, this regime was used with a dosing interval of 8 weeks between prime and boost to achieve optimal responses. Vaccine-induced antibody responses were measured from serum 2 weeks post-boost (day70) and used to determine their potential effects at inhibiting parasite intensity and prevalence as efficacy endpoints. The intramuscular route of immunisation has been shown to induce both cellular and humoral responses to transgenes [214, 391, 396]. Rodrigues et al. (1997) were able to show that a single dose of HuAdV5 expressing CSP induced greater antibody responses compared to other commonly used routes of administration such as intravenous and subcutaneous [391]. Therefore for this reason, intramuscular administration of the generated viral-vectors was used as the route of immunisation.

This chapter describes the head-to-head comparison of AnAPN1$_{fl}$, Pfs230-C, Pfs25, Pfs48/45 (Pfs48/45$_{-NGln}$ versus Pfs48/45$_{+NGln}$), and PfsHAP2 to: (a) induce antibodies in the ChAd63-MVA heterologous prime-boost regimen; and (b) ability to inhibit development of *P. falciparum* and thus prioritise candidate(s) for clinical development. The body of work outlined investigates the ability of vaccine-induced IgG responses to inhibit the infectivity not only of the lab strain of *P. falciparum* NF54 to its lab-adapted mosquito, *A. stephensi*, but also importantly, *P. falciparum* field isolates from Burkina Faso to a recently lab-adapted *A. gambiae* mosquito vector. This allowed the adequate assessment to enable a hierarchy to be determined on the basis of the efficacy endpoints. In order to obtain this hierarchy, they were tested in parallel with the same parasite exposure in the feeding assays.

4.2. Results

4.2.1. Preliminary immunogenicity and efficacy

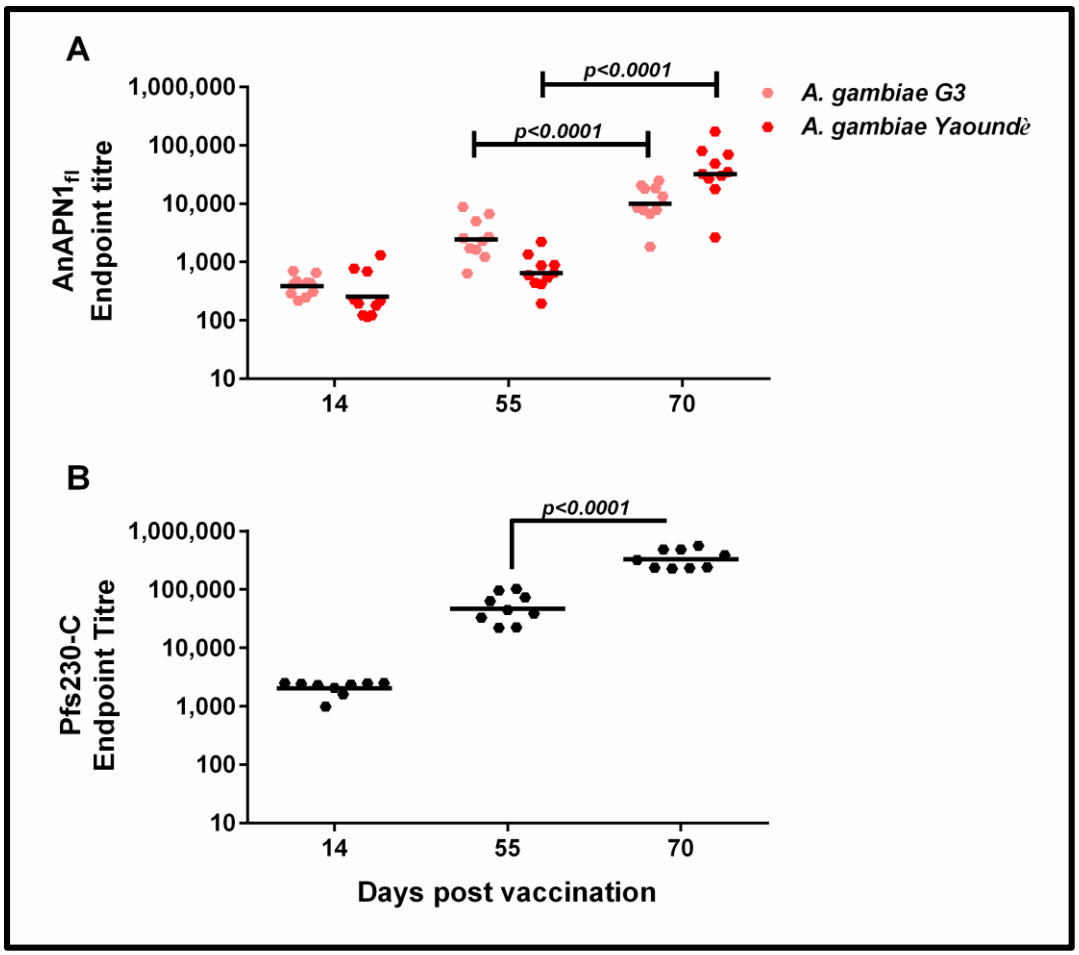
Pfs25 expressed in either HuAdV5 or ChAd63 has been previously shown to induce antibody responses [372]. HuAdV5 responses were boosted by MVA administration and Goodman et al. (2012) went on further to show that these responses inhibited *P. falciparum* NF54 parasite development in *A. stephensi*. This provided precedence to promote the testing of other TBV candidate antigens in the viral-vector heterologous prime-boost regimen. A pilot study was set up to determine the immunogenicity and efficacy of antibodies induced against AnAPN1$_{fl}$, Pfs230-C, Pfs48/45$_{-NGln}$, Pfs48/45$_{+NGln}$ and PfsHAP2 expressed as recombinant ChAd63-MVA viral-vectored vaccines. This section describes the assessment of antibody responses induced against these candidate antigens and their potential effect at inhibiting the infectivity of *P. falciparum* NF54 to *A. stephensi* in SMFA.

4.2.1.1. Antibody responses induced by ChAd63-MVA prime-boost.

Age-matched BALB/c mice were primed with ChAd63 and boosted on day56 with MVA expressing the respective antigen. Antigen-specific vaccine-induced IgG antibody titres were assessed on days 14, 55 and 70 by endpoint ELISA. All the animals seroconverted with induction of antibodies post-prime that were boosted by administration of MVA (**Figure 4.1**). Vaccine-induced IgG responses for AnAPN1$_{fl}$ were measured against midgut lysate from two mosquito strains, *A. gambiae* G3 and Yaoundè and in both instances a 4-fold (p<0.0001) and 50-fold (p<0.0001) increase was observed respectivelypost-boost (**Figure 4.1A**). IgG responses measured using recombinant protein increased against Pfs230-C (7-fold (p<0.0001), **Figure 4.1B**); Pfs48/45$_{-NGln}$ (4-fold (p=0.005), **Figure 4.1C**); and Pfs48/45$_{+NGln}$ (10-fold (p=0.0004), **Figure 4.1C**). Interestingly, Pfs48/45 with N-glycosylation sites substituted, Pfs48/45$_{-NGln}$, induced lower responses post-prime and post-boost compared to its

counterpart, Pfs48/45$_{+NGln}$ (day14 8-fold difference, p<0.0001; day55 7-fold difference, p=0.008; and day70 19-fold difference, p=0.0009 unpaired t-test).

Responses to PfsHAP2 however weren't boosted with only half of the animals having detectable responses post-prime and boost against antigen expressed in HEK293s in the LIPS assay (**Figure 4.1D**). On the other hand, animals with detectable responses in the LIPS assay had low to undetectable responses by ELISA measured against recombinant protein (195-684αα fragment (data not shown)). Considering that there had been no difficulties encountered in the generation of ChAd63-PfsHAP2 (**3.2.2.**), sequencing of both ChAd63 and MVA viruses was performed. The sequencing results revealed that PfsHAP2 sequence in ChAd63-PfsHAP2 had a deletion at the N terminus leaving a C terminal fragment of 150αα. However, the entire transgene was present in MVA. Based on this, PfsHAP2 wasn't considered in subsequent experiments to enable an unbiased comparative analysis.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AnAPN1fl
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1,000
100
10
14
55
70
p<0.0001
p<0.0001
A. gambiae G3
A. gambiae Yaoundè
B
Pfs230-C
Endpoint Titre
1,000,000
100,000
10,000
1,000
100
10
14
55
70
p<0.0001
Days post vaccination

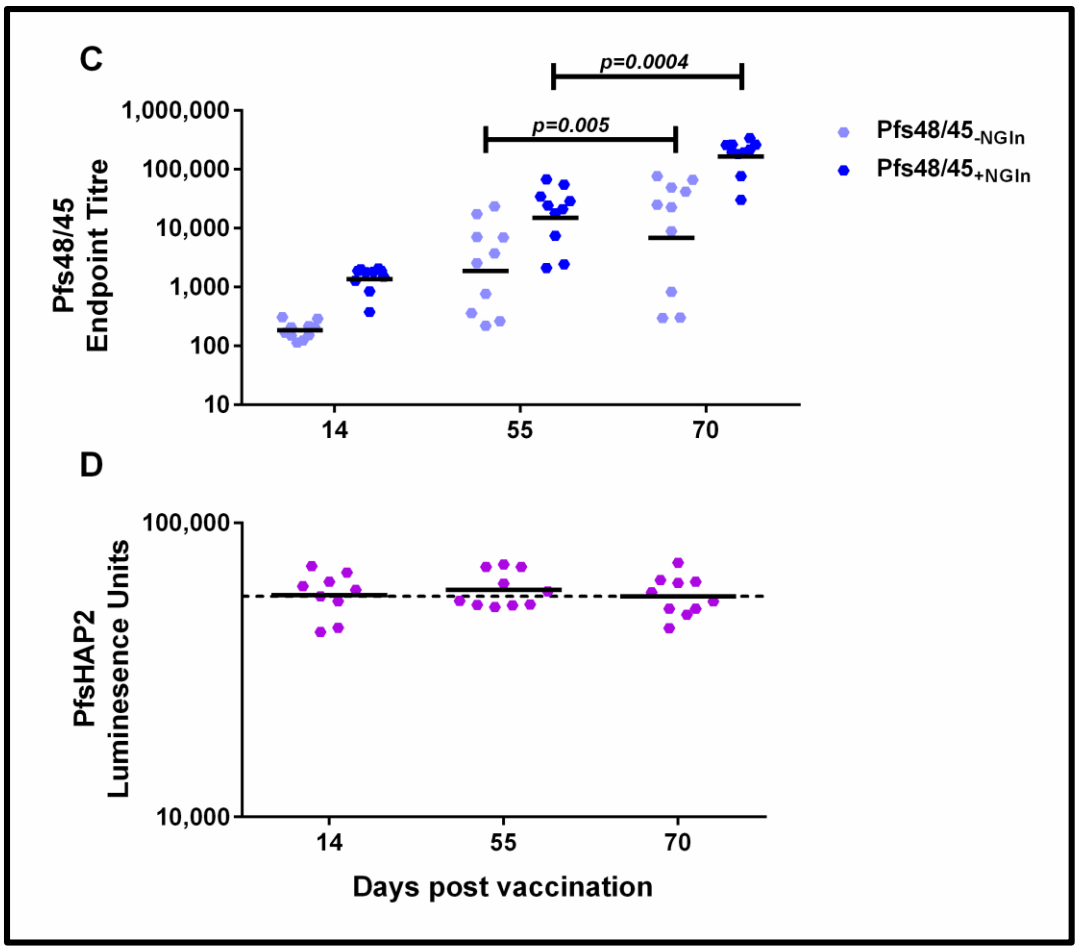


Figure 4.1. ChAd63-MVA prime-boost IgG responses to TBV candidate antigens.

BALB/c mice (n=9-10/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with respective MVA ($1x10^7$pfu, day56) expressing the individual antigens (**A**) AnAPN1$_{fl}$, (**B**) Pfs230-C, (**C**) Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$, and (**D**) PfsHAP2. Whole IgG responses were assayed on days 14, 55 and 70 either by endpoint ELISA using midgut lysate from *A. gambiae* G3 and Yaoundé strains (**A**) and recombinant protein (**B** and **C**), or by LIPS assay (**D**). The data was log-transformed and presented as geometric mean for each of the different time points. Dotted line in (**D**) indicates the cut-off for positive and negative responses with respect to responses measured against control antigen. Dots represent responses from individual mice. Significance considered at $p<0.05$.

4.2.1.2. Inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*

To test whether there was TBA against *P. falciparum* NF54 in SMFA, sera from animals vaccinated with AnAPN1$_{fl}$, Pfs230-C, Pfs48/45$_{-NGln}$, and Pfs48/45$_{+NGln}$ were pooled and sent to the NIH MFA Reference lab for assessment. In addition, sera from animals vaccinated with ChAd63 and MVA expressing irrelevant antigen (GFP) were used as control. Total IgG responses for pooled sera were determined by standardised ELISA (**Table 4.1**).

Pfs230-C has previously been shown to induce complement-dependent antibody-mediated TBA [171-173]. Therefore, sera were initially tested in the absence (**Figure 4.2A**) or presence (**Figure 4.2B**) of complement to assess whether there was any evidence of complement-mediated inhibition by antibodies induced using this regimen. In the absence of complement, oocyst intensity was greater than in the presence with antibody against Pfs230-C (p=0.03, Kruskal-Wallis test with Dunn's multiple comparison). Interestingly, the opposite was true with respect to the control (p=0.001). However, there was no evidence of an effect against the other antigens. Nevertheless, there was a 35% inhibition of intensity in the absence of complement attributed against Pfs230-C (p=0.01) with no observed effect on prevalence (**Figure 4.2A**).

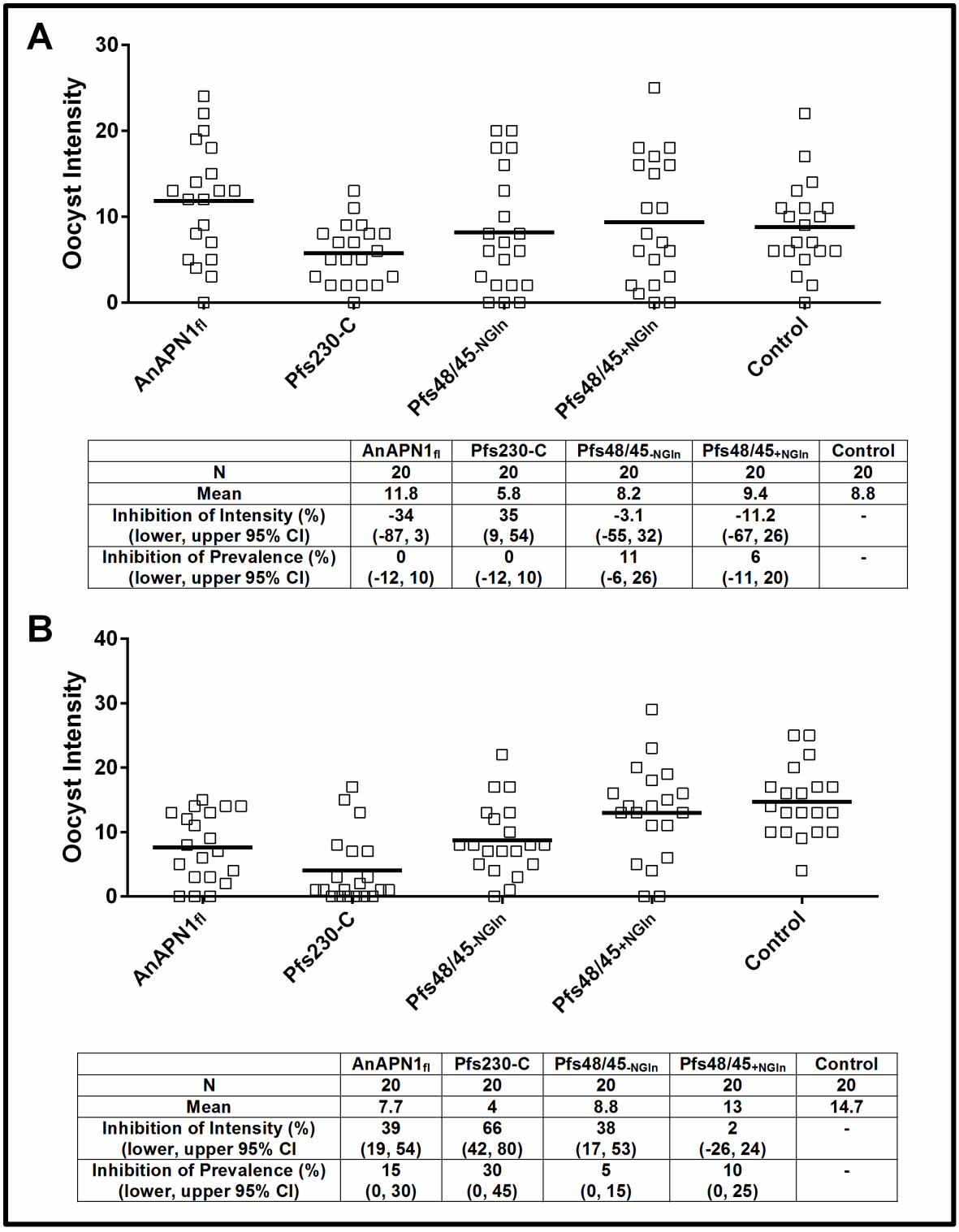
Interestingly, in the presence of complement inhibition of intensity, against AnAPN1$_{fl}$ (39%, p=0.001) and Pfs48/45$_{-NGln}$ (38%, p=0.002) was nearly half the effect observed against Pfs230-C (66%, p<0.0001). On the other hand, infection prevalence was only inhibited by 15% against AnAPN1$_{fl}$ (p=0.03) and 30% against Pfs230-C (p=0.002). Despite the higher antibody responses (7-fold higher, **Table 4.1**) of Pfs48/45$_{+NGln}$ compared to Pfs48/45$_{-NGln}$, the latter had a TB effect compared to the former in the presence of complement. To confirm inhibition in the presence of complement, a replicate SMFA was performed. In this experiment, only antisera from Pfs230-C vaccinated animals inhibited *P. falciparum* NF54

intensity by 43% (*p*=0.02, **Figure 4.3C**), a 1.5-fold reduction compared to that observed in **Figure 4.2B** with no observed effect on infection prevalence. However of note, the infection exposure was greater in this experiment compared to the former (mean control oocyst intensity of 40.7 vs. 14.7). There was no statistically significant inhibition observed by the other antisera.

Table 4.1. Antibody titres antisera used to determine TBA in SMFA

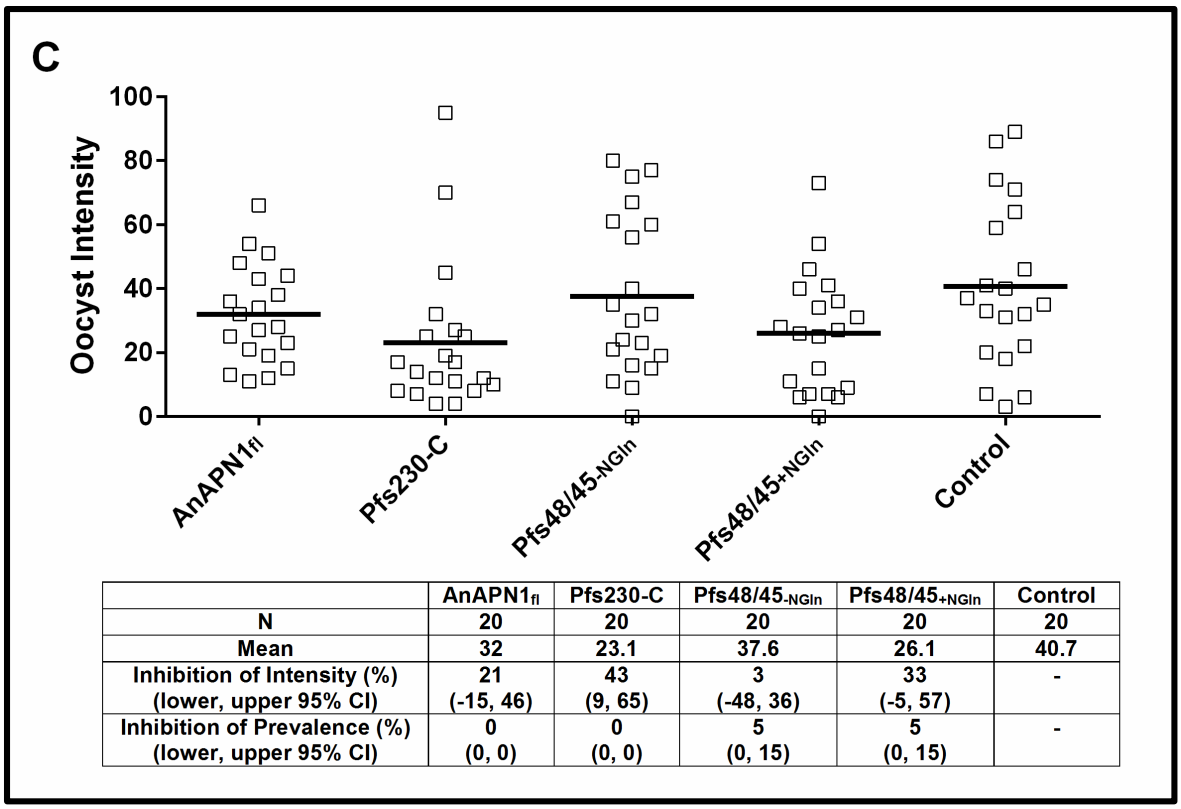
Antigen	Antibody titre	Titre in Feeder
$AnAPN1_{fl}$	5,995AU	280AU
Pfs230-C	18,797AU	880AU
$Pfs48/45_{-NGln}$	921AU	43AU
$Pfs48/45_{+NGln}$	6,568AU	308AU

Antibody titres were determined from an aliquot of sera from pooled vaccinated mice (n=5)



	AnAPN1$_{fl}$	Pfs230-C	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	20	20	20	20	20
Mean	11.8	5.8	8.2	9.4	8.8
Inhibition of Intensity (%) (lower, upper 95% CI)	-34 (-87, 3)	35 (9, 54)	-3.1 (-55, 32)	-11.2 (-67, 26)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	0 (-12, 10)	0 (-12, 10)	11 (-6, 26)	6 (-11, 20)	-

	AnAPN1$_{fl}$	Pfs230-C	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	20	20	20	20	20
Mean	7.7	4	8.8	13	14.7
Inhibition of Intensity (%) (lower, upper 95% CI	39 (19, 54)	66 (42, 80)	38 (17, 53)	2 (-26, 24)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	15 (0, 30)	30 (0, 45)	5 (0, 15)	10 (0, 25)	-



	AnAPN1$_{fl}$	Pfs230-C	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	20	20	20	20	20
Mean	32	23.1	37.6	26.1	40.7
Inhibition of Intensity (%) (lower, upper 95% CI)	21 (-15, 46)	43 (9, 65)	3 (-48, 36)	33 (-5, 57)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	0 (0, 0)	0 (0, 0)	5 (0, 15)	5 (0, 15)	-

Figure 4.2. Antisera inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*.

Sera pooled from mice (n=5/group, day70, **Figure 4.1**) vaccinated with each respective antigen or GFP (control) were mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* at a 1:21 dilution in SMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95%CI. Two independent experiments in the absence (**A)** or presence of complement (**B**) were conducted with a third experiment in the presence of complement (**C**). Squares represent oocyst numbers from each individual mosquito dissected.

4.2.2. Comparative assessment – P. falciparum NF54

Due to the observed induction of antibody responses to AnAPN1$_{fl}$, Pfs230-C, Pfs48/45$_{-NGln}$, and Pfs48/45$_{+NGln}$ and effects on *P. falciparum* NF54 parasite intensity and prevalence, a

comparative study was conducted including Pfs25. A standardised ELISA has been developed to accurately quantify the antibody responses to Pfs25 expressed as antibody units (AU) [452]. This ELISA allows the antibody concentration of each individual test sample to be extrapolated from a reference sample, as a standard, with known antibody concentration (in AU). Therefore, comparable standardised ELISAs were set up for AnAPN1$_{fl}$, Pfs230-C, Pfs48/45$_{-NGln}$, and Pfs48/45$_{+NGln}$ according to the published protocol using selected sera with high antibody titres generated from the pilot study (**Figure 4.1**). All subsequent vaccine-induced total IgG responses for these antigens were measured using this ELISA. To comparatively assess all the antigens, they were tested in the same feeding assay thus against the same parasite exposure.

4.2.2.1. Kinetics of the antibody response

To assess the immunogenicity of all the antigens assayed using the standardised ELISA, age-matched BALB/c mice were individually primed at day0 with ChAd63 and boosted at day56 with MVA expressing the different antigens. Sera were assayed for antibody induction days 14 and 55 with an additional time point post-prime (day35) to assess the kinetics of the ChAd63 priming response. Only in the case of AnAPN1$_{fl}$ and Pfs230-C did the priming IgG responses increase to time of boosting (p<0.05 repeated measures ANOVA, 3-fold and 2-fold increases respectively). In contrast, responses remained unchanged for Pfs25, Pfs48/45$_{-NGln}$, and Pfs48/45$_{+NGln}$ (**Figure 4.3**).

As observed in the pilot study, MVA vaccination boosted the post-prime responses for AnAPN1$_{fl}$ (11-fold increase, p=0.0003, paired t-test); Pfs230-C (16-fold increase, p=0.0002, paired t-test); Pfs48/45$_{-NGln}$ (14-fold increase, p=0.002, paired t-test); and Pfs48/45$_{+NGln}$ (12-fold increase, p=0.01, Wilcoxon matched-pairs test). However, responses to Pfs25 weren't statistically significantly boosted but a non-statistically significant trend was observed (7-fold

higher, p=0.10, Wilcoxon matched-pairs test) (**Figure 4.3**). There was an observed hierarchy of antibody responses with AnAPN1$_{fl}$, Pfs230-C, and Pfs48/45$_{+NGln}$ inducing the highest total IgG responses compared to Pfs48/45$_{-NGln}$ and Pfs25. Consistently, responses to Pfs48/45$_{+NGln}$ were greater than those measured against Pfs48/45$_{-NGln}$ (day70 responses, p=0.0001, unpaired t-test).

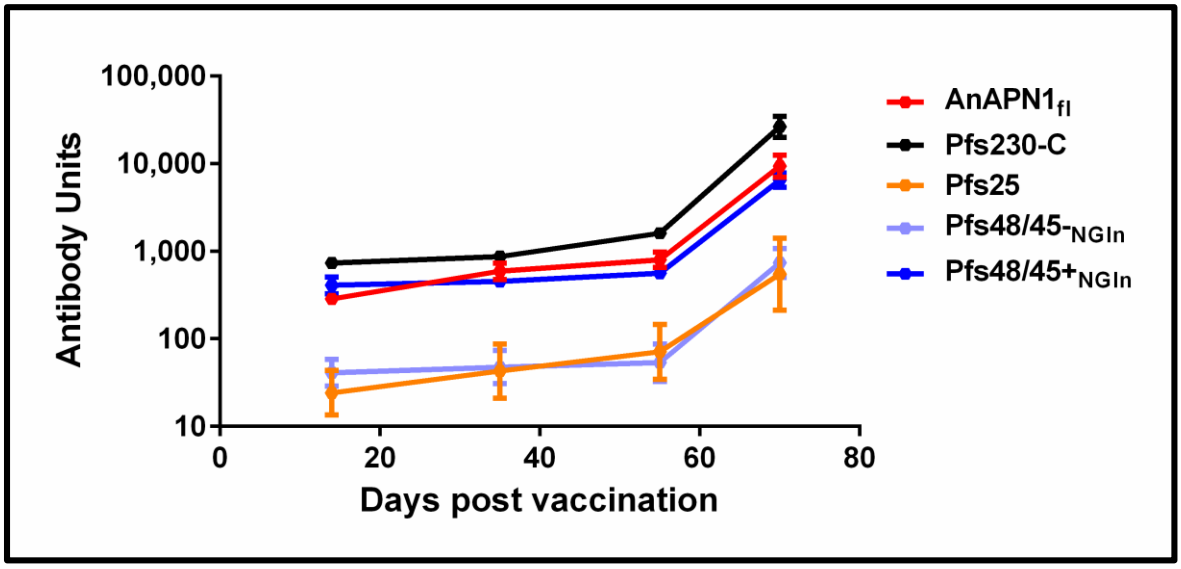


Figure 4.3. Kinetics of antibody priming responses following ChAd63 vaccination.

BALB/c mice (n=8/group) were vaccinated i.m. with ChAd63 (1x10^{8}iu, day0) and boosted with the respective MVA (1x10^{7}pfu, day56) expressing the individual antigens AnAPN1$_{fl}$, Pfs230-C, Pfs25, Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$. Whole IgG responses were assayed on days 14, 35, 55 post-prime and day70 by standardised ELISA using recombinant protein. The data was log-transformed and presented as geometric mean ± 95%CI for the different time points.

4.2.2.2. Expression of antigens in mammalian cells

To detect expression in mammalian cells, pENTR™4LPTOS plasmid DNA expressing each individual antigen was used to transfect HEK293 cells. As control, untransfected cells were used. Supernatant and cell lysates were harvested after 24hours and mixed with non-reducing

and reducing SDS buffer. The samples were run on polyacrylamide gels, transferred onto nitrocellulose membranes and day70 pooled sera used to detect protein expression.

Immunoblots revealed expression at the expected respective sizes: $AnAPN1_{fl}$ (112kDa, **Figure 4.4A**), Pfs230-C (83.5kDa, **Figure 4.4B**), and Pfs25 (22.4kDa, **Figure 4.4C**). In the case of the Pfs48/45 variants, $Pfs48/45_{-NGln}$ (**Figure 4.4D**) was observed weakly at 53kDa and $Pfs48/45_{+NGln}$ at ~50kDa (**Figure 4.4E**). High molecular weight species (between 80 and 100kDa) were observed for $Pfs48/45_{+NGln}$ (irrespective of the denaturation status) which might correspond to a 90kDa doublet reported by Kocken et al. (1993) when Pfs48/45 antisera reacted with gametocytes [292]. Pfs230-C antisera also reacted with a higher molecular weight species (~220kDa). The presence of these high molecular weight bands could be as a result of protein dimerisation or aggregation. Expression was seen mainly in the supernatant suggesting that the incorporation of the tPA leader sequence led to secretion of antigen, thought to be optimal for antibody induction [248, 443, 467].

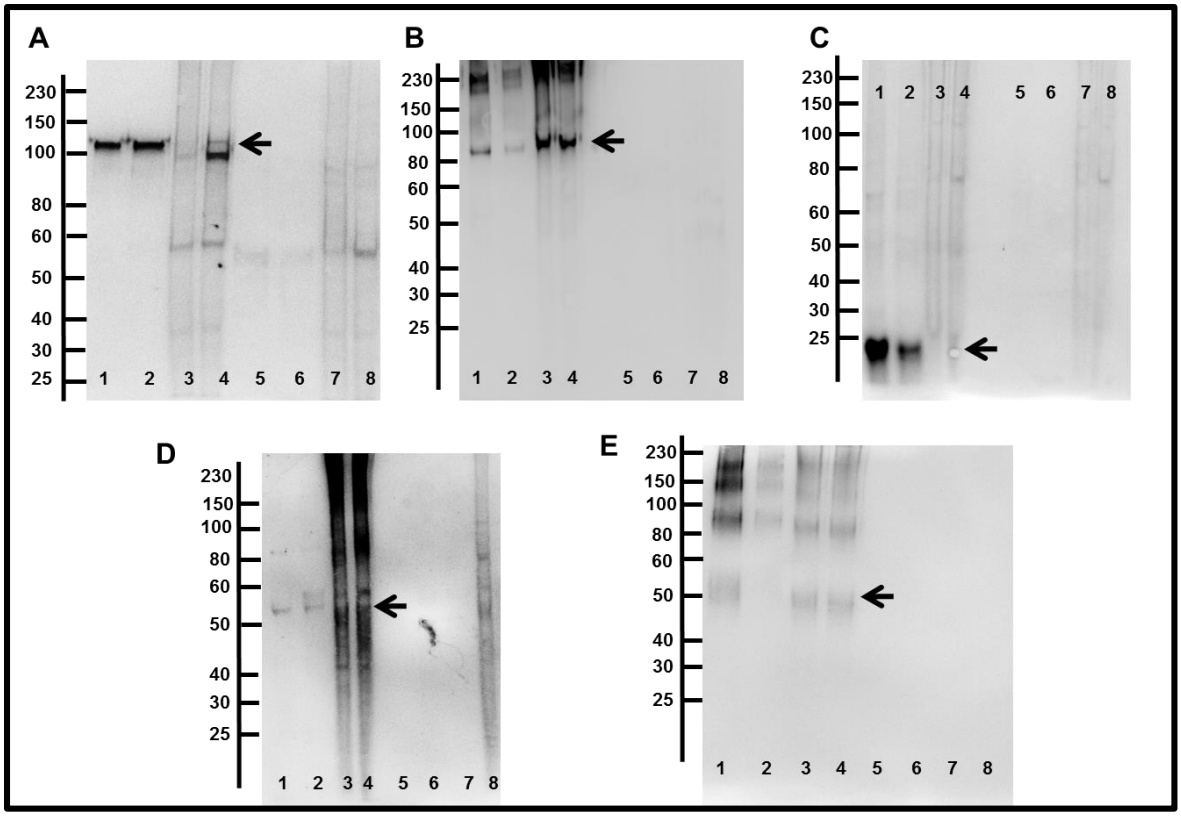


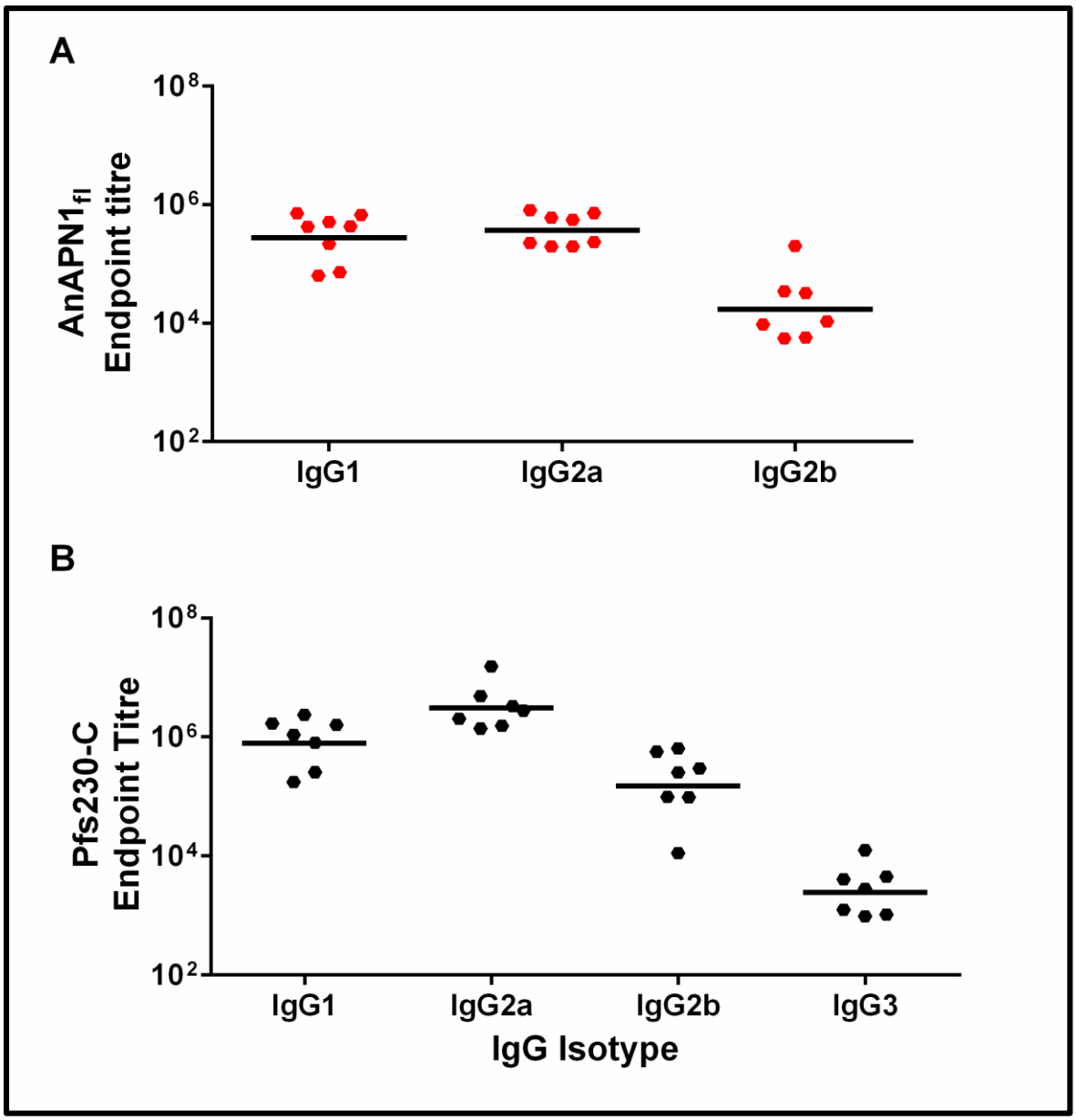
Figure 4.4. Expression of TBV candidate antigens in HEK239s.

pENTR4-LPTOS plasmid DNA expressing (**A**) AnAPN1$_{fl}$, (**B**) Pfs230-C, (**C**) Pfs25, (**D**) Pfs48/45$_{-NGln}$, and (**E**) Pfs48/45$_{+NGln}$ was used to transfect HEK293s for 24hours. Supernatant and cell lysate were harvested and mixed with either non-reducing or reducing SDS buffer. Samples were run on 10% (**A**) and 12% (**B-E**) polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using day70 pooled sera. Untransfected supernatant and cell lysate were used as negative controls. Lanes: 1 – antigen-specific supernatant in non-reducing buffer; 2 – antigen-supernatant in reducing buffer; 3 – antigen-specific lysate in non-reducing buffer; 4 – antigen-specific lysate in reducing buffer; 5 – 8 control supernatant or lysate respectively. Arrowheads indicate the relative migration mobility observed for each respective antigen.

4.2.2.3. IgG Isotype profiles after ChAd63-MVA vaccination

TBA attributed to antibodies against Pfs230-C have been shown to be IgG isotype-dependent with IgG2a and IgG2b isotypes mediating TBA whereas IgG1 has been shown to have no activity [172, 173]. Viral-vectors have previously been shown to induce a mixed Th1/Th2 type isotype profile that is dominated by cytophilic IgG2a [131]. Therefore, the induction of isotypes following ChAd63-MVA regimen was assayed to assess whether there was differential induction. Day70 sera from BALB/c mice previously vaccinated (**4.2.2.1**) were used to detect IgG1, IgG2a, IgG2b and IgG3 responses.

As observed by Douglas et al. (2010), a mixed Th1- and Th2-type isotype profile was observed as measured by IgG isotype endpoint ELISAs on day70 (**Figure 4.5**). The predominant isotypes induced were IgG1 and IgG2a for all the antigens with lower IgG2b responses. In contrast, there was an induction of IgG3 in the case of Pfs230-C (**Figure 4.5B**) whereas the other antigens didn't have detectable responses to this isotype. Pfs230-C has also been previously shown to induce all four isotypes when expressed in DNA [496]. The IgG isotype profile induced against rAnAPN1 has comprised of IgG1, IgG2a and IgG2b in the presence or absence of adjuvant [349] as observed with $AnAPNI_{fl}$ (**Figure 4.5A**). In the case of Pfs25 the isotype profile (**Figure 4.5C**) was reminiscent of that observed in a HuAd5-MVA prime-boost regimen [372]. For Pfs48/45 variants, there was a differential induction as not all animals had a detectable response with half of the animals having no induction of IgG1 and IgG2b against $Pfs48/45_{-NGln}$ (**Figure 4.5D**). The same animals with no detectable IgG1 responses also had no IgG2b. In contrast, all the animals in the $Pfs48/45_{+NGln}$ group had detectable responses to all three isotypes (**Figure 4.5D**). However, there was a comparable magnitude induced with no statistically significant differences detected in the isotype profiles between these two antigens.


A
AnAPN1fl Endpoint titre
10^8
10^6
10^4
10^2
IgG1
IgG2a
IgG2b
B
Pfs230-C Endpoint Titre
10^8
10^6
10^4
10^2
IgG1
IgG2a
IgG2b
IgG3
IgG Isotype

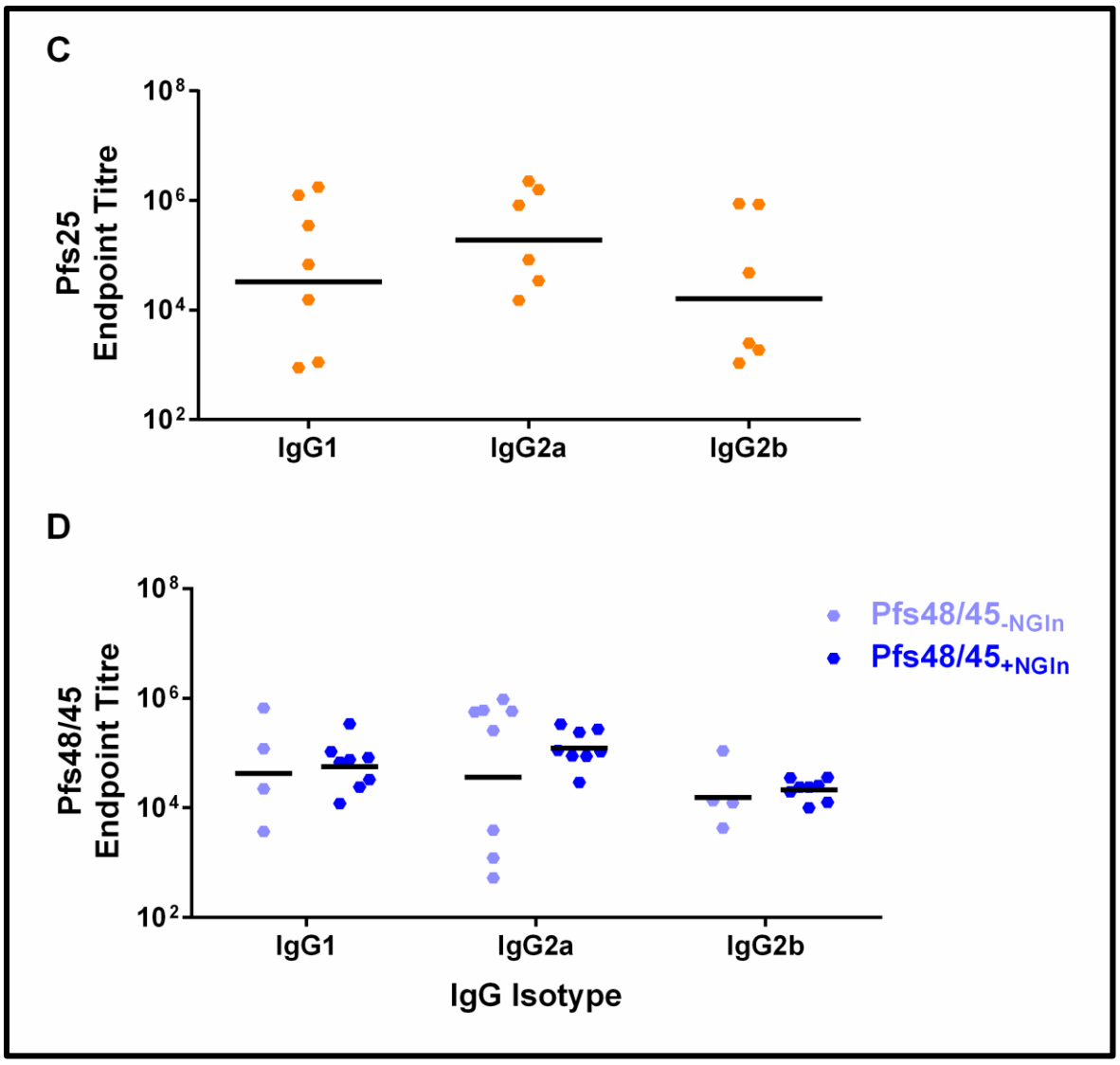


Figure 4.5. Induction of IgG isotypes following ChAd63-MVA vaccination.

Day70 sera from BALB/c mice (n=8/group) previously vaccinated (**4.2.2.1**) were used to assay IgG isotypes, IgG1, IgG2a, IgG2b and IgG3 for (**A**) AnAPN1$_{fl}$; (**B**) Pfs230-C; (**C**) Pfs25; and (**D**) Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$. Endpoint ELISAs were performed using recombinant protein. The data was log-transformed and presented as geometric mean for the different time points with dots representing responses from individual mice.

4.2.2.4. Longevity of antibody responses induced by ChAd63-MVA

To determine whether vaccine-induced IgG responses induced were maintained over time, age-matched BALB/c mice were prime-boost vaccinated (**4.2.2.1**). Total IgG was assayed every 28days by standardised ELISA up to 10 and a half months (day350) post-boost to determine the longevity of IgG responses. Vaccine-induced antibodies were maintained for all the antigens with no statistically significant differences between day70 and day350 responses (Friedman test with Dunn's multiple comparison test) (**Figure 4.6**).

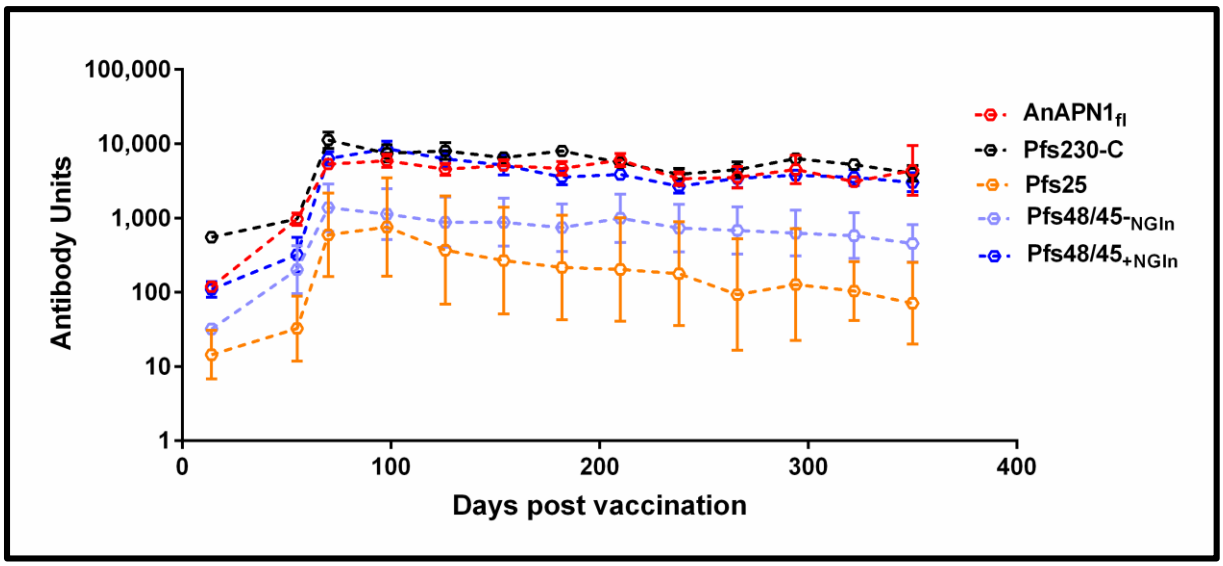


Figure 4.6. Longevity of antibody responses induced by ChAd63-MVA prime-boost.

BALB/c mice (n=4-5/group) were vaccinated with ChAd63 ($1x10^8$iu, day0) and boosted with the respective MVA ($1x10^7$pfu, day56) expressing the individual antigens $AnAPN1_{fl}$, Pfs230-C, Pfs25, $Pfs48/45_{-NGln}$ and $Pfs48/45_{+NGln}$. Total IgG responses were assayed on days 14, 55 and 70 and every 28days post-boost up to day350 by standardised ELISA using recombinant protein. The data was log-transformed and presented as geometric mean±SEM for the different time points.

4.2.2.5. Vaccine-induced antibodies recognise native antigen

To determine whether antibodies induced were able to recognise native antigen, indirect immunofluorescence assays were performed on parasite preparations to determine extent of localisation for parasite-based antigens (**Figure 4.7**). Surface staining of activated macrogametocytes and macrogametes (anti-Pfs230-C) and ookinetes (anti-Pfs25) additionally confirmed the recognised native antigen (**Figure 4.7**). Immunostained microgametocytes and exflagellating microgametes were observed using anti-Pfs48/45$_{+NGln}$ antisera whilst there was no labelling of parasites observed for Pfs48/45$_{-NGln}$. This suggested that antibodies raised against the Pfs48/45$_{+NGln}$ construct were able to recognise native parasite expressed on *P. falciparum* NF54 but not those raised against Pfs48/45$_{-NGln}$. For AnAPN1$_{fl}$, sera reactivity was measured using midgut lysate in ELISA and showed positive responses against two strains of *A. gambiae* (**Figure 4.1A**). Additionally, anti- sera recognised lysate from *A. gambiae* and *A. stephensi* on immunoblots (**Figure 4.8A**).

To further confirm the non-reactivity of Pfs48/45$_{-NGln}$ antisera, immunoblots of *P. falciparum* NF54 purified gametocytes (gift from Dr. Dinglasan, Johns Hopkins University) were used to assess reactivity. Antisera raised against Pfs48/45$_{-NGln}$ showed reactivity to gametocytes with a doublet observed in the gels run under both non-reducing and reducing conditions at 50kDA (**Figure 4.8A**). Immunobloting was able to detect reactivity of antisera against Pfs48/45$_{-NGln}$ to antigen in reduced form. Pfs48/45$_{+NGln}$ antisera on the other hand, strongly reacted with gametocytes in both non-reducing and reducing gels (**Figure 4.8B**). As observed in the immunoblots against mammalian expressed Pfs48/45$_{+NGln}$ (**Figure 4.4E**), there was weak staining of a species at ~100kDa, more evident in the reduced form, that might conform to the 90kDa doublet previously described [292].

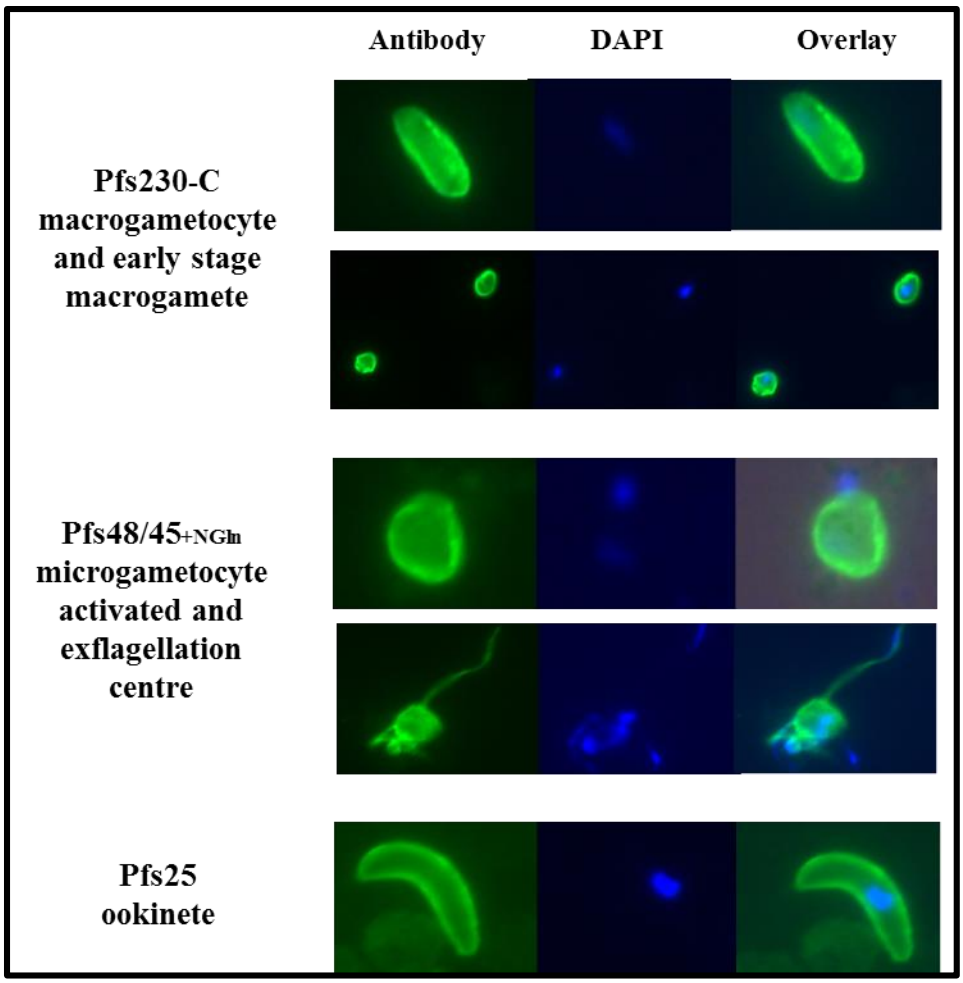


Figure 4.7. Reactivity of antisera against Pfs230-C, Pfs25 and Pfs48/45$_{+NGln}$.

In vitro cultured *P. falciparum* NF54 gametocytes (activated and non-activated) or Pfs25DR3 transgenic *P. berghei* ookinete were immunostained with antisera against Pfs230-C, Pfs48/45$_{+NGln}$ or purified total IgG against Pfs25 respectively. Antibody reactivity was detected by Alexa Fluor® 488-conjugated goat anti-mouse IgG (green) and nuclei were stained with DAPI (blue).

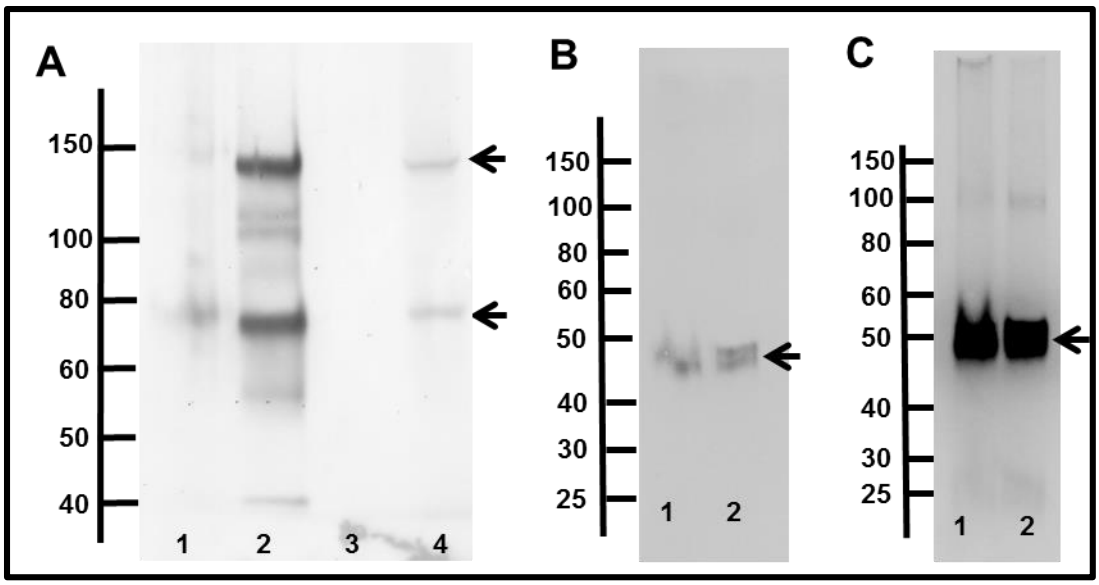


Figure 4.8. Reactivity of antisera against AnAPN1$_{fl}$, Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$.

A. gambiae (Yaoundé) and *A. stephensi* (SDA500) midgut lysate and *P. falciparum* NF54 gametocytes grown in culture purified by magnetic beads were mixed into either non-reducing or reducing SDS buffer. 5µg/lane and 10µg/lane of either midgut lysate or gametocyte preparation was loaded onto 8% and 12% polyacrylamide gels respectively, and transferred onto nitrocellulose membranes and immunoblots obtained using day70 pooled sera from (**A**) AnAPN1fl, (**B**) Pfs48/45$_{-NGln}$ and (C) Pfs48/45$_{+NGln}$ vaccinated animals. Lanes: (**A**) 1 – non-reducing and 2 – reducing buffer *A. gambiae*; 3 – non-reducing; 4 – reducing buffer *A. stephensi*; (**B** and **C**) 1 – non-reducing; 2 – reducing gametocyte preparation. Arrowheads indicate relative migration mobility observed.

4.2.2.6. Inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*

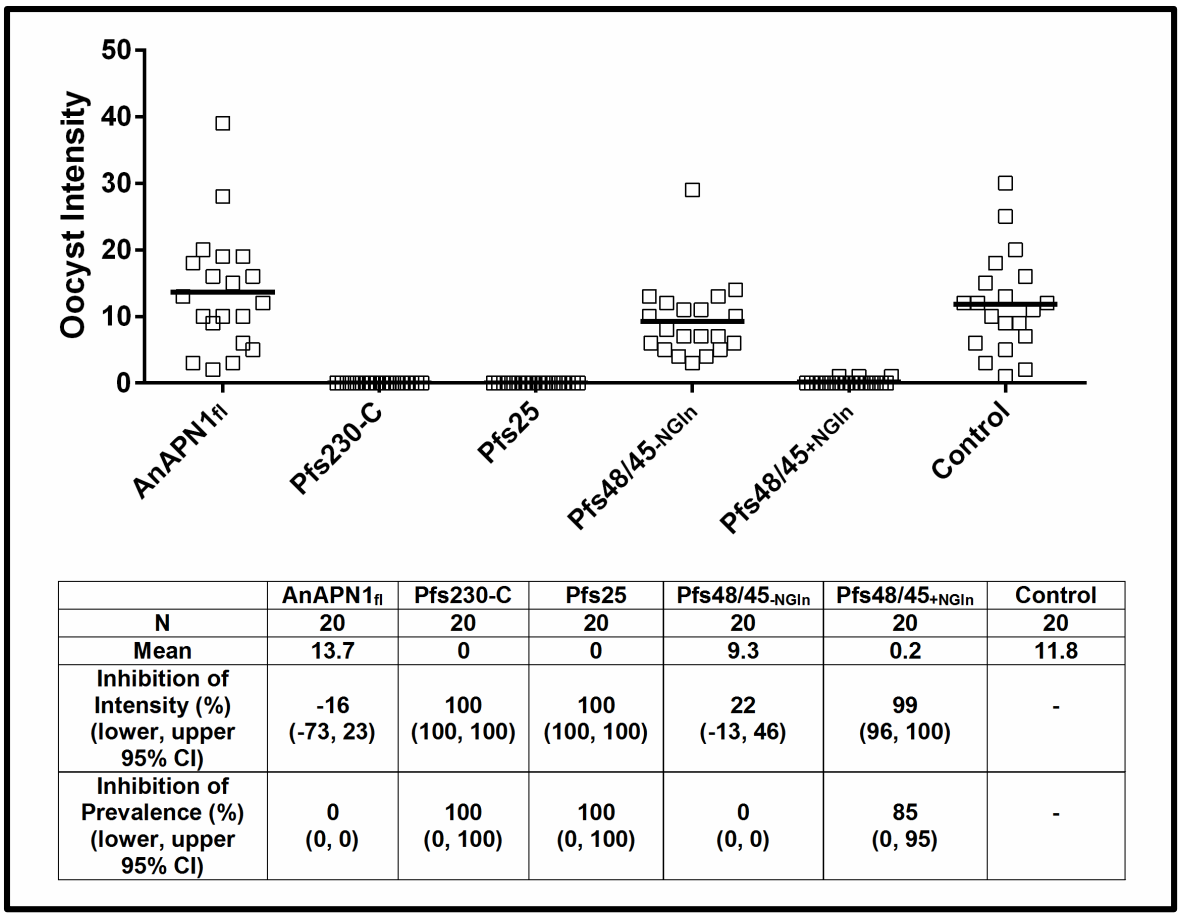
Antibodies raised against Pfs230-C, Pfs25, and Pfs48/45 have been shown to have varying TBA against *P. falciparum* depending on the expression system employed with the level of antibody titre largely driving efficacy in the case of Pfs25 [191]. Efficacy using anti-rAnAPN1 has been shown against both *P. berghei* and *P. falciparum* [348, 349]. However, direct antigen-to-antigen comparison on the ability to inhibit infectivity of lab-adapted *P. falciparum* NF54 parasites to its lab-adapted mosquito *A. stephensi* has never been conducted. To distinguish antibody-specific effects from possible non-antibody mediated effects in sera, total IgG was purified using protein G columns from pooled day70 sera (**4.2.2.1.**). The antibody titres for the individual pooled sera were determined by standardised ELISAs (**Table 4.2**). To enable comparative analysis, the same concentration (655μg/ml, 1:5.34 dilution) of total IgG against each of the antigens was subjected to the same parasite exposure in SMFA.

Complete TBE, inhibition of intensity and prevalence, was mediated against Pfs230-C (100%, $p<0.0001$) and Pfs25 (100%, $p<0.0001$); whilst efficacy against Pfs48/45$_{+NGln}$ (99% and 85%, $p<0.0001$) was insufficient to completely inhibit parasite intensity and prevalence (**Figure 4.9**). In comparison, IgG against AnAPN1$_{fl}$ and Pfs48/45$_{-NGln}$ had no inhibitory effects on either intensity or prevalence, with; intensity being enhanced in the case of AnAPN1$_{fl}$. As observed for whole IgG responses, efficacy against Pfs48/45$_{+NGln}$ was superior to that mediated against Pfs48/45$_{-NGln}$.

Table 4.2. Antibody titres of antisera used to purify total IgG for SMFA

Antigen	Antibody titre
$AnAPN1_{fl}$	11,587AU
Pfs230-C	15,206AU
Pfs25	3,287AU
$Pfs48/45_{-NGln}$	1,538AU
$Pfs48/45_{+NGln}$	7,136AU

Antibody titres were determined from an aliquot of sera from pooled vaccinated mice (n=8)



	AnAPN1$_{fl}$	Pfs230-C	Pfs25	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	20	20	20	20	20	20
Mean	13.7	0	0	9.3	0.2	11.8
Inhibition of Intensity (%) (lower, upper 95% CI)	-16 (-73, 23)	100 (100, 100)	100 (100, 100)	22 (-13, 46)	99 (96, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	0 (0, 0)	100 (0, 100)	100 (0, 100)	0 (0, 0)	85 (0, 95)	-

Figure 4.9. Comparative inhibition of *P. falciparum* NF54 infectivity to *A. stephensi.*

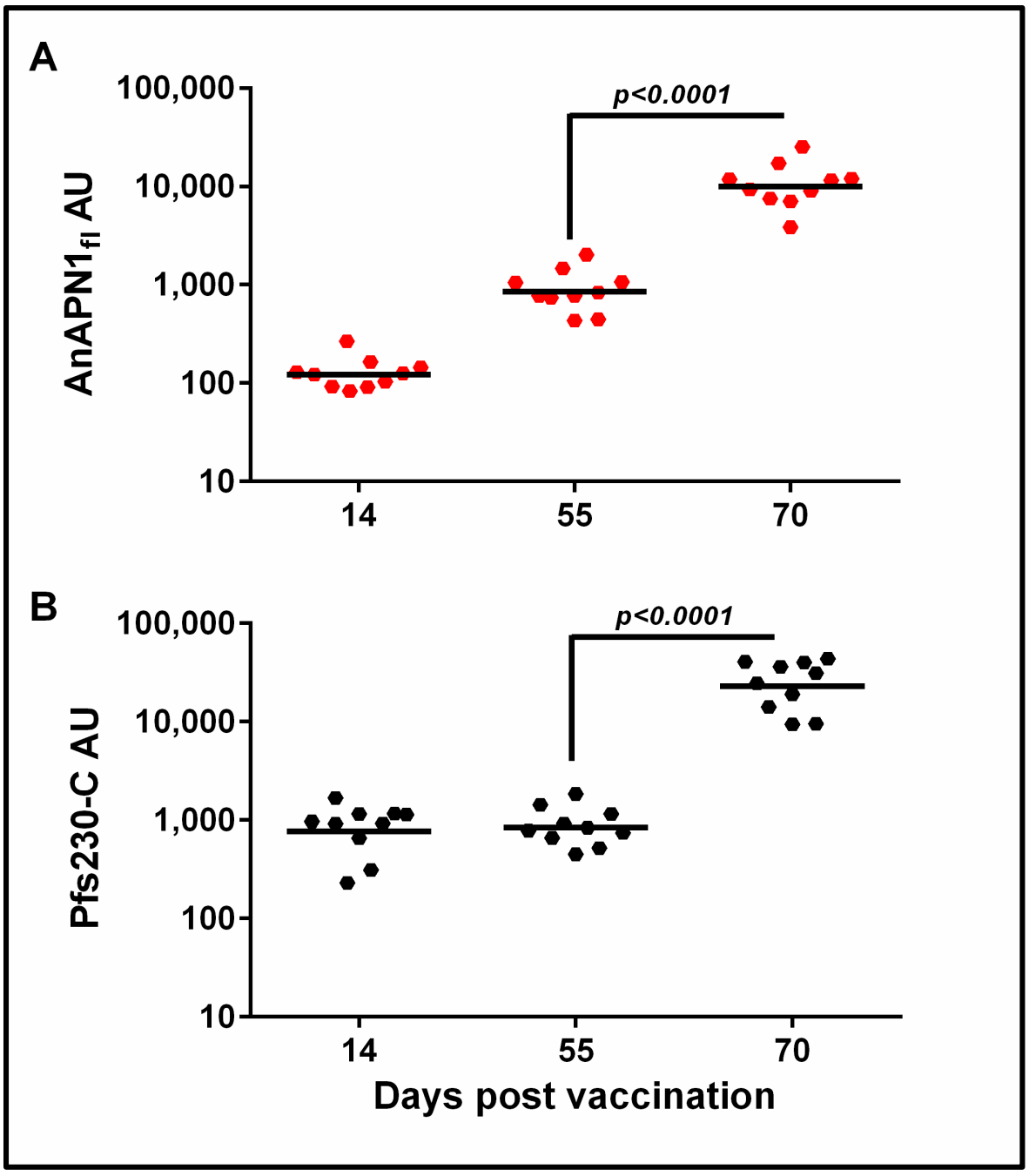
Total IgG purified (3.5mg/ml) from pooled sera from vaccinated mice with each respective antigen or GFP (control) (**4.2.2.1.**) was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* at 1:5.34 dilution (~655µg/ml) in SMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to control as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.

4.2.3. Comparative assessment – P. falciparum field isolates

4.2.3.1. Inhibition of parasite infectivity to *A. gambiae*

Based on the comparative assessment against *P. falciparum* NF54 (**4.2.2**), it was evident that Pfs230-C, Pfs25 and Pfs48/45$_{+NGln}$ were the leading candidates. However, if candidate(s) are to be prioritised for clinical product development, it is also imperative to test efficacy using parasite-vector combinations relevant to endemic settings. There have only been two published studies reporting the testing of efficacy of vaccine-induced antibodies against *P. falciparum* field isolates [460, 503]. Therefore, a comparative assessment to determine whether efficacy observed against NF54 *P. falciparum* would be extended to circulating *P. falciparum* parasites in *A. gambiae*, a predominant mosquito species relevant for transmission in sub-Saharan Africa, was conducted [460, 511].

A mouse experiment was set up as source of purified IgG for this assessment. BALB/c mice were prime-boost vaccinated with ChAd63-MVA expressing the individual respective antigens. In addition, a group of mice was further vaccinated with ChAd63 and MVA expressing GFP as viral-vector control. Whole IgG responses were determined by standardised ELISA (**Figure 4.10**). As previously observed, the ChAd63 priming responses were boosted by MVA administration at varying magnitudes ascertained by paired *t*-test ($p<0.0001$ in each case) against AnAPN1$_{fl}$ (12-fold); Pfs230-C (27-fold); Pfs48/45$_{-NGln}$ (6-fold) and Pfs48/45$_{+NGln}$ (27-fold). In this instance, responses against Pfs25 were also boosted 15-fold ($p<0.0001$, paired *t*-test). The superiority of Pfs48/45$_{+NGln}$ responses in comparison to those of Pfs48/45$_{-NGln}$ were further validated in this experiment with a 4-fold difference between day70 responses ($p=0.0002$, unpaired t-test).


A
100,000
10,000
1,000
100
10
AnAPN1fl AU
p<0.0001
14
55
70
B
100,000
10,000
1,000
100
10
Pfs230-C AU
p<0.0001
14
55
70
Days post vaccination

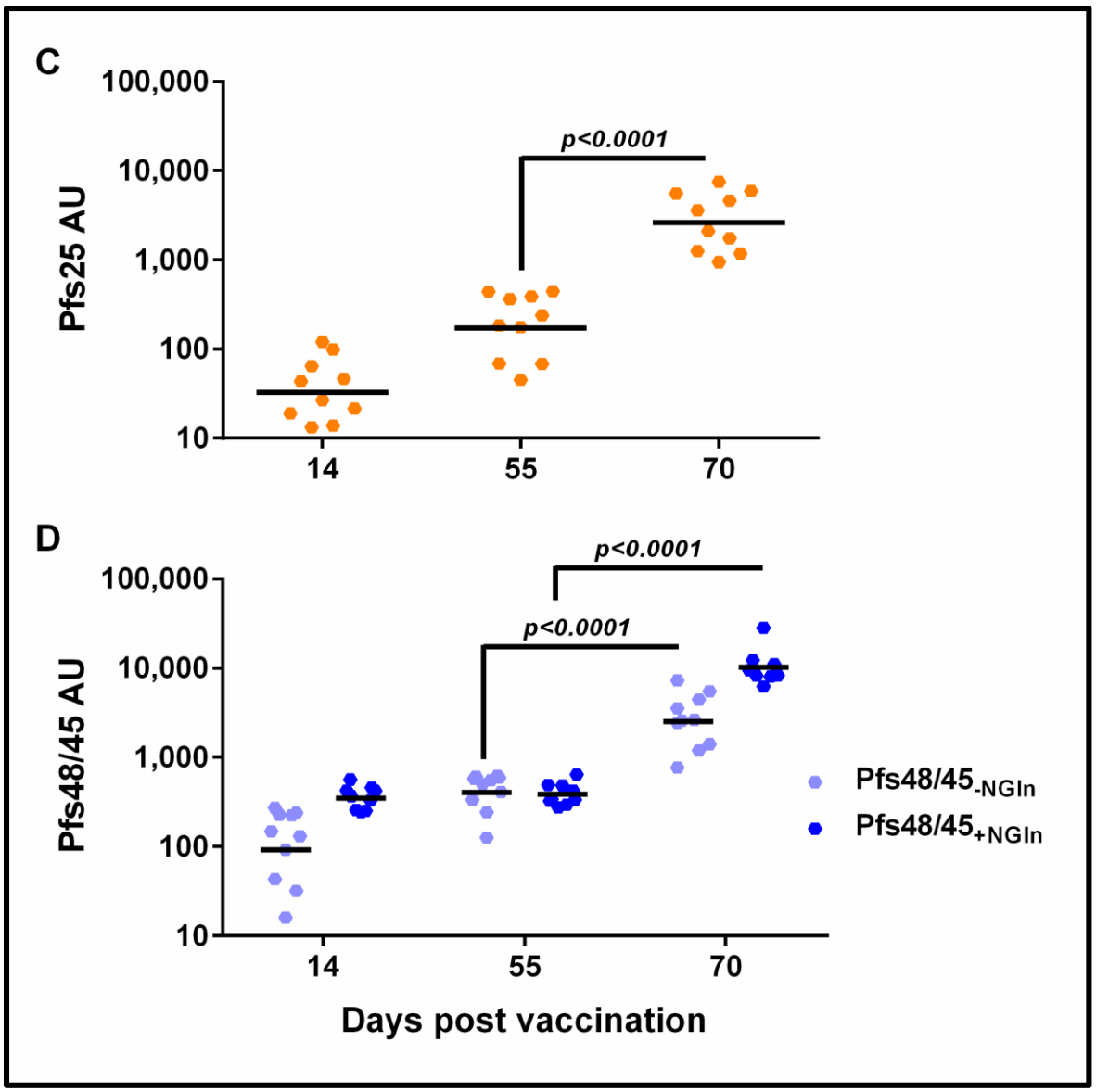


Figure 4.10. Induction of IgG after ChAd63-MVA prime-boost vaccination.

BALB/c mice (n=10/group) were vaccinated as previously described (**Figure 4.3.**). Whole IgG responses were assayed on days 14, 55 and 70 by standardised ELISA using sera from; (**A**) AnAPN1$_{fl}$, (**B**) Pfs230-C, (**C**) Pfs25, and (**D**) Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$. The data was log-transformed and presented as geometric mean for each of the different time points. Dots represent responses from individual mice. Significance considered at $p<0.05$.

Sera from day70 samples was pooled from each group including viral-vector control and used to purify total IgG (2.5mg/ml). Antibody titres of pooled sera was determined by standardised ELISA (**Table 4.3**). Purified total IgG was sent to Dr. Anna Cohuet, Burkina Faso to be tested in DMFA. Feeding assays were performed with gametocyte-containing blood as a source of parasites sampled from children positive for gametocytes by microscopy. Individual gametocyte carriers were used in each DMFA and all the antigens simultaneously assessed at an IgG concentration of 500μg/ml. Overall, there was no statistically significant difference in oocyst intensity between using IgG purified from viral-vector control or AB^+ serum in the assays, therefore, all DMFA data was analysed relative to AB^+ serum as control.

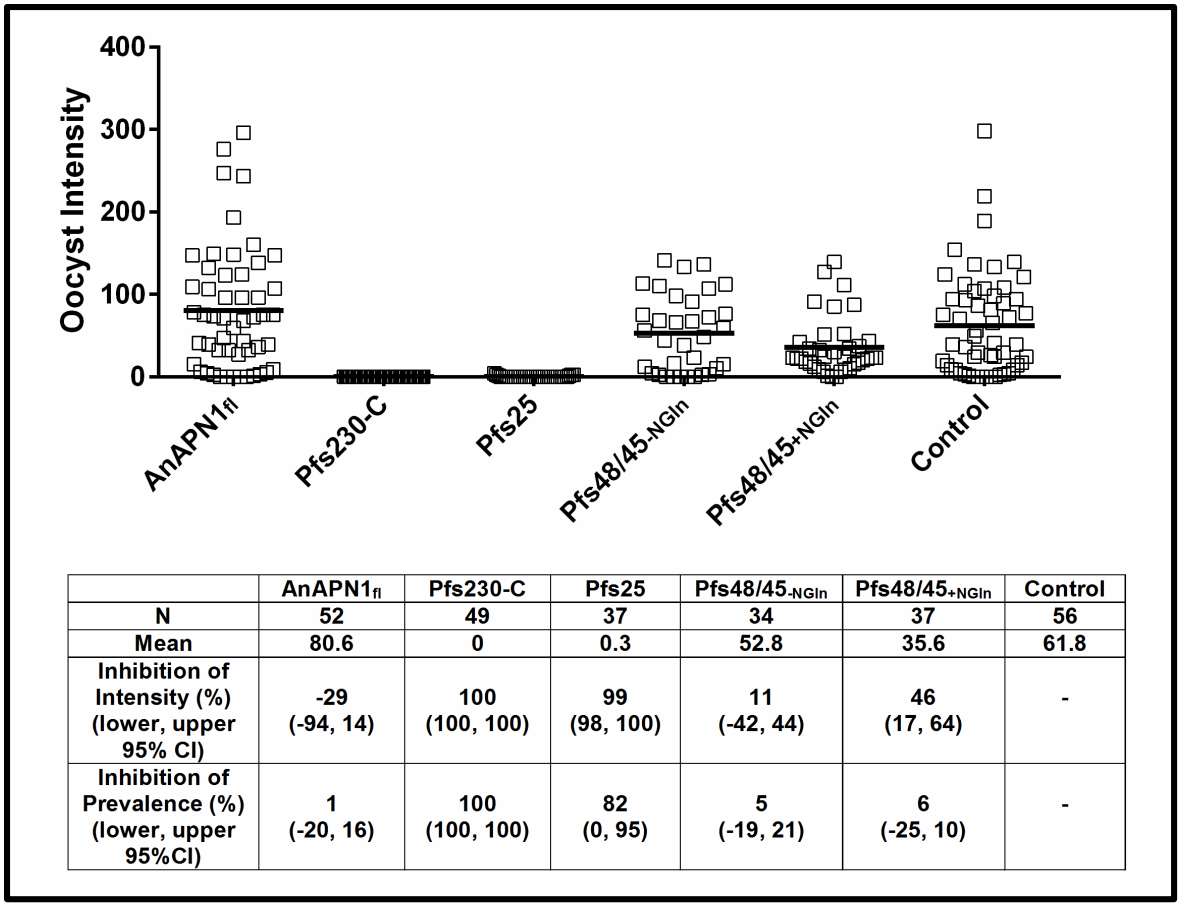
Table 4.3. Antibody titres of antisera used to purify total IgG for DMFA

Antigen	Antibody titre
$AnAPN1_{fl}$	10,853AU
Pfs230-C	26,266AU
Pfs25	4,662AU
$Pfs48/45_{-NGln}$	3,420AU
$Pfs48/45_{+NGln}$	ND

Antibody titres were determined from an aliquot of sera from pooled vaccinated mice (n=10). ND: not determined.

As observed with efficacy against *P. falciparum* NF54, a hierarchy of inhibition was observed against the first gametocyte carrier. IgG against Pfs230-C achieved complete efficacy (100%, $p<0.0001$) followed by that of Pfs25 (99% and 82%, $p<0.0001$) whilst that against Pfs48/45$_{+NGln}$ only inhibited parasite intensity (46%, $p=0.007$) about half that observed against *P. falciparum* NF54 (**Figure 4.11**). As previously observed against NF54, IgG against AnAPN1$_{fl}$ and Pfs48/45$_{-NGln}$ had no effect at inhibiting either intensity or prevalence with the former enhancing intensity. Thus, it was evident that Pfs230-C and Pfs25 resulted in responses with the most efficacy.

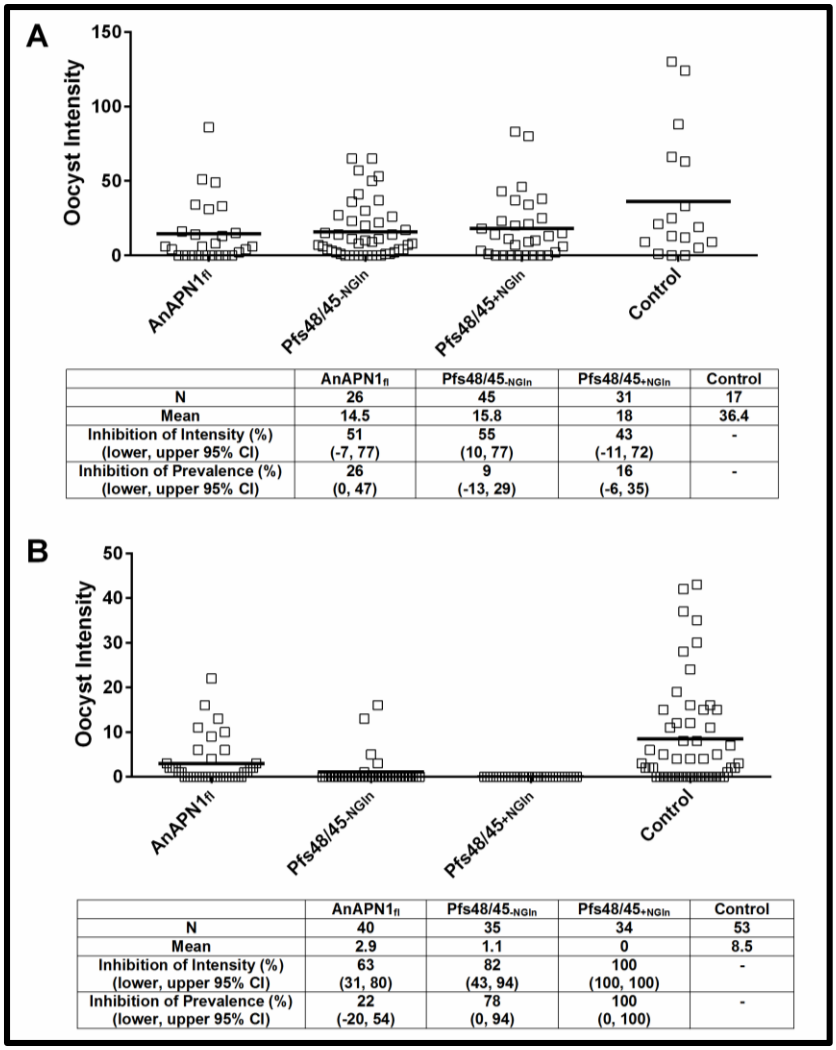
To completely obviate the need to pursue AnAPN1$_{fl}$, Pfs48/45$_{-NGln}$ and Pfs48/45$_{+NGln}$, further assessment was carried out against two different gametocyte carriers (**Figure 4.12**). Only anti-Pfs48/45$_{-NGln}$ inhibited intensity by 55% ($p=0.02$) with no effect on prevalence against the one gametocyte carrier (**Figure 4.12A**). On subsequent parasite exposure from the other gametocyte carrier (**Figure 4.12B)**, potent activity was observed with; anti-Pfs48/45$_{+NGln}$ giving complete efficacy at 100% ($p<0.0001$, both intensity and prevalence), and anti-Pfs48/45$_{-NGln}$ (82% and 78%, $p<0.009$ and $p<0.0001$, intensity and prevalence respectively), whilst anti-AnAPN1$_{fl}$ only exhibited activity at inhibiting intensity (63%, $p=0.003$).



	AnAPN1$_{fl}$	Pfs230-C	Pfs25	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	52	49	37	34	37	56
Mean	80.6	0	0.3	52.8	35.6	61.8
Inhibition of Intensity (%) (lower, upper 95% CI)	-29 (-94, 14)	100 (100, 100)	99 (98, 100)	11 (-42, 44)	46 (17, 64)	-
Inhibition of Prevalence (%) (lower, upper 95%CI)	1 (-20, 16)	100 (100, 100)	82 (0, 95)	5 (-19, 21)	6 (-25, 10)	-

Figure 4.11. Comparative inhibition of *P. falciparum* field isolate infectivity to *A. gambiae*.

Total IgG purified (2.5mg/ml) from day70 pooled sera (**Figure 4.9**) was mixed with gametocyte-containing blood from a single donor. The donor plasma was replaced with a 1:5 dilution of IgG (500µg/ml) and immediately fed to *A. gambiae* in DMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to AB^+ serum (control) as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.



	AnAPN1$_{fl}$	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	26	45	31	17
Mean	14.5	15.8	18	36.4
Inhibition of Intensity (%) (lower, upper 95% CI)	51 (-7, 77)	55 (10, 77)	43 (-11, 72)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	26 (0, 47)	9 (-13, 29)	16 (-6, 35)	-

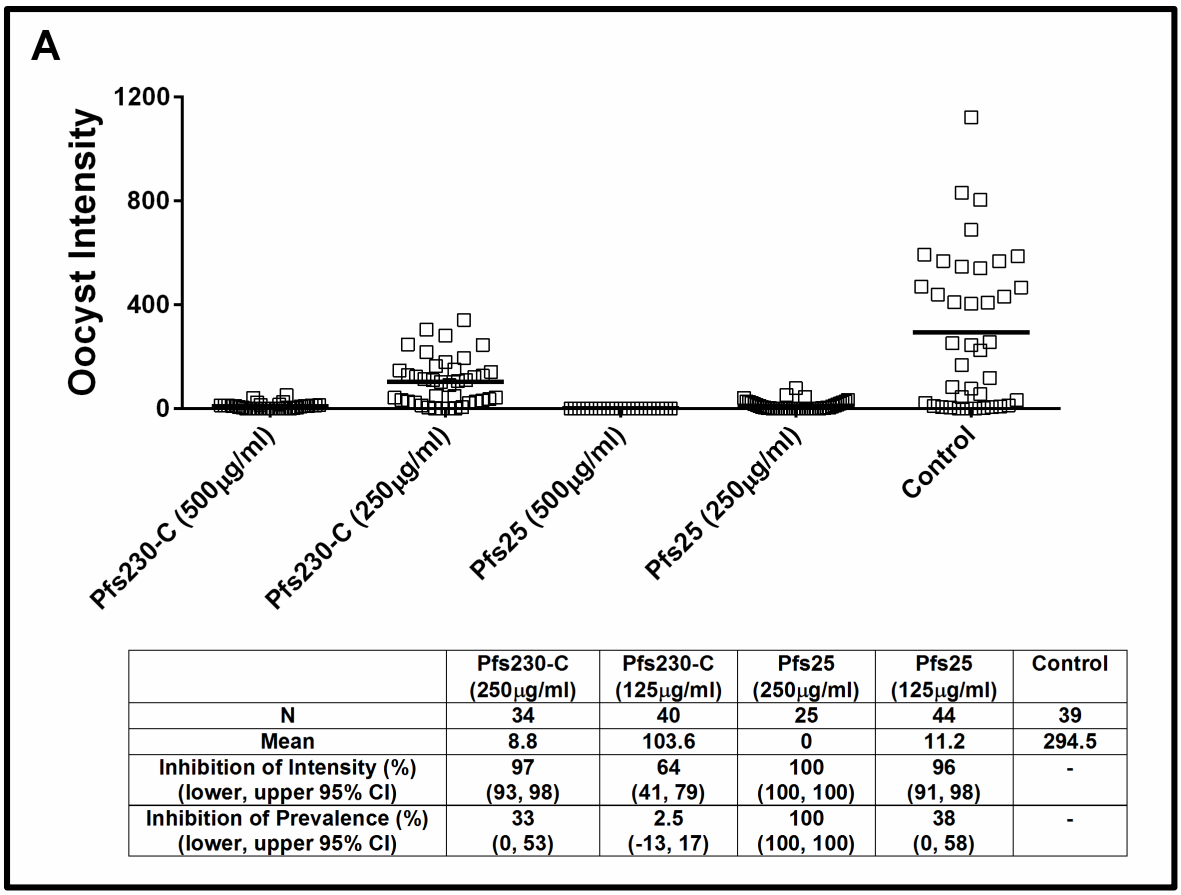
	AnAPN1$_{fl}$	Pfs48/45$_{-NGln}$	Pfs48/45$_{+NGln}$	Control
N	40	35	34	53
Mean	2.9	1.1	0	8.5
Inhibition of Intensity (%) (lower, upper 95% CI)	63 (31, 80)	82 (43, 94)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	22 (-20, 54)	78 (0, 94)	100 (0, 100)	-

Figure 4.12. Differential inhibition of infectivity by anti-AnAPN1$_{fl}$, anti-Pfs48/45$_{-NGln}$ and anti-Pfs48/45$_{+NGln}$ IgG.

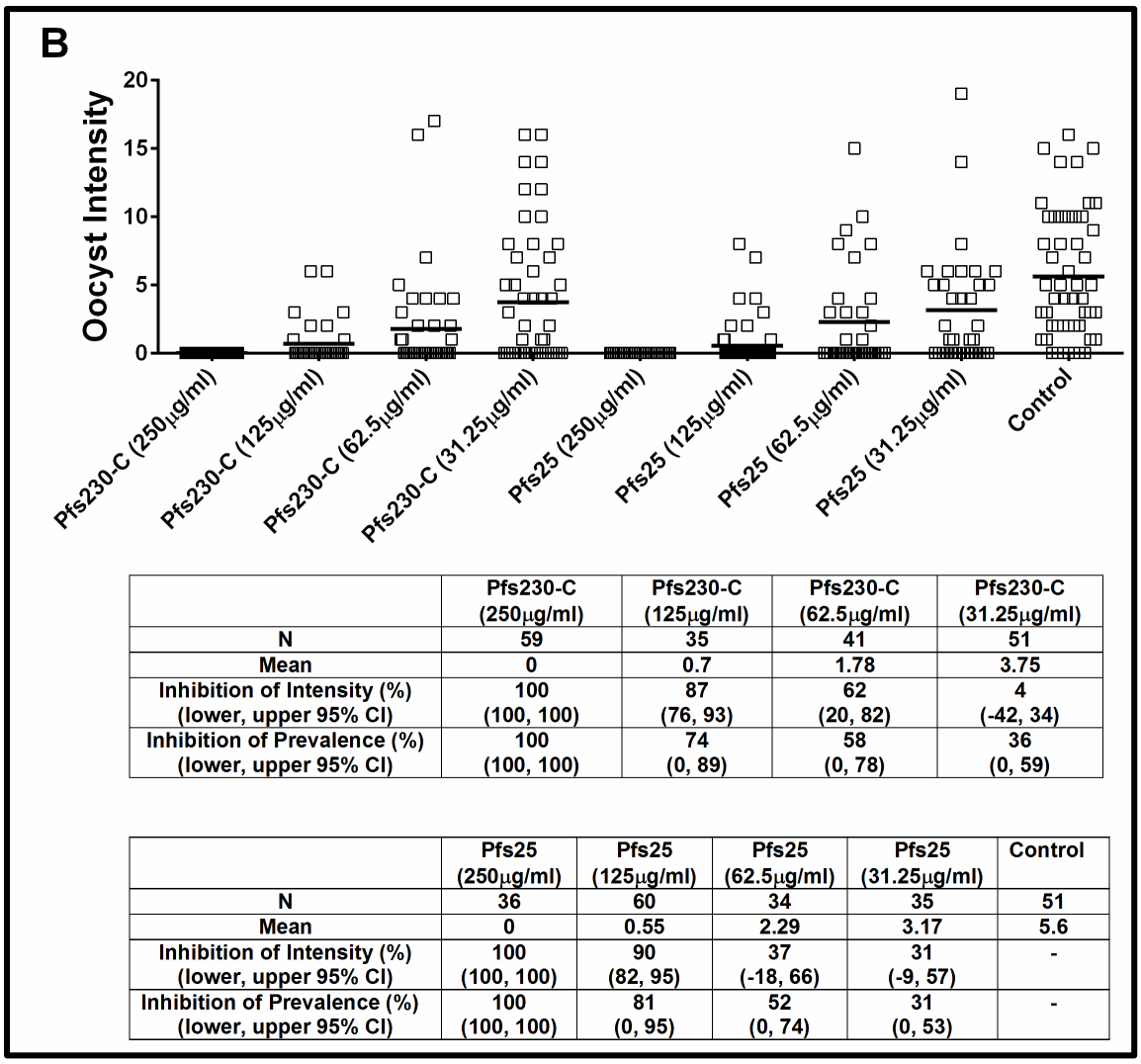
Total IgG purified (2.5mg/ml) from day70 pooled sera (**Figure 4.9**) was mixed with gametocyte-containing blood from two separate donors (**A** and **B**). The donor plasma was replaced IgG (500µg/ml) and immediately fed to *A. gambiae* in DMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to AB^+ serum (control) as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.

4.2.3.2. Additional effect of anti-Pfs230-C and anti-Pfs25 IgG

Vaccine-induced IgG antibodies against both Pfs230-C and Pfs25 consistently showed higher TBE compared to the other candidate antigens whether exposed to the homologous parent strain or field isolates. Therefore, inhibition exhibited by total IgG was further investigated to determine if there was any evidence of concentration-dependent efficacy. Initial testing at 500μg/ml and 250μg/ml against a gametocyte carrier with a high gametocyte infection (mean control infection of 294.5), resulted in a reduction of 97% (p<0.0001) to 64% (p=0.0002) in infection intensity for anti-Pfs230-C IgG whilst prevalence was only inhibited by 33% at the higher concentration (p=0.001) (**Figure 4.13A**). In the case of anti-Pfs25 IgG, the highest concentration tested resulted in complete inhibition of both intensity and prevalence (100%, p<0.0001), whilst the lower concentration had an impact of 96% and 38% reduction in intensity and prevalence respectively (p<0.0001) (**Figure 4.13A**). Both anti-Pfs230-C and anti-Pfs25 IgG were able to mediate TBA in a concentration-dependant manner. To further confirm the concentration-dependent efficacy, additional concentrations were further tested against another gametocyte donor. Both anti-Pfs230-C and anti-Pfs25 IgG resulted in significant concentration-dependent inhibition of infectivity (**Figure 4.13B**). Anti-Pfs230-C IgG mediated inhibition of intensity up to 62.5μg/ml whilst anti-Pfs25 IgG had activity only up to 125μg/ml whilst inhibition of prevalence was evident with a concentration of up to 31.25μg/ml in both cases. Interestingly, anti-Pfs230-C IgG were more potent at inhibiting parasite infectivity with a lower infection (mean control infection of 5.6) compared to a higher infection (mean control infection of 294.5) as the same concentration of 250μg/ml resulted in complete inhibition in the former compared to the latter. On the other hand, anti-Pfs25 IgG had comparable impact at either concentrations regardless of the level of infection.



	Pfs230-C (250µg/ml)	Pfs230-C (125µg/ml)	Pfs25 (250µg/ml)	Pfs25 (125µg/ml)	Control
N	34	40	25	44	39
Mean	8.8	103.6	0	11.2	294.5
Inhibition of Intensity (%) (lower, upper 95% CI)	97 (93, 98)	64 (41, 79)	100 (100, 100)	96 (91, 98)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	33 (0, 53)	2.5 (-13, 17)	100 (100, 100)	38 (0, 58)	-



	Pfs230-C (250μg/ml)	Pfs230-C (125μg/ml)	Pfs230-C (62.5μg/ml)	Pfs230-C (31.25μg/ml)
N	59	35	41	51
Mean	0	0.7	1.78	3.75
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	87 (76, 93)	62 (20, 82)	4 (-42, 34)
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	74 (0, 89)	58 (0, 78)	36 (0, 59)

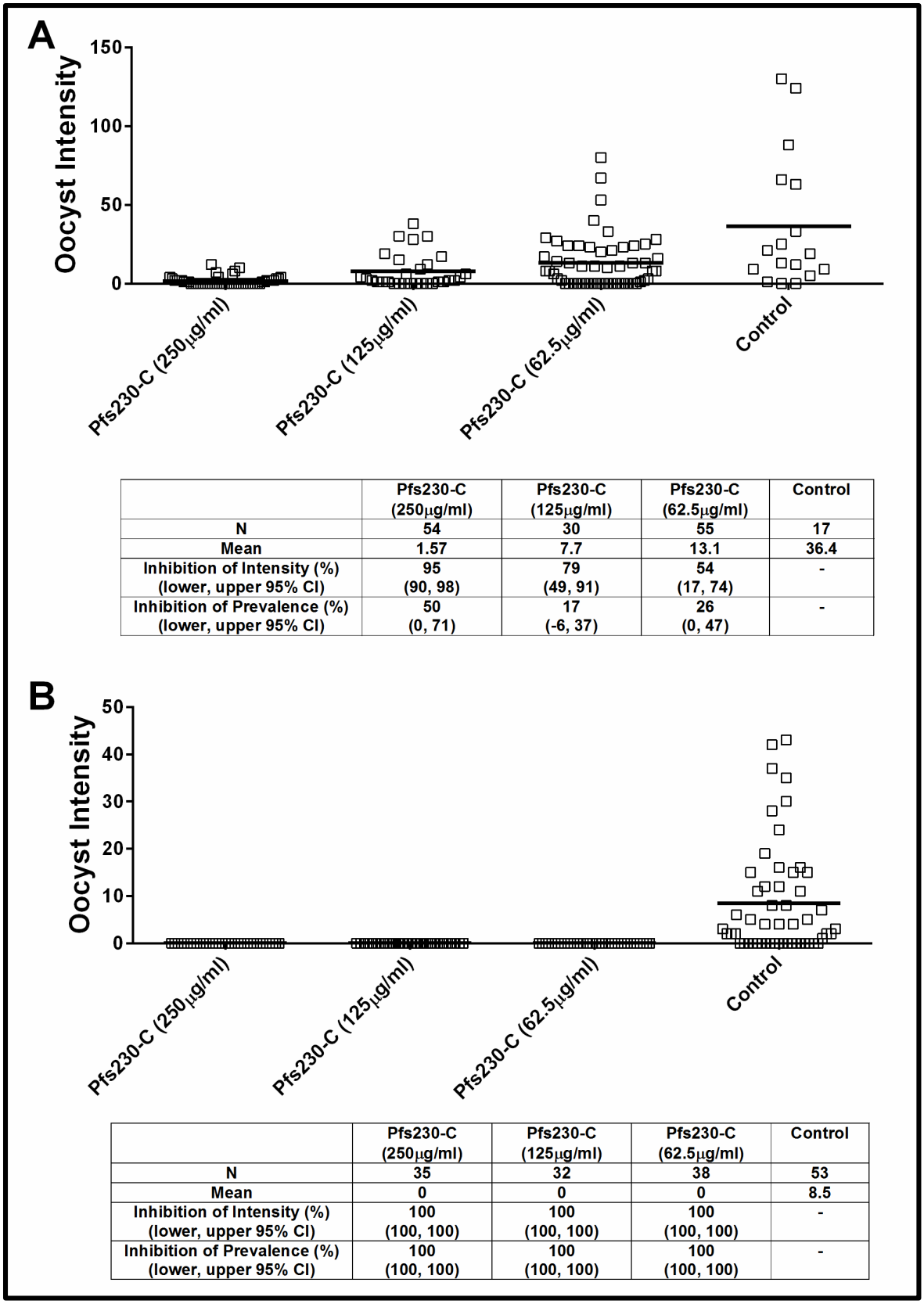
	Pfs25 (250μg/ml)	Pfs25 (125μg/ml)	Pfs25 (62.5μg/ml)	Pfs25 (31.25μg/ml)	Control
N	36	60	34	35	51
Mean	0	0.55	2.29	3.17	5.6
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	90 (82, 95)	37 (-18, 66)	31 (-9, 57)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	81 (0, 95)	52 (0, 74)	31 (0, 53)	-

Figure 4.13. Concentration-dependent inhibition of parasite infectivity and prevalence.

Total IgG purified (2.5mg/ml) from day70 pooled sera (**Figure 4.9**) was mixed with gametocyte-containing blood from two separate donors (**A** and **B**). IgG from Pfs230-C and Pfs25 vaccinated mice was diluted to various concentrations (shown in parenthesis). The donor plasma was replaced with the IgG and immediately fed to *A. gambiae* in DMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to AB^+ serum (control) as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.

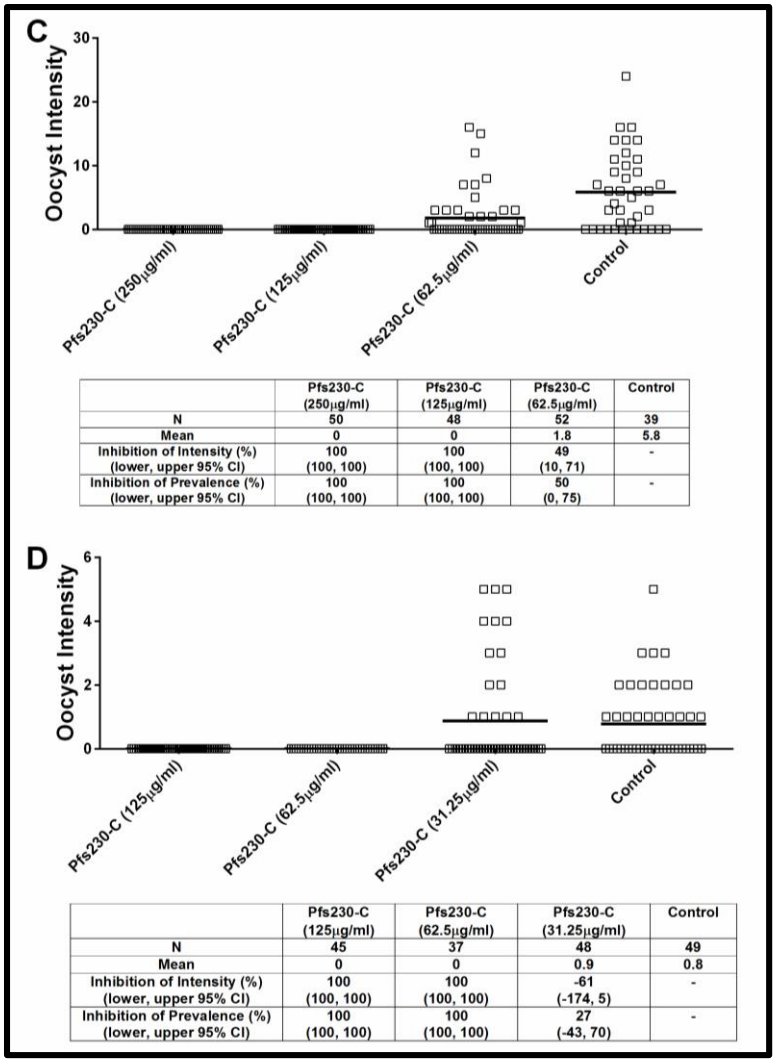
Taking into consideration the level of efficacy observed by anti-Pfs230-C and anti-Pfs25 IgG in two different infections (**Figure 4.13**), additional parasites from different gametocyte carriers were tested for their infectivity at different concentrations. Considering that anti-Pfs230-C IgG was more potent at inhibiting infectivity at lower infection levels, four different infection levels were tested from different gametocyte carriers (**Figure 4.14)**. There was concentration-dependent inhibition of intensity against parasites from gametocyte carrier in **Figure 4.14A**, whereas complete inhibition of both intensity and prevalence was observed regardless of the concentration against parasites in **Figure 4.14B**. Irrespective of the mean oocyst in the control, there was consistent complete inhibition of both intensity and prevalence observed at 250μg/ml and 125μg/ml in three out of four of the experiments (**Figure 4.14B-D**). Additional testing of anti-Pfs25 IgG at 500μg/ml and 250μg/ml against parasites from another gametocyte carrier, resulted in complete efficacy at both IgG concentrations (**Figure 4.15**).

Taken together, assessment against parasites from different gametocytes carriers for both anti-Pfs230-C and anti-Pfs25 IgG showed that these antibodies were potent at inhibiting parasite infectivity. The impact on infectivity was dependent on the amount of IgG present and the level of parasite infection. The more intense the parasite exposure (as observed by the mean control oocyst numbers), the more total antigen-specific IgG was required to completely inhibit both intensity and prevalence.



	Pfs230-C (250µg/ml)	Pfs230-C (125µg/ml)	Pfs230-C (62.5µg/ml)	Control
N	54	30	55	17
Mean	1.57	7.7	13.1	36.4
Inhibition of Intensity (%) (lower, upper 95% CI)	95 (90, 98)	79 (49, 91)	54 (17, 74)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	50 (0, 71)	17 (-6, 37)	26 (0, 47)	-

	Pfs230-C (250µg/ml)	Pfs230-C (125µg/ml)	Pfs230-C (62.5µg/ml)	Control
N	35	32	38	53
Mean	0	0	0	8.5
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	100 (100, 100)	-

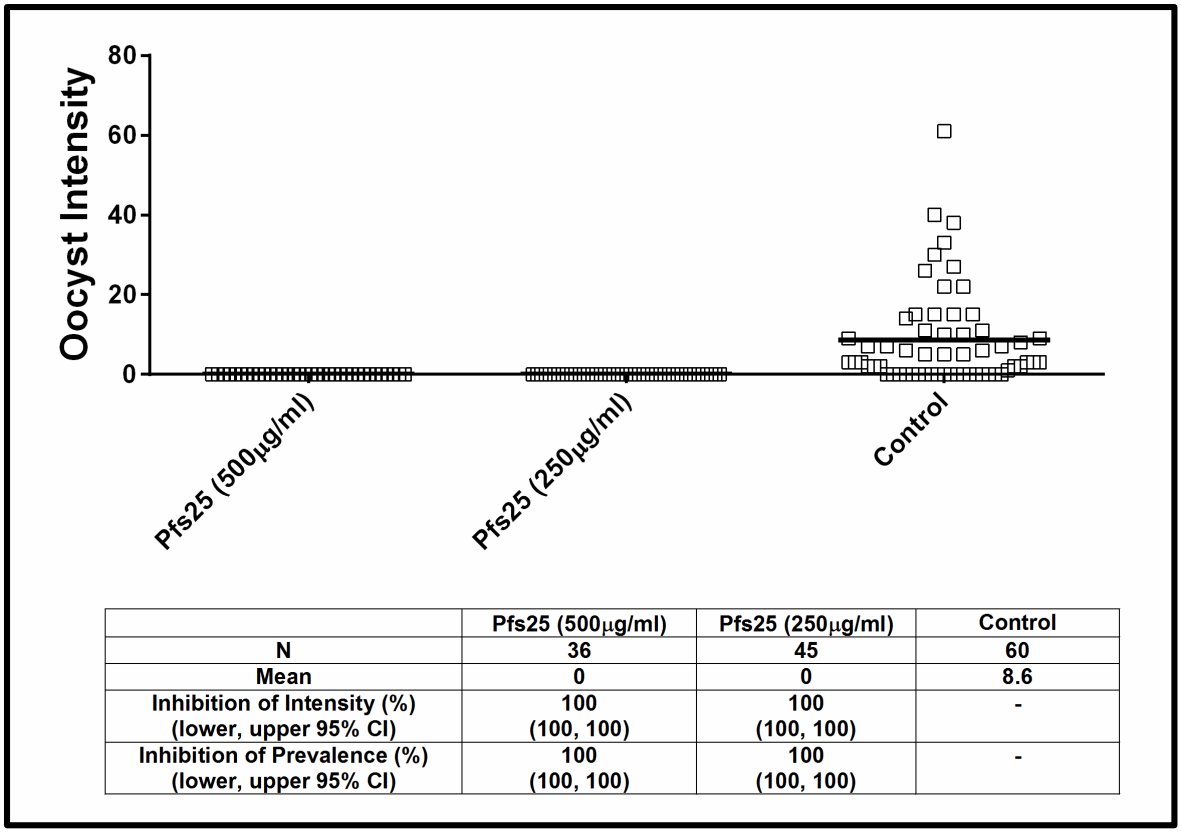


	Pfs230-C (250µg/ml)	Pfs230-C (125µg/ml)	Pfs230-C (62.5µg/ml)	Control
N	50	48	52	39
Mean	0	0	1.8	5.8
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	49 (10, 71)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	50 (0, 75)	-

	Pfs230-C (125µg/ml)	Pfs230-C (62.5µg/ml)	Pfs230-C (31.25µg/ml)	Control
N	45	37	48	49
Mean	0	0	0.9	0.8
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	-61 (-174, 5)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	27 (-43, 70)	-

Figure 4.14. Inhibition mediated by anti-Pfs230-C IgG against different parasite donors.

Total IgG purified (2.5mg/ml) from day70 pooled sera (**Figure 4.9**) was diluted at different concentrations (shown in parenthesis) and mixed with gametocyte-containing blood from four independent gametocyte carriers (**A**), (**B**), (**C**), and (**D**). The donor plasma was replaced with IgG and immediately fed to *A. gambiae* in DMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to AB^+ serum (control) as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.



	Pfs25 (500µg/ml)	Pfs25 (250µg/ml)	Control
N	36	45	60
Mean	0	0	8.6
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	100 (100, 100)	-

Figure 4.15. Additional evidence of inhibition mediated by anti-Pfs25 IgG.

Total IgG purified (2.5mg/ml) from day70 pooled sera (**Figure 4.9**) was mixed with gametocyte-containing blood. The donor plasma was replaced with IgG at two different concentrations (shown in parenthesis) and immediately fed to *A. gambiae* in DMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to AB^{+} serum (control) as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.

4.3. Discussion

The work described demonstrates that ChAd63-MVA viral-vectored delivery platform, expressing TBV candidate antigens, was able to induce antibodies and can be used to comparatively assess their ability to inhibit *P. falciparum* infectivity. The head-to-head comparison of these antigens provides evidence that vaccine-induced antibodies have an impact against two parasite sources in two hosts, *A. stephensi* and *A. gambiae*. This provides further evidence that the viral-vectored delivery platform can be used to induce TB antibodies against these candidate antigens as observed previously with the expression of Pfs25 [340, 372]. Overall, there has been demonstrable TBA exhibited by vaccine-induced IgG response thus making this regimen a useful platform for the screening and development of antibody-inducing vaccines that can be translated into the clinic.

The ChAd63 priming response was unable to induce measurable antibody responses in the PfsHAP2 construct tested due to a deletion. This was unexpected as recombinant ChAd63-PfsHAP2 was the only construct that didn't have any evidence of gene rearrangements (**Chapter 3**). The screening of recombinant preparations showed bands that were of correct size with no outgrowths as observed for the other constructs and previously published [492]. Even though the recombinant was re-engineered in ChAd63 under transgene repression, transgene deletion still resulted upon subsequent screening by sequencing. However, PfsHAP2 is still an attractive candidate antigen due to its conserved role in parasite development [295]. Therefore, appropriate design modifications to include only the region that is conserved across species might enable improved expression of this candidate antigen.

Development of effective parasite-based TBVs has been hampered by the inability to express full-length correctly folded immunogenic proteins, notably Pfs230 and Pfs48/45, cysteine-rich proteins with immunodominant conformational-dependent epitopes [172, 322, 509].

Expression of full-length Pfs48/45 in *E. coli* has been successful after codon harmonisation [504]. On the other hand, correctly folded protein consisting of the C-terminus has also been expressed in this system [385]. Expression of full-length Pfs48/45 in viral-vectors provides evidence that these antigens can be expressed and used to induce antigen-specific antibodies that have functional activity against the homologous parent strain NF54 and field isolates.

In this instance, substitution of N-glycosylation sites resulted in a less effective immunogen that was inferior at inducing antigen-specific IgG responses. These antibodies were less effective at inhibiting parasite infectivity compared to the immunogen with intact N-glycosylation sites. Thus, there might be a possible role of N-glycosylation site substitution in the expression and immunogenicity of Pfs48/45. Evidence of the potential impact of N-glycosylation site substitution on immunogenicity and efficacy has been observed with MSP1-42 [512-514]. Stowers et al. (2002) showed that N-glycosylation site substitution of PfMSP1-42 lead to enhanced antibody responses [513], an observation confirmed when the same antigen was expressed in a DNA vector [514]. These findings are contrary to what has been observed in this study, with the opposite being evident. Substitution of N-glycosylation sites led to inferior antibody responses and leads one to speculate that N-glycosylation site usage in *P. falciparum* might be antigen dependent. However, it has been postulated that *Plasmodium* doesn't modify its proteins with N-glycans [515, 516]. On the other hand, Bushkin et al. (2010) have recently reported that these can be expressed however there is evidence that the number of sugar residues is minimal [489]. Nevertheless, even though Pfs48/45 possesses these sites, it has been shown that the parasite doesn't produce a glycosylated protein [490]. Expression of Pfs48/45 in vaccinia virus resulted in a glycosylated product that considerably induced less antibody responses in mice with a consequence on the ability to inhibit parasite infectivity to *A. stephensi* [490]. Milek et al.

(1998) observed that this glycosylated product wasn't secreted suggesting possible incorrect folding which is crucial for an antigen as Pfs48/45 that relies on conformation for antibody recognition [385, 490]. On the other hand, expression of non-glycosylated Pfs48/45 has been achieved by co-expression with PNGase, a deglycosylating enzyme, in plants with intact conformational-dependent epitopes responsible for mediating TBA, even though efficacy wasn't reported [517]. Therefore, determining whether N-glycosylation site substitution had an effect on expression, immunogenicity and efficacy [as the mammalian system used for viral-vector vaccine testing allows for N-glycosylation] was imperative. Hence from the two versions of Pfs48/45, it is evident that there are differences in expression, immunogenicity and TB profile of these two constructs. Milek et al. (1998) additionally observed non-reactivity of antibodies raised against the glycosylated product to native antigen and the lack of recognition of native antigen by antibodies against Pfs48/45$_{\text{-NGln}}$ provides some evidence that the protein is in an incorrect conformation and that TBA in this case, is dependent on native conformation [490]. Differential TBA against Pfs48/45 variants observed in this study could be due to variations in field isolates but this needs to be explored, looking at whether polymorphisms exist in the different circulating isolates tested. However, the Pfs25 gene in this population of parasites has been shown to be conserved irrespective of the parasite diversity [460]. More so, antibodies against P25 are able to mediate TBA despite evidence of parasite diversity, [384, 460].

It is pertinent to determine whether vaccine-induced responses are long-lasting, as for any TBV to be successful in effectively reducing transmission in an endemic areas, antibodies induced need to persist at least through the transmission season, which varies in different regions of sub-Saharan Africa where *P. falciparum* malaria is most prevalent, but typically lasts between 5-7months [518-520]. A prolonged vaccine-induced antibody response using

this delivery system has been demonstrated for all the antigens without the need for a second booster shot. Microparticle delivery of rAnAPN1 has also been shown to induce long-term antibody responses (6months post-prime) with the highest responses observed with incomplete Freund's adjuvant [495]. Responses in other pre-clinical animal models against Pfs25 and Pfs48/45 have been shown to be maintained 16 and 7months post-booster shots respectively [453, 504]. However, Pfs25 is a poor immunogen and therefore to improve immunogenicity, it has been conjugated to the outer-membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B inducing TB antibodies [453]. This immunogen, Pfs25-OMPC, resulted in antibody responses in rhesus monkeys that were nonetheless maintained. In comparison, Wu et al. (2006) further showed that administration with Montanide ISA-51 in rhesus monkeys induced antibody responses that declined, required administration of a second booster shot to reach Pfs25-OMPC, and diminished to about half the peak responses after 6months of follow up in mice [453]. On the contrary, Pfs25 antibody responses in this study have been maintained for over 10months with only a ChAd63-MVA prime-boost regimen.

The acquisition of antibodies to gametocyte and/or gamete antigens after natural infection [even after a single infection [170, 181]] is an added advantage for enhancing vaccine-induced responses to TBVs due to the potential to either boosting these responses and/or augmenting natural transmission-blocking responses. Theoretically, vaccine-induced responses to antigens such as Pfs230 and Pfs48/45 can be boosted by natural infection. However, this long-held assertion requires further experimental verification with the only evidence observed with the use of a DNA vaccine encoding Pbs48/45 showing boostability after two *P. berghei* infections [521]. Moreover, there is evidence that gametocyte/gamete exposure over time leads to boostability of responses after repeated parasite challenge [522].

However, it still remains to be validated whether: (a) other regimes that can induce equal or better responses; (b) vaccine-induced responses against other gametocyte/gamete antigens would be boosted after infection; and (c) responses induced after infection would themselves be boosted by vaccination. It would be ideal to elicit not only antibodies but also an effective memory B-cell pool and long lived plasma cells that would be able to provide a sustained and effective antibody circulation. Evidence of CD4 T-cell involvement in the induction of antibodies against Pfs48/45 could provide a mechanism for longevity of the antibody response and the use of viral-vectors that are able to induce T-cells would thus help sustain this response [192-195]. However, there is also evidence that long-term antibody responses can be induced independently of T-cells [523].

There is weak evidence that antibodies raised against full-length AnAPN1 are able to inhibit parasite development even though there's evidence of induction of antigen-specific antibodies using viral-vectors. There has been no evidence published to date of complete inhibitory effects against either *P. berghei* or *P. falciparum* using rAnAPN1 with human-compatible adjuvants [348, 349, 495]. Thus there is possibility that antibodies raised to different forms of the antigen might be able to completely inhibit parasite development in a human-compatible delivery system. Therefore, it would be essential to ascertain whether vaccine-induced antibodies from viral-vectored delivery system would have differential impact on parasite infectivity against rAnAPN1 and $AnAPN1_{fl}$. This would validate the use of this antigen as a TBV candidate due to effect on diverse *Plasmodium* species in different anopheline species.

This chapter has described the use of viral-vectors as a delivery platform for inducing antibodies to candidate TBV antigens. Vaccine-induced antibodies against $AnAPN1_{fl}$, $Pfs48/45_{-NGln}$, and $Pfs48/45_{+NGln}$ have exhibited variable but incomplete TBA. In contrast,

Pfs230-C and Pfs25 have consistently shown high-level (>95%) impact on parasite infectivity using lab and field parasites in two of the most prominent mosquito vectors. This level of efficacy observed against field isolates is encouraging for the further development of these two candidate antigens due to recently described evidence of clonality and multiplicity of infection (MOI) in this population of parasites [460]. Da et al. (2013) have reported a high parasite diversity with a high rate of MOI compared to parasites in Thailand [460], in the specific region of Burkina Faso where the TBV candidate antigens in this study were tested.

Efficacy against field isolates has been observed depending on the IgG concentration and the mean oocyst intensity and number of infected mosquitoes in the control group. Churcher et al. (2012) have recently re-evaluated the relationship between infection intensity and prevalence in relation to the control [378]. According to this re-analysis, variations in the mean parasite intensity in the control has an impact on an interventions' ability to inhibit prevalence of infection. Therefore, the ability to assess all the candidate antigens in the same experiment has allowed an effective comparison as each comparator had the same parasite exposure. This comparative assessment is novel, testing against both lab and field isolates, and is a demonstration that such an approach be adopted for the screening of potential TBV candidate antigens as the measurement of vaccine efficacy is highly dependent on the levels and number of infection in the control [360, 378].

Summary of overall findings:

- ChAd63-MVA heterologous prime-boost regimen was able to induce antigen-specific antibodies against leading TBV candidate antigens that were consistently prime-boosted in a number of experiments. Therefore this human-compatible delivery platform proved useful for the expression of antibody-inducing vaccines.
- A hierarchy based on inhibition of parasite infectivity was evident against a homologous parasite strain in *A. stephensi*: Pfs230≥Pfs25>Pfs48/45$_{+NGln}$.
- The observed hierarchy in lab conditions was replicated in representative field conditions with antibodies against Pfs230-C and Pfs25 mediating a concentration-dependent inhibition of parasite infectivity in *A. gambiae*.
- N-glycosylation site substitution was observed to influence the immunogenicity and in turn efficacy of Pfs48/45 with the wild type being superior to the mutant.

5. Narrowing Down Candidate Antigens: Evaluation of Antigen Combination Approaches

5.1. Introduction

Results from the comparative screening of antibodies induced against leading TBV candidate antigens (**Chapter 4**), revealed a hierarchy based on efficacy with antibodies to Pfs230-C, Pfs25, and Pfs48/45$_{+NGln}$. Thus it was pertinent to narrow down to at least two candidate antigens by evaluating antigen combination strategies. There is growing evidence that subunit vaccines can be developed to target multiple candidate antigens of the same [249, 267, 524-531] and/or different [423, 424, 532-536] stages of the parasites life cycle; more than one serotype [537, 538]; and more than one pathogen [539-541]. This approach allows for induction of possibly additive and/or synergistic responses that target the susceptible organism(s) and for the possibility of overcoming antigenic variability.

The evidence for the possibility of a multi-antigen and multi-stage subunit malaria vaccine approach involved a fusion of seven antigens including Pfs25 expressed in the poxviruses, NYVAC and ALVAC, referred to as Pf7 [423, 424, 532]. In non-human primates, antibody responses were demonstrated to be induced to four of the antigens including Pfs25 [424]. Tine et al. (1996) further showed that all the individual antigens were expressed *in vitro* and vaccine-induced responses were able to cross-react with native parasite surface antigen [424]. In humans, there was a differential induction of antibody responses, depending on the antigen, with only 30% of the vaccinees responding to six out of the seven antigens when administered at a high dose [423]. Upon homologous parasite challenge, only one individual was protected with evidence of delay in the other vaccinees compared to the control group.

On the contrary, it is argued that these multi-antigen and/or multi-stage approaches for malaria are more likely to be more efficacious than single antigen approaches due to the

repertoire of vaccine-induced responses [542]. There are a number of studies that demonstrate combination of liver- and blood-stage antigens either co-administered or as fusion vaccines [138, 438, 532, 534, 543-549]. However, in some cases, especially when vaccines were co-administered, there was evidence of either immune interference and/or antigen competition from immunodominant antigens masking vaccine-induced responses to the less dominant antigens [138, 544-546]. Thus, for the selection and narrowing down of potential TBV candidates, it would be pertinent to determine whether co-administration or fusion of candidate antigens has an impact on antibody responses and in turn efficacy.

The only published evidence of transmission-blocking antigen combinations has been with P25 and P28 [529-531]. Gozar et al. (1998) were able to demonstrate that a protein-fusion vaccine construct expressed in yeast, with the antigens separated by a glycine-proline (GP) linker, induced antibodies, in mice, that had inhibitory effects against *P. falciparum* infectivity to *A. freeborni* [529]. They further went on to demonstrate that the same was true when antibodies were raised in rabbits [530]. The use of a GP linker sequence is reported to allow individual antigens within a fusion construct to maintain conformation which is vital for antigen-specific conformation-dependent responses [529, 530, 550]. However, it is yet to be demonstrated whether any other TBV combination can induce transmission-blocking antibodies expressed as a glycine-proline linker fusion antigen.

An alternative approach for co-expression to GP linker sequences is expression of multiple proteins using self-cleaving peptides. Self-cleaving peptides were first described in foot-and-mouth disease virus (FMDV) [551, 552] and have since been described in other viruses, and the parasite *Trypanosoma* [553-555]. These have been used extensively in a number of applications and in a number of expression systems (reviewed in [556-558]). Self-cleaving peptides allow multiple proteins encoded as a single transcript to be expressed as individual

proteins [558]. FMDV encodes a 19αα self-cleaving protease, referred to as 2A (FMDV-2A (F2A), the most widely used self-cleaving peptide), which self-cleaves at the C-terminus between the last two amino acids, glycine and proline residues [551, 552, 559]. The mechanism of cleavage occurs in the ribosome during translation via a ribosomal skip over, between the glycine and proline residues, resulting in translation of the downstream transcript [560-562].

A number of studies have used 2A or 2A-like sequences to express antigens inducing protective responses [541, 563-566]. These studies have either expressed two or more proteins. Mir et al. (2009) have demonstrated comparable expression profiles between single and triple antigen constructs, in a DNA vaccine, at both transcript and protein levels [564]. On the other hand, Li et al. (2009) demonstrated comparable viral neutralising antibody responses induced with recombinant HuAd5 expressing glycoprotein 3 and 5 of porcine reproductive and respiratory syndrome virus fused to heat shock protein 70 from *Heamophilus parasuis* either by GP linker or F2A peptide [541]. Thus it would be important to determine whether the most efficacious TBV antigen combinations could be expressed in ChAd63 and MVA. It would also be ideal to determine whether expressing multiple antigens using F2A would be comparable to a fusion construct (separated by a GP linker) in terms of inducing antibodies with transmission-blocking potential. These comparisons would enable advancement of the most efficacious platform for a combination TBV approach.

This chapter explored the possibility of narrowing down three of the most efficacious TBV candidate antigens for potential evaluation as clinical candidates. Vaccine-induced responses to Pfs230-C, Pfs25 and Pfs48/45$_{+NGln}$ were previously demonstrated to be potent at inhibiting infectivity of *P. falciparum* NF54 to *A. stephensi*, and thus were taken forward for further testing. Therefore, it was important to down select these candidate antigens to achieve

optimal efficacy; test whether combinations of the targets might be feasible; and also lead to the advancement of the best candidate antigen(s) or antigen combination for possible clinical testing. Thus this chapter, primarily demonstrates, the possibility of: (a) additivity and/or synergism of vaccine-induced antibodies in SMFA; (b) immune interference as a result of co-administration; and (c) testing antigen fusion combination(s) based on (a) and (b).

5.2. Results

5.2.1. Assessment of efficacy: combining IgG in SMFA

The potential combined effects of Pfs25 and Pfs28 were initially informed from experiments mixing anti-sera in SMFA [328]. Duffy et al. (1997) were able to show that mixing anti-Pfs25 and anti-Pfs28 antibodies in a membrane feeder resulted in greater inhibition of parasite infectivity compared to individual anti-sera [328]. Thus to assess whether antibodies to either Pfs230-C, Pfs25, and/or Pfs48/45$_{+NGln}$ would exhibit additive and/or synergistic inhibitory effects against parasite infectivity, total IgG (**4.2.2.1**) was either tested singly or mixed, at varying concentrations in SMFA (**Figure 5.1**).

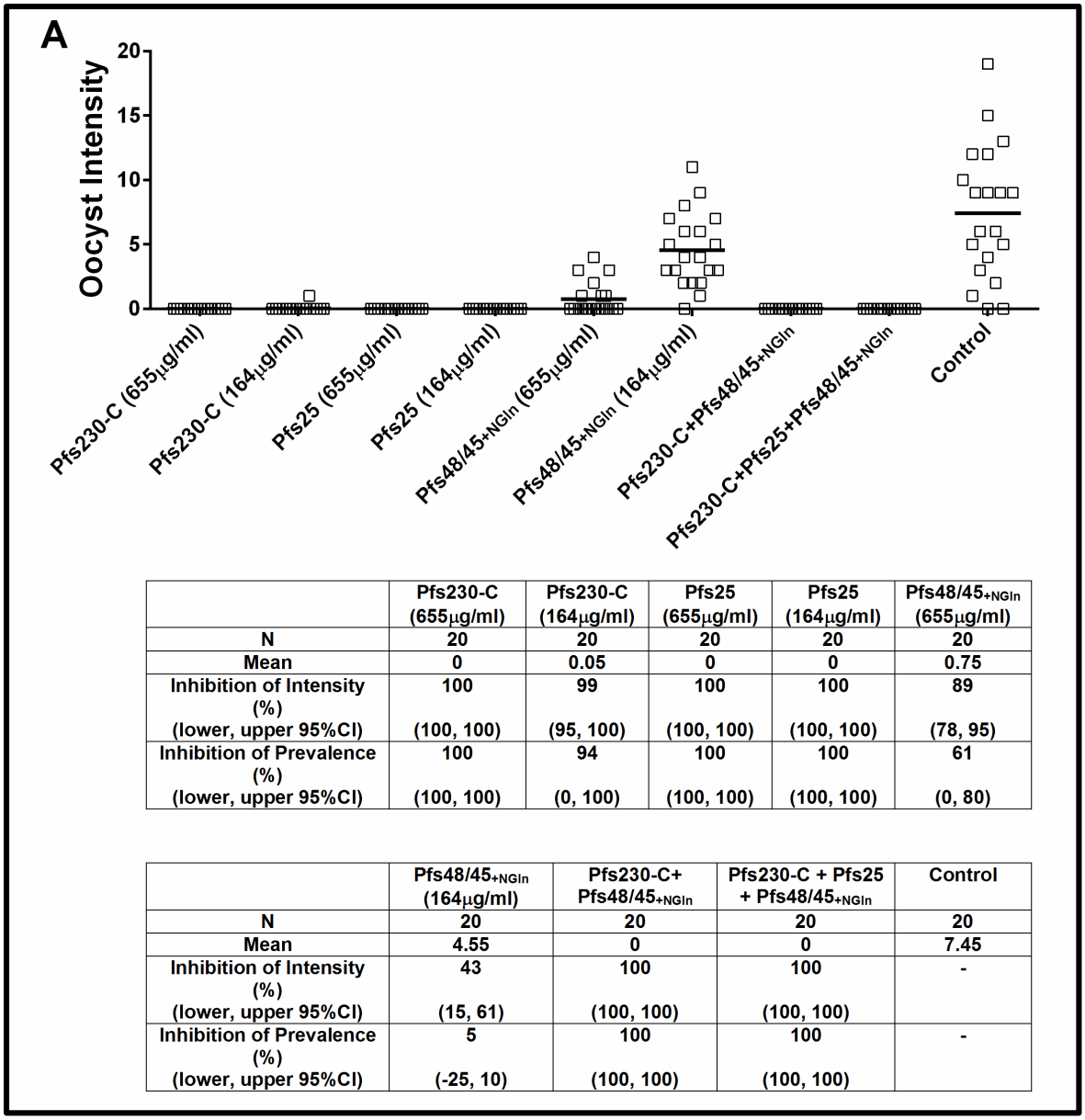
As previously observed [at 655μg/ml of IgG] (**Figure 4.9**), anti-Pfs230-C and anti-Pfs25 IgG gave complete TBE (100%, $p<0.0001$); whilst anti-Pfs48/45$_{+NGln}$ IgG was insufficient at completely inhibiting parasite intensity and prevalence (89% and 61% respectively, $p<0.0001$) (**Figure 5.1A**). At 164μg/ml, anti-Pfs230-C IgG almost completely inhibited parasite intensity and prevalence (99% and 94% respectively, $p<0.0001$) whilst anti-Pfs25 IgG had complete TBE (100%, $p<0.0001$). For anti-Pfs48/45$_{+NGln}$ IgG at this concentration, only inhibition of parasite intensity was statistically significantly (reduced to about half, 43%, $p=0.009$) with no observed effect on infection prevalence. Due to the expression of Pfs230 and Pfs48/45 on the same developmental stage, the respective total IgGs were mixed at

164μg/ml, to test any evidence of additive and/or synergistic effects. This resulted in complete inhibition of parasite intensity and prevalence (100%, p<0.0001). However, it wasn't evident whether anti-Pfs48/45$_{+NGln}$ IgG had any synergistic role due to the potent inhibition exhibited by anti-Pfs230-C IgG at this IgG concentration in this experiment. The same was true when anti-Pfs230-C, anti-Pfs25, and anti-Pfs48/45$_{+NGln}$ IgG were all mixed at the same concentration (**Figure 5.1A**).

To determine possible additive and/or synergistic effects, respective total IgG were tested at a lower IgG concentrations both singly and in combination (**Figure 5.1B**). In this experiment, mixing of anti-Pfs230-C and anti-Pfs48/45$_{+NGln}$ IgG exhibited possible synergistic effects at inhibiting parasite intensity (75%, p=0.0001) whilst there was no synergistic effect at inhibiting parasite prevalence. However, anti-Pfs48/45$_{+NGln}$ IgG singly didn't have any detectable statistically significant effect at inhibiting parasite intensity. Combination of anti-Pfs25 and anti-Pfs48/45$_{+NGln}$ IgG had a possible sub-additive effect on parasite intensity (85% vs. 94%, p<0.0001). On the other hand, mixing anti-Pfs230-C and anti-Pfs25 IgG or all three anti-IgGs together had the same effect on parasite intensity as when anti-Pfs25 IgG was individually tested.

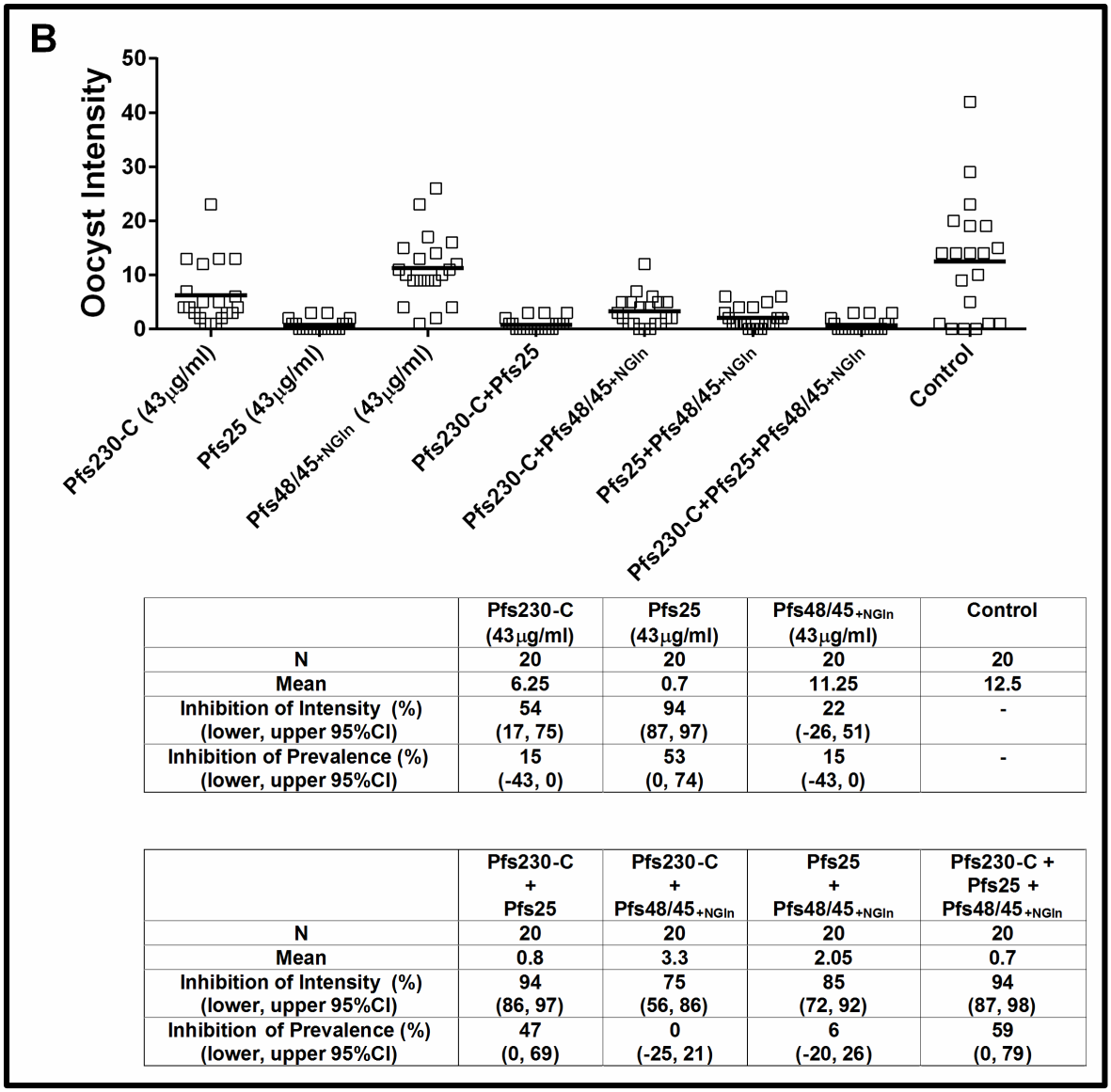
To adequately distinguish any additivity and/or synergism, the concentration of anti-Pfs25 IgG required to give an inhibitory effect of 50% (IC_{50}) was evaluated (**Figure 5.1C**). In this experiment, the IC_{50} was estimated using a nonlinear regression analysis (with robust fit). The relative IC_{50} concentration was determined to be ~8.75μg/ml. In the mixing experiment, anti-Pfs230-C and anti-Pfs48/45$_{+NGln}$ IgG were tested at 43μg/ml and 164μg/ml concentrations respectively (~50% observed inhibition of intensity, **Figure 5.1A and B**). However, in this experiment at these IgG concentrations, anti-Pfs25 and anti-Pfs48/45$_{+NGln}$ IgG, used singly, didn't statistically significantly inhibit intensity or prevalence whilst anti-

Pfs230-C IgG had a higher observed inhibition of intensity (77%, p=0.0003) than expected (**Figure 5.1D**). On the other hand, combining anti-Pfs25 and anti-Pfs48/45$_{+NGln}$ IgG appeared to have an additive effect at inhibiting parasite intensity (however this wasn't statistically significant) with no observed effect at inhibiting parasite prevalence. Combination of anti-Pfs230-C and anti-Pfs25 IgG had a synergistic effect at inhibiting both parasite intensity (82%, p<0.0001) and prevalence (35%, p=0.001) (**Figure 5.1D**). When anti-Pfs230-C and anti-Pfs48/45$_{+NGln}$ IgGs were mixed, a sub-additive effect on parasite intensity and prevalence (64%, p<0.0001 and 20%, p=0.01 respectively) was observed whilst when all the three IgGs were mixed, the effect on inhibition intensity mirrored that observed with the individual effect of anti-Pfs230-C IgG (79% vs. 77%, p<0.0001). It thus appeared that when all IgGs were mixed in all the experiments, the effect on parasite infectivity was likely attributed to the most potent.



	Pfs230-C (655µg/ml)	Pfs230-C (164µg/ml)	Pfs25 (655µg/ml)	Pfs25 (164µg/ml)	Pfs48/45+NGln (655µg/ml)
N	20	20	20	20	20
Mean	0	0.05	0	0	0.75
Inhibition of Intensity (%) (lower, upper 95%CI)	100 (100, 100)	99 (95, 100)	100 (100, 100)	100 (100, 100)	89 (78, 95)
Inhibition of Prevalence (%) (lower, upper 95%CI)	100 (100, 100)	94 (0, 100)	100 (100, 100)	100 (100, 100)	61 (0, 80)

	Pfs48/45+NGln (164µg/ml)	Pfs230-C+ Pfs48/45+NGln	Pfs230-C + Pfs25 + Pfs48/45+NGln	Control
N	20	20	20	20
Mean	4.55	0	0	7.45
Inhibition of Intensity (%) (lower, upper 95%CI)	43 (15, 61)	100 (100, 100)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95%CI)	5 (-25, 10)	100 (100, 100)	100 (100, 100)	-



	Pfs230-C (43μg/ml)	Pfs25 (43μg/ml)	Pfs48/45+NGln (43μg/ml)	Control
N	20	20	20	20
Mean	6.25	0.7	11.25	12.5
Inhibition of Intensity (%) (lower, upper 95%CI)	54 (17, 75)	94 (87, 97)	22 (-26, 51)	-
Inhibition of Prevalence (%) (lower, upper 95%CI)	15 (-43, 0)	53 (0, 74)	15 (-43, 0)	-

	Pfs230-C + Pfs25	Pfs230-C + Pfs48/45+NGln	Pfs25 + Pfs48/45+NGln	Pfs230-C + Pfs25 + Pfs48/45+NGln
N	20	20	20	20
Mean	0.8	3.3	2.05	0.7
Inhibition of Intensity (%) (lower, upper 95%CI)	94 (86, 97)	75 (56, 86)	85 (72, 92)	94 (87, 98)
Inhibition of Prevalence (%) (lower, upper 95%CI)	47 (0, 69)	0 (-25, 21)	6 (-20, 26)	59 (0, 79)

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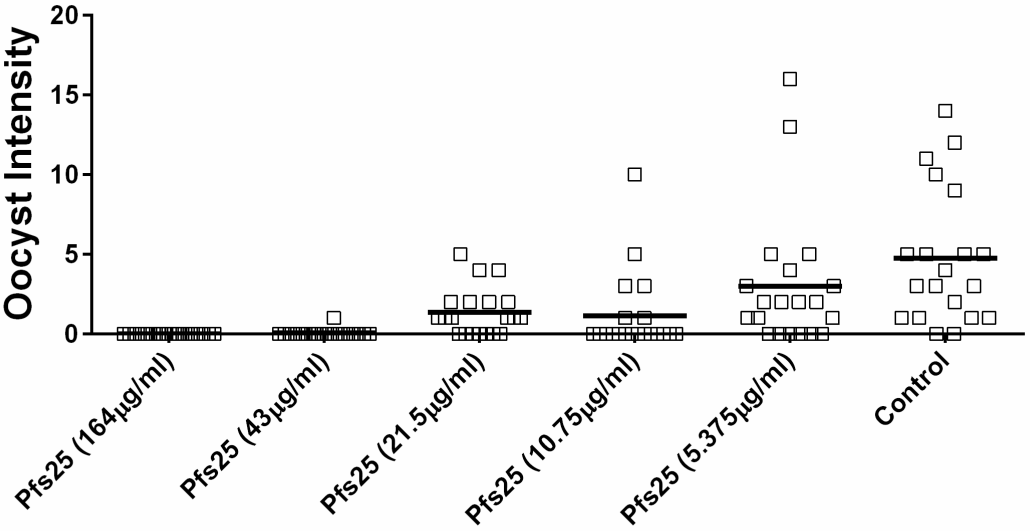
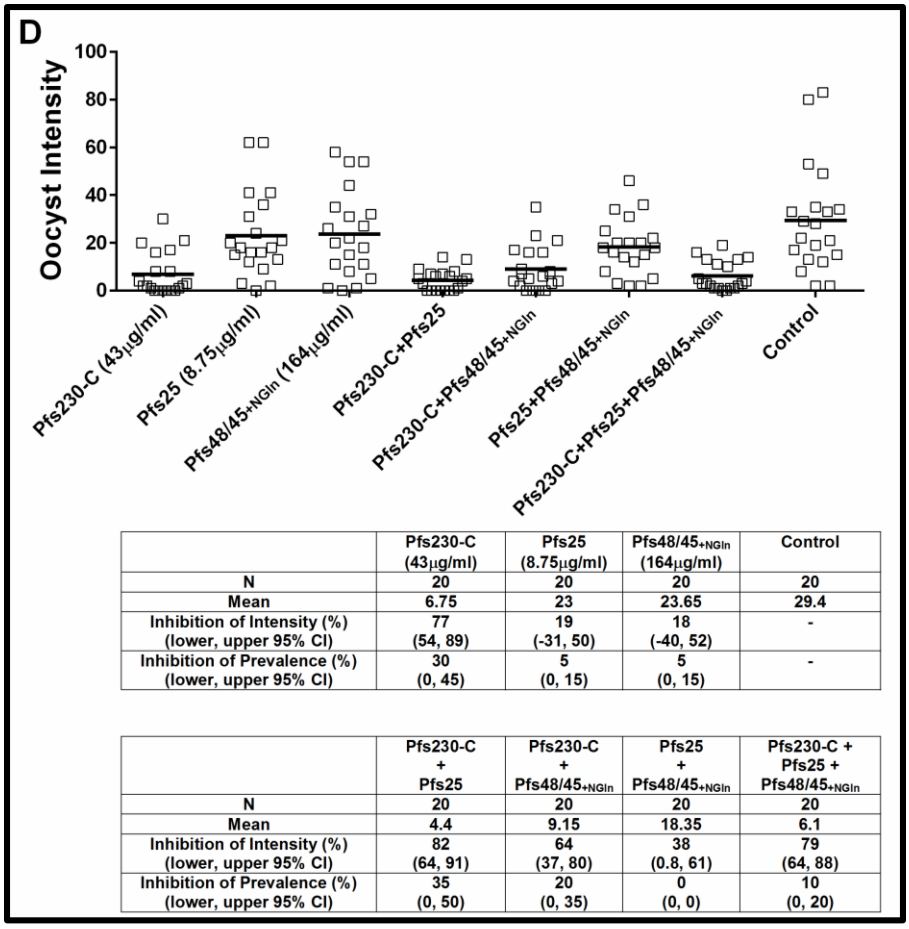


	Pfs25 (164µg/ml)	Pfs25 (43µg/ml)	Pfs25 (21.5µg/ml)
N	20	20	20
Mean	0	0.05	1.35
Inhibition of Intensity (%) (lower, upper 95%CI)	100 (100, 100)	99 (92, 100)	72 (46, 85)
Inhibition of Prevalence (%) (lower, upper 95%CI)	100 (100, 100)	94 (0, 100)	28 (0, 50)

	Pfs25 (10.75µg/ml)	Pfs25 (5.375µg/ml)	Control
N	20	20	20
Mean	1.15	3	4.75
Inhibition of Intensity (%) (lower, upper 95%CI)	76 (44, 90)	37 (-25, 68)	-
Inhibition of Prevalence (%) (lower, upper 95%CI)	67 (0, 84)	22 (0, 42)	-



	Pfs230-C (43μg/ml)	Pfs25 (8.75μg/ml)	Pfs48/45+NGln (164μg/ml)	Control
N	20	20	20	20
Mean	6.75	23	23.65	29.4
Inhibition of Intensity (%) (lower, upper 95% CI)	77 (54, 89)	19 (-31, 50)	18 (-40, 52)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	30 (0, 45)	5 (0, 15)	5 (0, 15)	-

	Pfs230-C + Pfs25	Pfs230-C + Pfs48/45+NGln	Pfs25 + Pfs48/45+NGln	Pfs230-C + Pfs25 + Pfs48/45+NGln
N	20	20	20	20
Mean	4.4	9.15	18.35	6.1
Inhibition of Intensity (%) (lower, upper 95% CI)	82 (64, 91)	64 (37, 80)	38 (0.8, 61)	79 (64, 88)
Inhibition of Prevalence (%) (lower, upper 95% CI)	35 (0, 50)	20 (0, 35)	0 (0, 0)	10 (0, 20)

Figure 5.1. Combination of anti-Pfs230-C, anti-Pfs25 and anti-Pfs48/45+NGln IgG.

Total IgG (3.5mg/ml) from pooled sera (day70) from vaccinated mice with each respective antigen or GFP (control) (**4.2.2.1.**) was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* in SMFA. The total IgG was diluted in PBS at varying concentrations either shown in parenthesis or as follows: (**A**) mixing at 164μg/ml for each respective antigen; (**B**) all diluted at 43μg/ml and mixed either singly or in combination; and, (**D**) mixing at the respective concentrations shown for each individual antigen. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95%CI. Squares represent oocyst numbers from each individual mosquito dissected.

5.2.2. ChAd63-MVA prime-boost vaccine combinations

Due to the difficulty of predicting efficacy [with a certain IgG concentration] in SMFA and to test whether the different antigens work together in synergy, the possibility of co-administering the vaccines was investigated. This was done to evaluate whether co-administration of the vaccines would have an impact on antibody responses which could impact efficacy. A number of studies have tested the co-administration of heterologous viral-vectors expressing a single antigen [420, 567] or viral-vectors expressing two different antigens [549] in the same syringe. On the other hand, Sheehy et al. (2012) have evaluated co-administering viral-vectors expressing MSP1 with either those expressing AMA1 or ME-TRAP in separate sites in clinical trials and observed immune interference due to dominant MSP1 T-cell and antibody responses [138]. However, assessment of either mixing viral-vectors in the same syringe or co-administering in separate sites hasn't been addressed to determine impact on antibody responses. In addition to determining the impact of co-administration on antibody responses, the impact on the induction of antigen-specific antibody-secreting cell (ASC) responses were investigated. Induction of antigen-specific ASCs have been shown to be necessary for sustaining antibody responses [568-570]. ASCs have been shown to migrate from the spleen to the bone marrow after the peak response [570, 571]. Thus their presence in the spleen was determined at the peak of the antibody response.

Vaccinations with different antigen combinations were administered either as mixtures in the same syringe or individually in separate sites (**Table 5.1**). To determine the possibility of immune interference, comparisons were made between method of administration (separate sites vs. same syringe); and effect of antigen (antigen vs. control). To detect statistically significant differences in the antibody responses within groups across time points (boosting of responses), multiple *t*-tests with Holm-Sidak multiple comparisons post-test were

performed. For differences between groups within time points, one-way ANOVA with Holm-Sidak's multiple comparison post-test was performed. To detect any differences in the ASC responses between the groups, either Kruskal-Wallis test with Dunn's multiple comparisons post-test was performed whilst association with antibody responses was determined by Spearman's rank correlation test (r_s); or One-way ANOVA with Holm-Sidak's multiple comparison post-test whilst association with antibody responses was determined by Pearson's correlation test (r).

Table 5.1. ChAd63-MVA prime-boost vaccine combinations.

Combinations	Prime	Boost
Pfs230-C and Pfs25	ChAd63-Pfs230-C (/+)	MVA-Pfs230-C (/+)
	ChAd63-Pfs25	MVA-Pfs25
Pfs230-C and Pfs48/45$_{+NGln}$	ChAd63-Pfs230-C (/+)	MVA-Pfs230-C (/+)
	ChAd63-Pfs48/45$_{+NGln}$	MVA-Pfs48/45$_{+NGln}$
Pfs25 and Pfs48/45$_{+NGln}$	ChAd63-Pfs25 (/+)	MVA-Pfs25 (/+)
	ChAd63-Pfs48/45$_{+NGln}$	MVA-Pfs48/45$_{+NGln}$
Pfs230-C and control	ChAd63-Pfs230-C (/+)	MVA-Pfs230-C (/+)
	ChAd63-VC	MVA-VC
Pfs25 and control	ChAd63- Pfs25 (/+)	MVA- Pfs25 (/+)
	ChAd63-VC	MVA-VC
Pfs48/45$_{+NGln}$ and control	ChAd63-Pfs48/45$_{+NGln}$ (/+)	MVA-Pfs48/45$_{+NGln}$ (/+)
	ChAd63-VC	MVA-VC

BALB/c mice were vaccinated i.m. with the same dose of virus for each individual viral-vector ($1x10^8$IU and $1x10^7$PFU of ChAd63 and MVA respectively) in a total volume of 50µl, 25µl in each quadricep. To control for viral load, each individual antigen had a vaccination administered with viral-vectors expressing GFP (viral-vector control (VC)). (/+) indicates whether vaccines were administered in separate sites (/) or mixed in the same syringe (+).

5.2.2.1. Pfs230-C and Pfs25 co-administration

Age-matched BALB/c mice were primed with ChAd63 and eight weeks later boosted with MVA with combinations of Pfs230-C and Pfs25 either administered in separate sites or mixed in the same syringe (**Table 5.1**). Control combinations involved prime-boost with ChAd63-MVA expressing the respective antigen and GFP (VC). Antigen-specific vaccine-induced total IgG was assessed on days 14, 55 and 70 by standardised ELISA. There was significant boosting, regardless of the regimen, of Pfs230-C antibody responses in all groups after priming at varying magnitudes (p<0.0001, **Figure 5.2A**). Ditto, Pfs25 antibody responses were boosted in all groups also at varying magnitudes (p=0.006 for Pfs25/VC group and p<0.0001 for all the other groups, **Figure 5.2B**).

However, Pfs230-C IgG responses at day14 post-prime were remarkably reduced when co-administered with Pfs25 in the same syringe (p<0.0001, 73-fold lower, Pfs230-C+VC vs. Pfs230-C+Pfs25, **Figure 5.2A**). The downstream effect was a 49-fold significant difference detected by co-administering Pfs25 in separate sites (p<0.0001, Pfs230-C/Pfs25 vs. Pfs230-C+Pfs25). On the other hand, vaccination in separate sites at this time point was observed to give significantly better responses even in the presence of VC (p<0.001, 1.5-fold higher, Pfs230-C/VC vs. Pfs230-C+VC,). This phenomenon was however not observed either at day55 or day70. Thus, co-administration of Pfs230-C with Pfs25 had an effect on anti-Pfs230-C responses when the two were co-administered in the same syringe in comparison to separate sites only at day14 post-prime.

IgG responses to Pfs25 at all time points were significantly higher when co-administered with Pfs230-C in separate sites (Pfs25/Pfs230-C vs. Pfs25+Pfs230-C, p<0.001, 8-fold; p<0.05, 7-fold; and p<0.05, 8-fold; at days 14, 55 and 70 respectively, **Figure 5.2B**). The trend was similar with respect to VC but only up to day55 (Pfs25/VC vs. Pfs25+VC, day14 p<0.001, 9-

fold; and day55 $p<0.05$, 6-fold). Overall comparison of day14 responses revealed that co-administering Pfs25 with Pfs230-C resulted in a decrease in the magnitude of the anti-Pfs25 antibody response whether the vaccination was given in separate sites (Pfs25/VC vs. Pfs25/Pfs230-C) or mixed in the same syringe (Pfs25+VC vs. Pfs25+Pfs230-C) (3-fold and 8-fold respectively, $p<0.05$). In comparison to anti-Pfs230-C responses, vaccination by mixing in the same syringe had an impact on the anti-Pfs25 antibody priming response and thus affected the magnitude of the boosting responses.

To determine the relationship between antigen-specific ASCs and antibody responses, ELISPOT assays were performed using spleens from vaccinated mice (n=5/group) at day70 **(Figure 5.3)**. Antigen-specific ASCs were detected in all groups for both antigens with a few low responders in Pfs25 mixing groups (Pfs25+VC and Pfs25+Pfs230-C). For Pfs230-C, as observed for antibody responses at the peak time point, there was no statistically significant difference across all the groups (**Figure 5.3A**). Additionally, there was no correlation observed between the antibody and ASC responses irrespective of the group and even when the responses were pooled (**Figure 5.3B**, $r_s=0.2$, $p=0.4$). In regards to Pfs25, groups receiving vaccinations mixed in the same syringe had lower median numbers of ASCs, however, there were no statistically significant detectable differences across all the groups (**Figure 5.3C**). On the other hand, there was a strong positive correlation across all the groups between ASCs and the peak antibody responses (**Figure 5.3D**, $r_s=0.72$, $p=0.0003$).

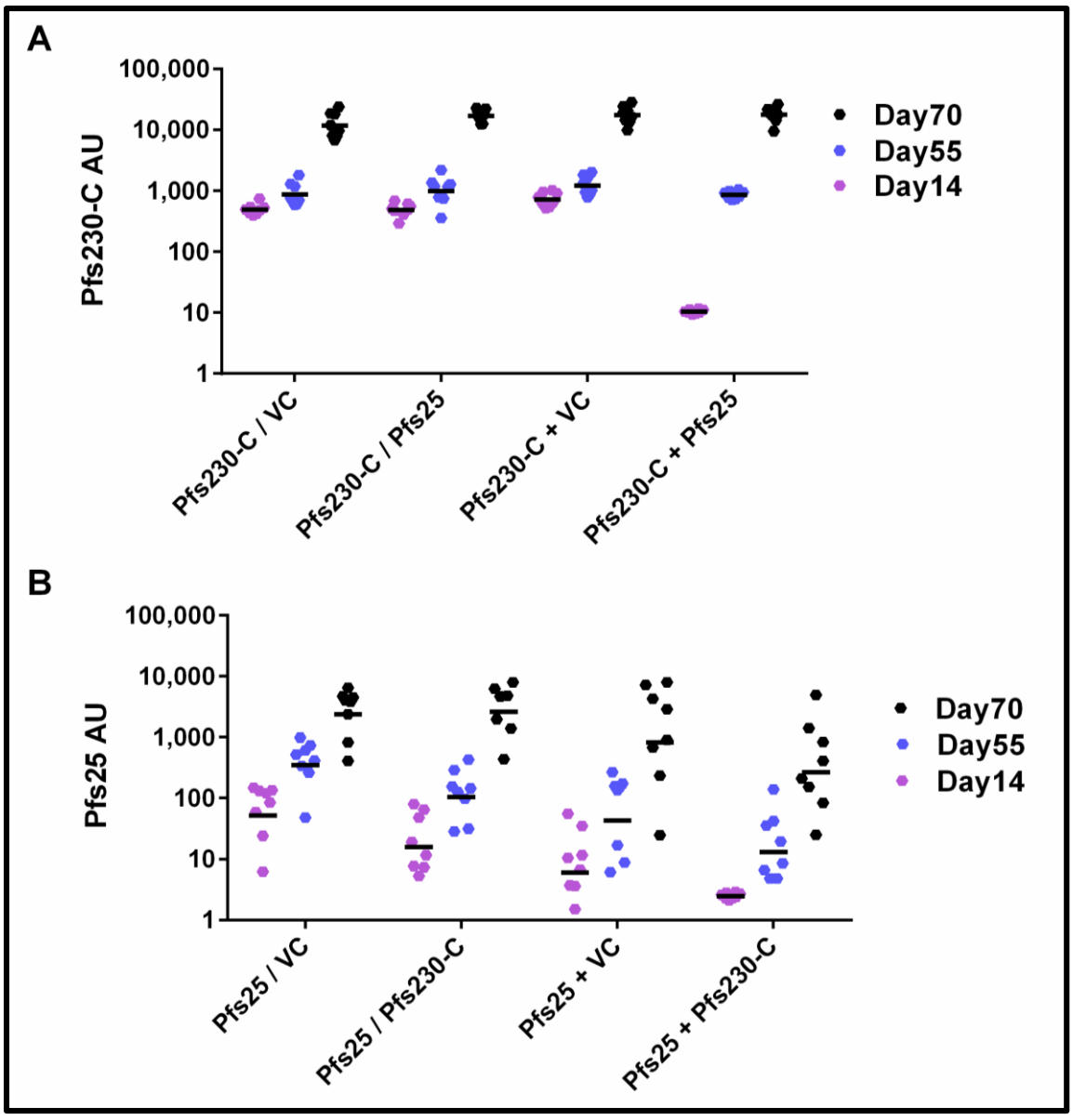
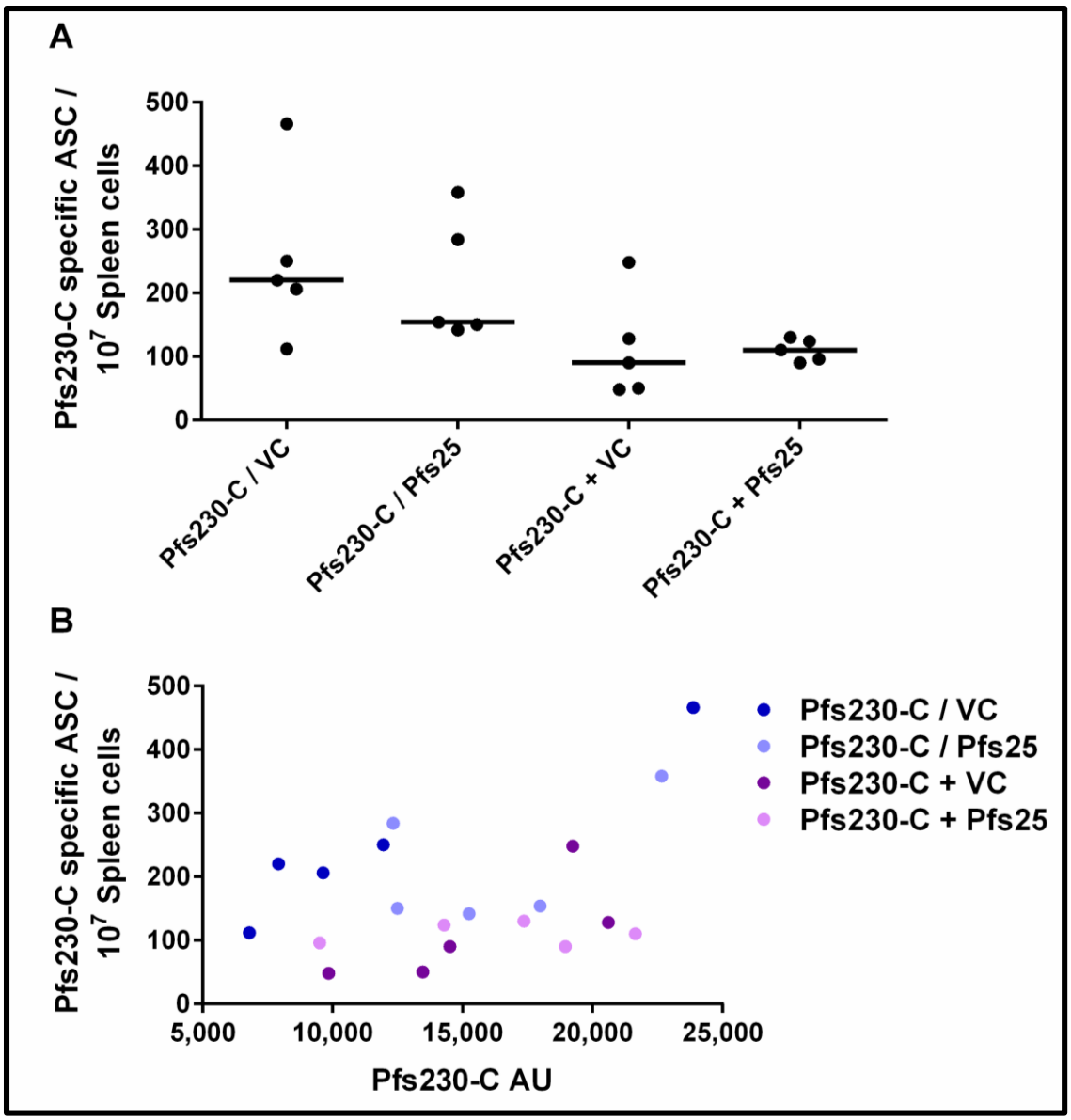


Figure 5.2. IgG responses to Pfs230-C and Pfs25 co-administration.

BALB/c mice (n=8/group) were vaccinated i.m. with ChAd63 ($1x10^8$IU, day0) and MVA ($1x10^7$PFU, day56) expressing either Pfs230-C, Pfs25 or GFP administered in separate sites (denoted by /) or mixed in the same syringe (denoted by +). IgG responses were measured on days 14, 55 and 70 by standardised ELISA using (**A**) Pfs230-C and (**B**) Pfs25 recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points per group. Dots represent responses from individual mice. VC: viral-vector control expressing GFP.


A
Pfs230-C specific ASC / 10^7 Spleen cells
500
400
300
200
100
0
Pfs230-C / VC
Pfs230-C / Pfs25
Pfs230-C + VC
Pfs230-C + Pfs25
B
Pfs230-C specific ASC / 10^7 Spleen cells
500
400
300
200
100
0
5,000
10,000
15,000
20,000
25,000
Pfs230-C AU
Pfs230-C / VC
Pfs230-C / Pfs25
Pfs230-C + VC
Pfs230-C + Pfs25

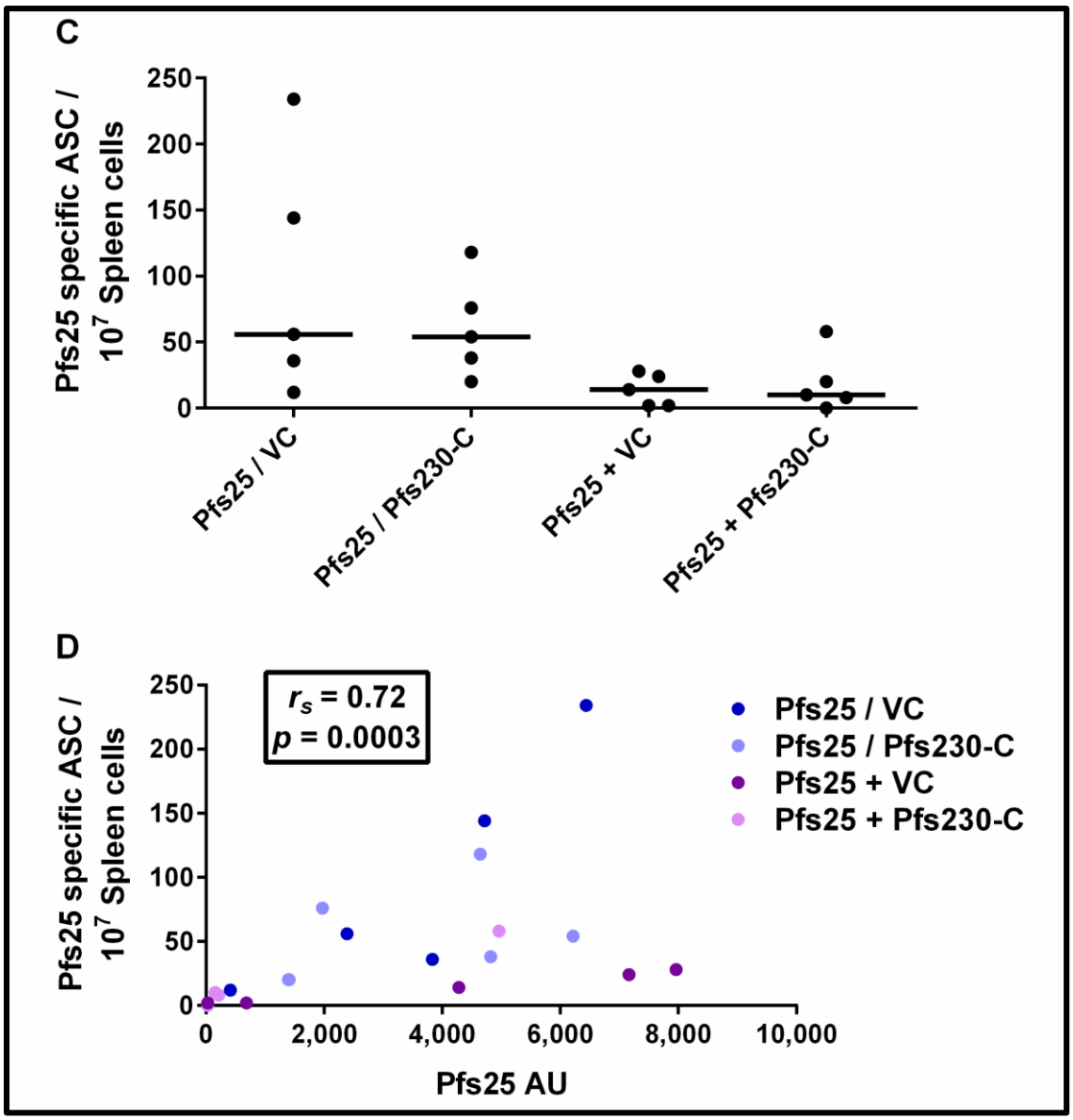


Figure 5.3. ASC responses to Pfs230-C and Pfs25 after co-administration.

BALB/c mice (n=5/group) were vaccinated as described in **Figure 5.2.** At day70, spleens from each individual mouse were used to isolate splenocytes from which ASCs specific for Pfs230-C (**A and B**) and Pfs25 (**C and D**) were measured by ELISPOT. (**A and C**) shows antigen-specific ASC responses/10^7 splenocytes; and (**B and D**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as medians and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. ASC: antibody-secreting cell; VC: viral-vector control expressing GFP.

5.2.2.2. Pfs230-C and Pfs48/45$_{+NGln}$ co-administration

Age-matched BALB/c mice were vaccinated as described in **5.2.2.1** with Pfs230-C and Pfs48/45$_{+NGln}$ combinations either administered in separate sites or mixed in the same syringe (**Table 5.1**). Control combinations involved prime-boost with the respective antigen and VC. Antigen-specific total IgG was assessed on days 14, 55 and 70 by standardised ELISA. There was significant boosting, regardless of the regimen, of antigen-specific responses in all groups after priming at varying magnitudes for both Pfs230-C and Pfs48/45$_{+NGln}$, (p<0.0001, **Figure 5.4**).

With this combination, anti-Pfs230-C IgG responses were comparable in all the groups post-prime (days 14 and 55) with no statistically significant difference detected between the method of administration and combination (**Figure 5.4A**). However, post-boost, there was an effect of co-administering with Pfs48/45$_{+NGln}$ in separate sites resulting in a two-fold lower antibody response to Pfs230-C (Pfs230-C/VC vs. Pfs230-C/Pfs48/45$_{+NGln}$, p<0.05). On the other hand, anti-Pfs48/45$_{+NGln}$ antibody responses at day14 post-prime were two-fold lower when co-administered with Pfs230-C either in the same syringe or in separate sites (, p<0.05, **Figure 5.4B**). However, at all the other time points, there was no statistically significant difference whether Pfs48/45$_{+NGln}$ was co-administered in separate sites, in the same syringe, or with either Pfs230-C or control.

To increase the statistical power of detecting any relationship between antibody and ASC responses, ELISPOT assays were performed on all vaccinated animals (n=8/group) (**Figure 5.5**). Antigen-specific ASCs against Pfs230-C were detected in all the groups irrespective of the method or the antigen used in co-administration (**Figure 5.5A**). The mean ASC responses in the group receiving Pfs230-C and control in separate sites were superior in comparison to co-administering with Pfs48/45$_{+NGln}$ (Pfs230-C/VC vs. Pfs230-C/Pfs48/45$_{+NGln}$, 3-fold higher,

p<0.0001). There was a five-fold reduction in responses when the vaccination was administered mixed in the same syringe in the presence of the control (Pfs230-C/VC vs. Pfs230-C+VC) (*p*<0.0001). Additionally, there was no association between the peak antibody and mean ASC responses when all groups were compared (r=0.09, *p*=0.6, **Figure 5.5B**). There was a trend towards a negative correlation, though not statistically significant in all groups with the exception of the Pfs230-C/Pfs48/45$_{+NGln}$ group. The antibody response was strongly associated with ASCs in this group (r=0.72, *p*=0.04).

In regards with Pfs48/45$_{+NGln}$, similar trends were observed with detectable ASCs in most of the groups and overall superior mean responses in the Pfs48/45$_{+NGln}$/VC group (**Figure 5.5C**). Moreover, groups receiving vaccinations in the same syringe, irrespective of the antigen, had comparable responses with the presence of both low and non-responders. There was a 5-fold reduction in the mean ASC response when Pfs230-C was co-administered in separate sites (Pfs48/45$_{+NGln}$/VC vs. Pfs48/45$_{+NGln}$/Pfs230-C, *p*<0.01). The magnitude was further reduced by 16-fold with regards to the method of co-administration in relation to the control (Pfs48/45$_{+NGln}$/VC vs. Pfs48/45$_{+NGln}$+VC, *p*<0.001). Overall, there was no observed association between antibody and ASC responses (r=0.19, *p*=0.3, **Figure 5.5D**). However, when the groups were individually analysed, there were strong positive associations in the Pfs48/45$_{+NGln}$/VC (r=0.83, *p*=0.01) and Pfs48/45$_{+NGln}$/Pfs230-C groups (r=0.76, *p*=0.03).

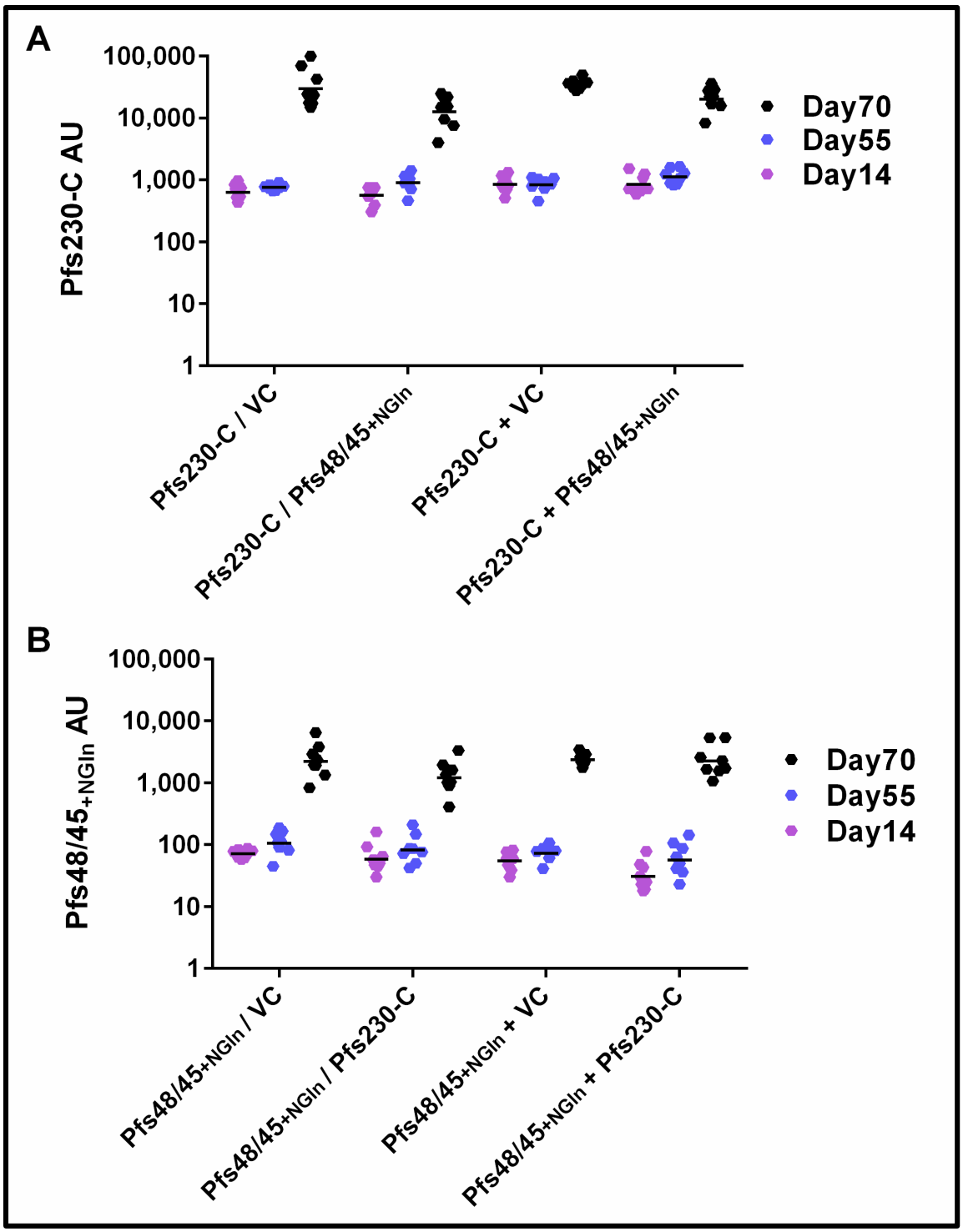
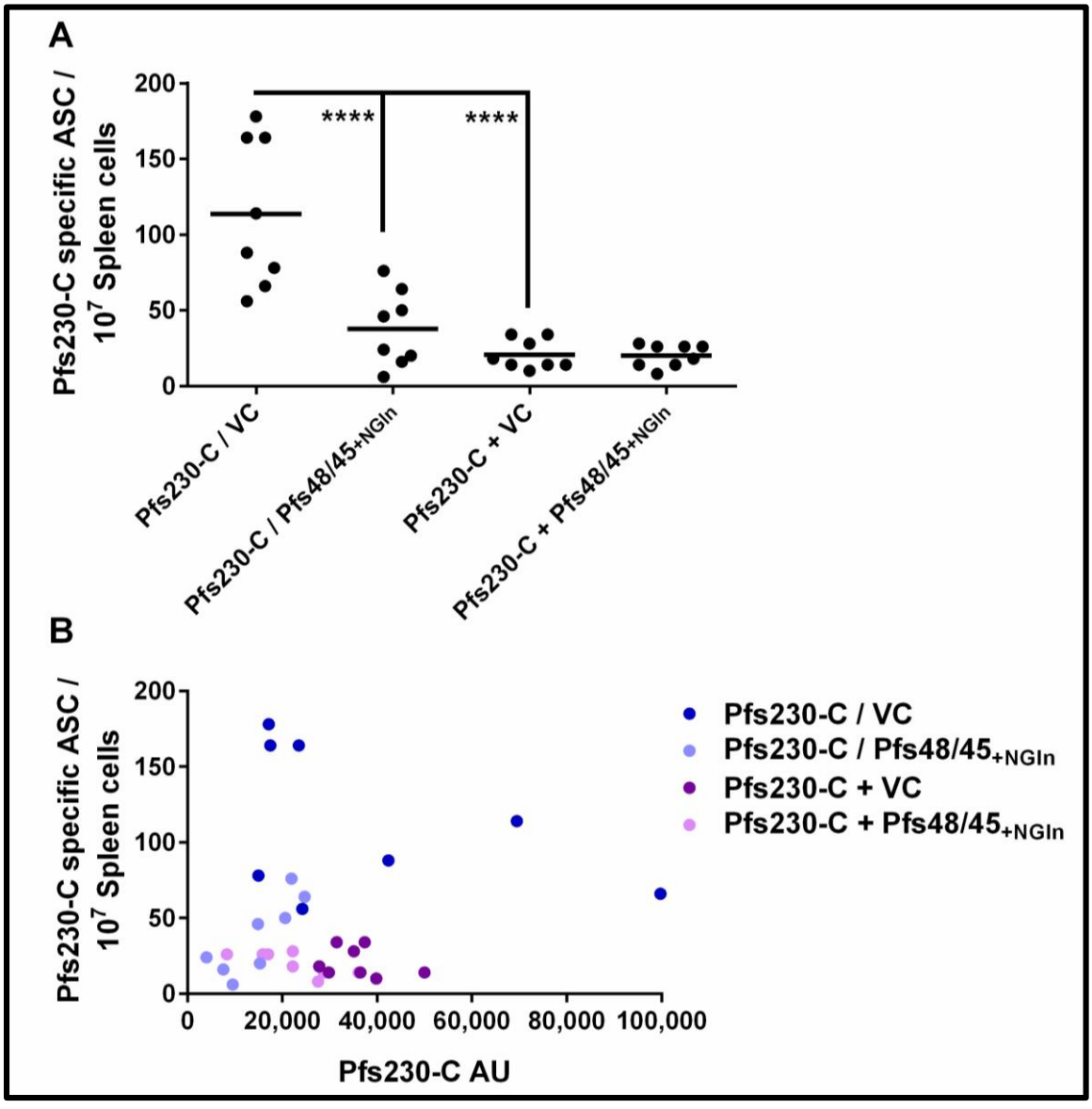

A
Pfs230-C AU
100,000
10,000
1,000
100
10
1
Day70
Day55
Day14
Pfs230-C / VC
Pfs230-C / Pfs48/45+NGln
Pfs230-C + VC
Pfs230-C + Pfs48/45+NGln
B
Pfs48/45+NGln AU
100,000
10,000
1,000
100
10
1
Day70
Day55
Day14
Pfs48/45+NGln / VC
Pfs48/45+NGln / Pfs230-C
Pfs48/45+NGln + VC
Pfs48/45+NGln + Pfs230-C

Figure 5.4. IgG responses to Pfs230-C and Pfs48/45$_{+NGln}$ co-administration.

BALB/c mice (n=8/group) were vaccinated i.m. with ChAd63 (1x10^{8}IU, day0) and MVA (1x10^{7}PFU, day56) expressing either Pfs230-C, Pfs48/45$_{+NGln}$ or GFP administered in separate sites (denoted by /) or mixed in the same syringe (denoted by +). IgG responses were measured on days 14, 55 and 70 by standardised ELISA using (**A**) Pfs230-C and (**B**) Pfs48/45$_{+NGln}$ recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points per group. Dots represent responses from individual mice. VC: viral-vector control expressing GFP.



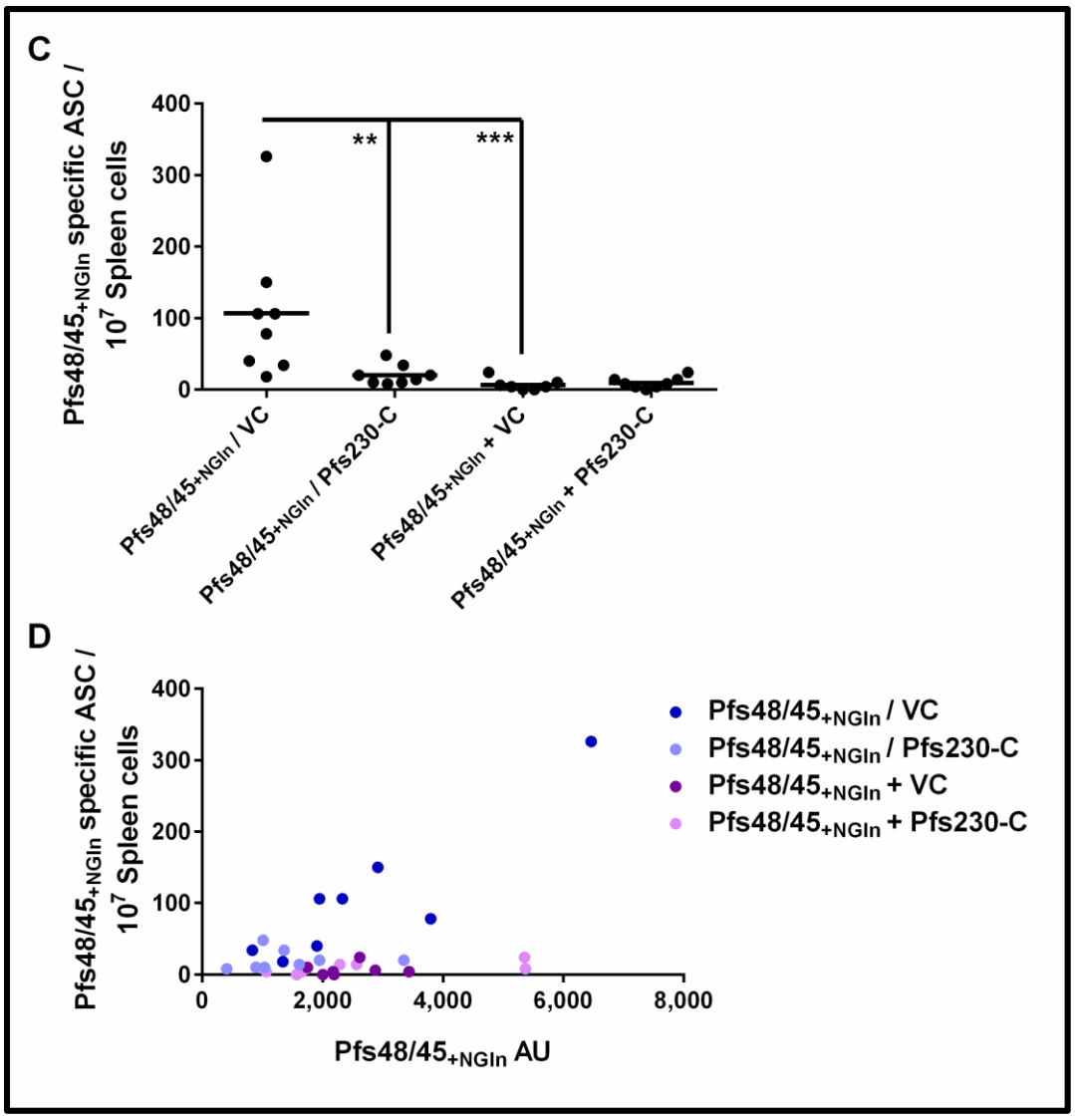


Figure 5.5. ASC responses to Pfs230-C and Pfs48/45$_{+NGln}$ after co-administration.

BALB/c mice (n=8/group) were vaccinated as described in **Figure 5.4.** At day70, spleens from each individual mouse were used to isolate splenocytes from which ASCs specific for Pfs230-C (**A and B**) and Pfs48/45$_{+NGln}$ (**C and D**) were measured by ELISPOT. (**A and C**) shows antigen-specific ASC responses/10^7 splenocytes; and (**B and D**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as mean and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. Asterisks represent statistical significance. ASC: antibody-secreting cell; VC: viral-vector control expressing GFP.

5.2.2.3. Pfs25 and Pfs48/45$_{+NGln}$ co-administration

Age-matched BALB/c mice were vaccinated as described in **5.2.2.1** with Pfs25 and Pfs48/45$_{+NGln}$ combinations either administered in separate sites or mixed in the same syringe (**Table 5.1**). Control combinations involved prime-boost with respective antigen and VC. Antigen-specific total IgG was assessed on days 14, 55 and 84 (due to unforeseeable circumstances) by standardised ELISA. The boosting of antigen-specific antibody responses was consistent as previously demonstrated (**5.2.2.1** and **5.2.2.2**), regardless of the regimen in all groups at varying magnitudes for Pfs25 and Pfs48/45$_{+NGln}$, (p<0.0001, **Figure 5.6**).

Anti-Pfs25 specific antibodies were comparable between all the groups at all the time points assessed with no statistically significant difference (**Figure 5.6A**). Thus there was no effect of co-administration of Pfs48/45$_{+NGln}$ on Pfs25 antibody responses whether administered in separate sites or mixed in the same syringe. On the contrary, differences in anti-Pfs48/45$_{+NGln}$ responses were detected at day14 post-prime (**Figure 5.6B**). There was a 2-fold reduction in responses when vaccination was administered mixed in the same syringe (p<0.01, Pfs48/45$_{+NGln}$+VC vs. Pfs48/45$_{+NGln}$+Pfs25) whereas no statistically significant effect of co-administration with Pfs25 in separate sites (Pfs48/45$_{+NGln}$/VC vs. Pfs48/45$_{+NGln}$/Pfs25) was observed. Furthermore, anti-Pfs48/45$_{+NGln}$ responses in the group receiving vaccinations in the same syringe significantly higher than in the group receiving vaccination in separate sites (2-fold higher, p<0.01, Pfs48/45$_{+NGln}$/VC vs. Pfs48/45$_{+NGln}$+VC). Nonetheless, responses at days 55 and 84 were all comparable irrespective of the antigen combination and method of co-administration (**Figure 5.6B**).

Pfs25-specific ASC responses were detected in the majority of vaccinated animals with a few non-responders (**Figure 5.7A**). The median antigen-specific ASC responses were comparable in each group irrespective of the antigen mixture or method of co-administration with no

statistically significant difference observed even though the Pfs25/Pfs48/45$_{+NGln}$ group appeared to have higher responses. Overall, there was a positive correlation between antibody and ASC responses (**Figure 5.7B**, r_s=0.58, p=0.0007). However, when the groups were individually analysed, a positive association was only observed in the group vaccinated with Pfs25/Pfs48/45$_{+NGln}$ (r_s=0.81, p=0.02).

Ditto, anti-Pfs48/45$_{+NGln}$ ASCs were also detected in all vaccinated animals irrespective of the antigen mixture or method of co-administration with a few non-responders (**Figure 5.7C**). The median antigen-specific ASC responses in the group co-administered with Pfs25 in separate sites had superior responses resulting in statistically significant differences whether Pfs48/45$_{+NGln}$ was co-administered with (a) control (Pfs48/45$_{+NGln}$/VC, 6-fold higher, p<0.001); or (b) Pfs25 but by mixing in the same syringe (Pfs48/45$_{+NGln}$+Pfs25, 5-fold higher, p<0.01). Furthermore, there was no statistically significant association between antibody and ASC responses across all groups whether analysed individually or pooled (**Figure 5.7D**).

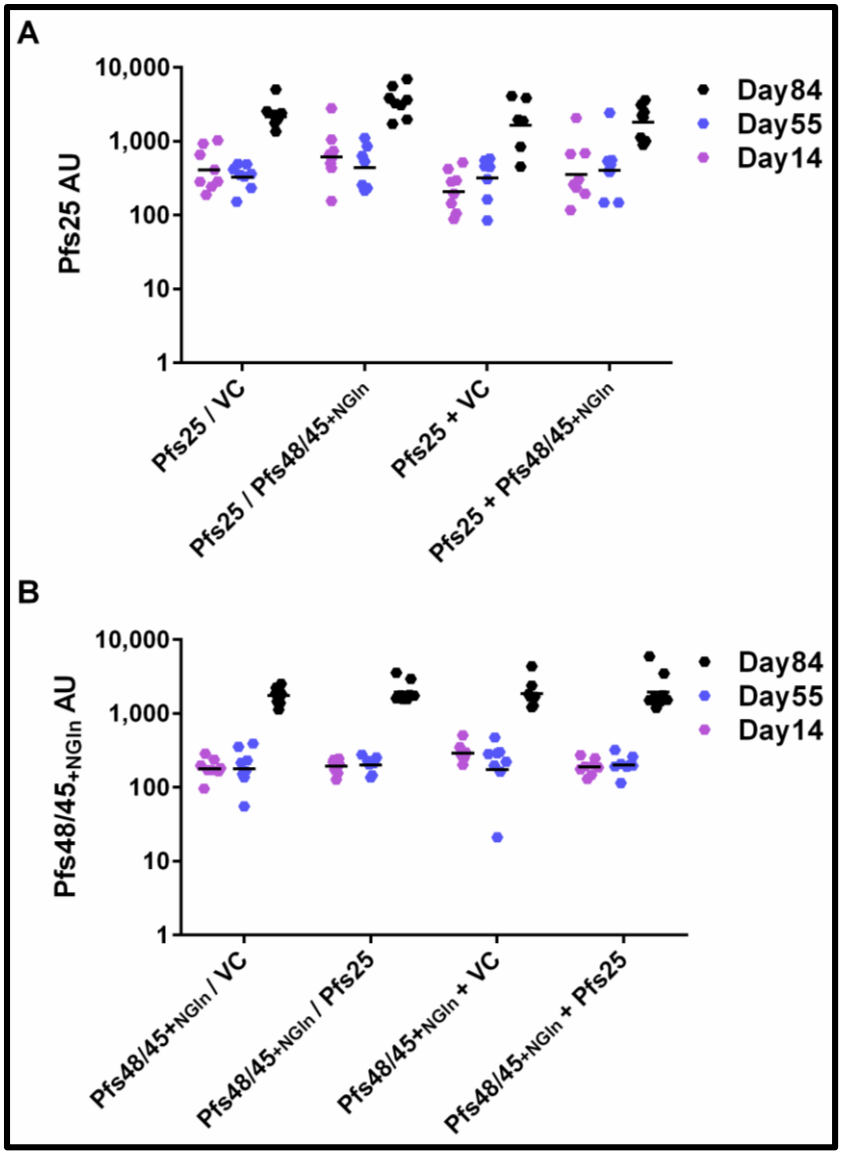
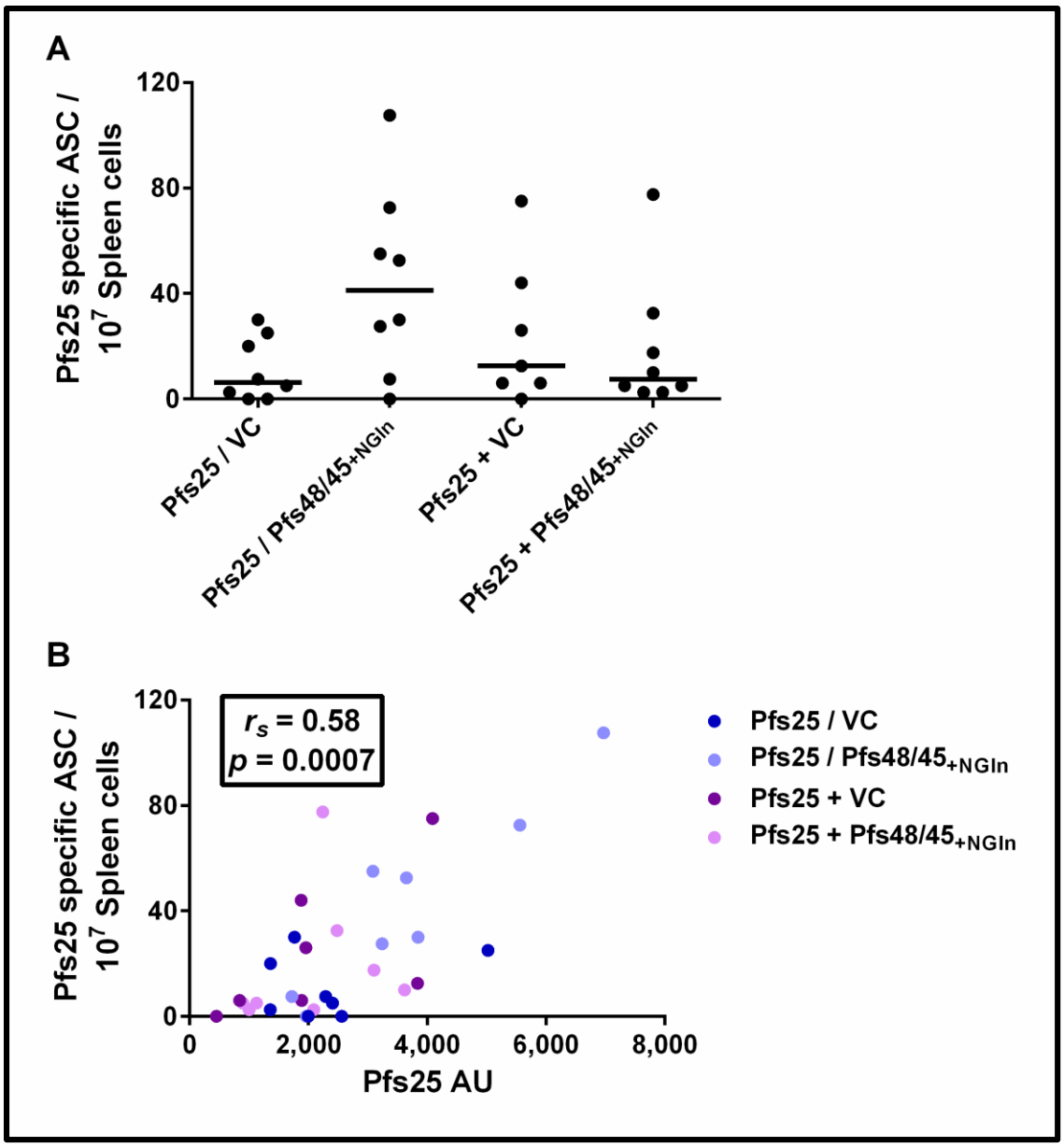


Figure 5.6. IgG responses to Pfs25 and Pfs48/45$_{+NGln}$ co-administration.

BALB/c mice (n=8/group) were vaccinated i.m. with ChAd63 (1x10^{8}IU, day0) and MVA (1x10^{7}PFU, day56) expressing either Pfs25, Pfs48/45$_{+NGln}$ or GFP administered in separate sites (denoted by /) or mixed in the same syringe (denoted by +). IgG responses were assayed on days 14, 55 and 84 by standardised ELISA using (**A**) Pfs25 and (**B**) Pfs48/45$_{+NGln}$ recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points per group. Dots represent responses from individual mice. VC: viral-vector expressing GFP.


A
Pfs25 specific ASC / 10^7 Spleen cells
120
80
40
0
Pfs25 / VC
Pfs25 / Pfs48/45+NGln
Pfs25 + VC
Pfs25 + Pfs48/45+NGln
B
Pfs25 specific ASC / 10^7 Spleen cells
r_s = 0.58
p = 0.0007
120
80
40
0
0
2,000
4,000
6,000
8,000
Pfs25 AU
Pfs25 / VC
Pfs25 / Pfs48/45+NGln
Pfs25 + VC
Pfs25 + Pfs48/45+NGln

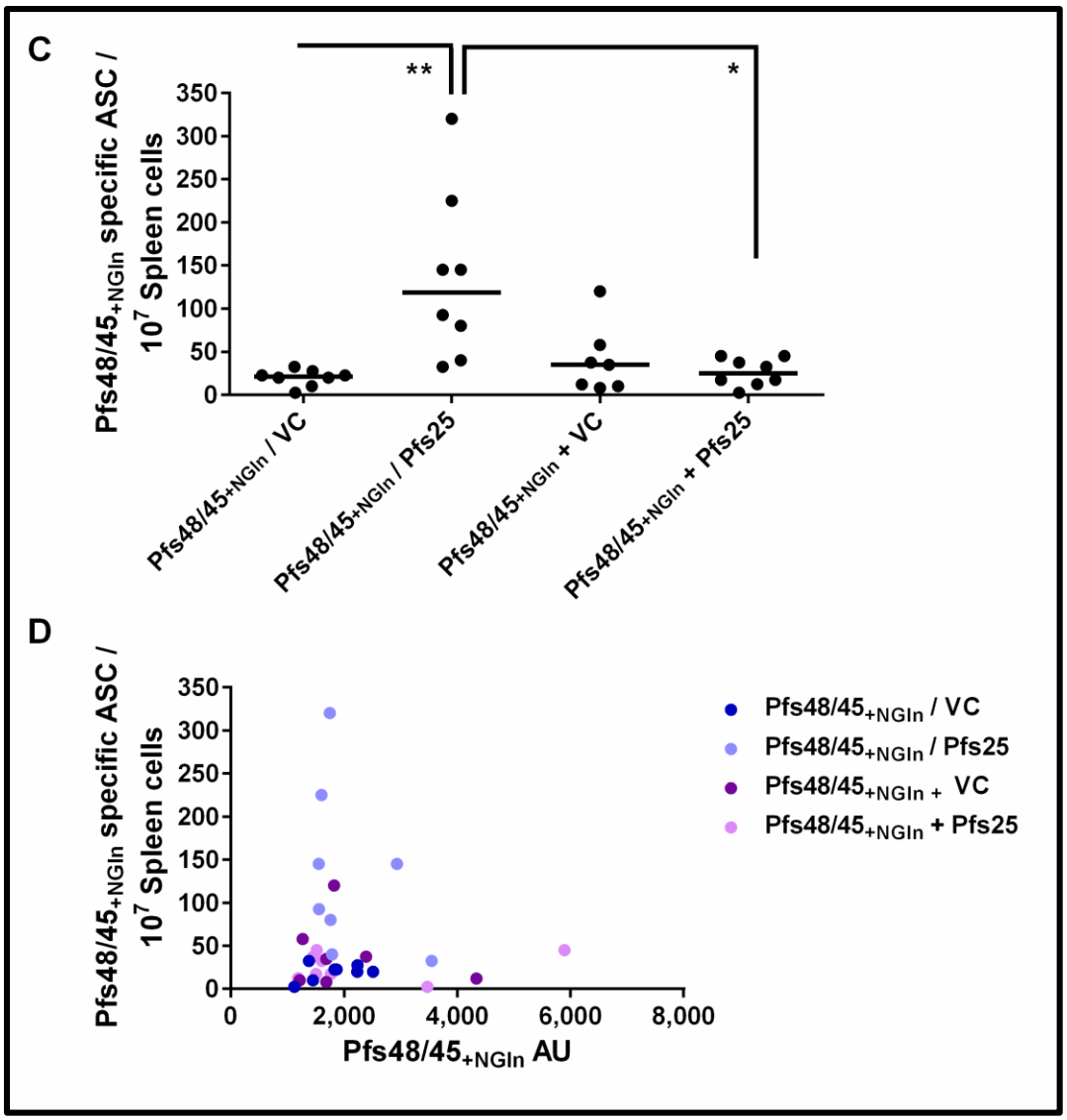


Figure 5.7. ASC responses to Pfs25 and Pfs48/45$_{+NGln}$ after co-administration.

BALB/c mice (n=8/group) previously vaccinated as described in **Figure 5.6.** At day70, spleens from each individual mouse were used to isolate splenocytes from which ASCs specific for Pfs25 (**A and B**) and Pfs48/45$_{+NGln}$ (**C and D**) were measured by ELISPOT. (**A and C**) shows antigen specific ASC responses/10^7 splenocytes; and (**B and D**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as medians and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. Asterisks represent statistical significance. ASC: antibody-secreting cell; VC: viral-vector expressing GFP.

5.2.3. Assessment of efficacy in rabbits

5.2.3.1. Vaccine-induced antibody responses

To assess immunogenicity, age-matched New Zealand white rabbits were primed at day 0 with ChAd63 and boosted at day 56 with MVA expressing Pfs230-C, Pfs25, Pfs48/45$_{+NGln}$ or control. Sera were assayed for total IgG responses days 14 and 55 post-prime and day70 post-boost by either endpoint or standardised ELISA (**Figure 5.8**). Anti-Pfs25 antibodies were assayed in a standardised ELISA using pooled anti-sera that was previously described [572]. This pool of anti-sera has been shown to have an IC_{50} antibody titre of 382AU.

The ChAd63 priming responses were boosted by the administration of MVA at varying magnitudes in all the animals ascertained by Wilcoxon matched-pairs test. Anti-Pfs230-C total IgG were 9-fold higher after boosting (**Figure 5.8A**, p=0.01), whilst anti-Pfs48/45$_{+NGln}$ responses were boosted 50-fold (**Figure 5.8B**, p=0.002); and anti-Pfs25 24-fold (**Figure 5.8C**, p=0.02). Pfs48/45$_{+NGln}$ post-prime responses appeared to decrease to time of boosting however the trend wasn't statistically significant (day14 vs. day55, p=0.06). The geometric mean responses in the Pfs25 vaccinated group were 2-fold higher than that required to inhibit 50% of parasite infectivity. One rabbit in this group had low antibody responses which were 1 fold lower than that required to inhibit parasite infectivity by 50% (**Figure 5.8C**).

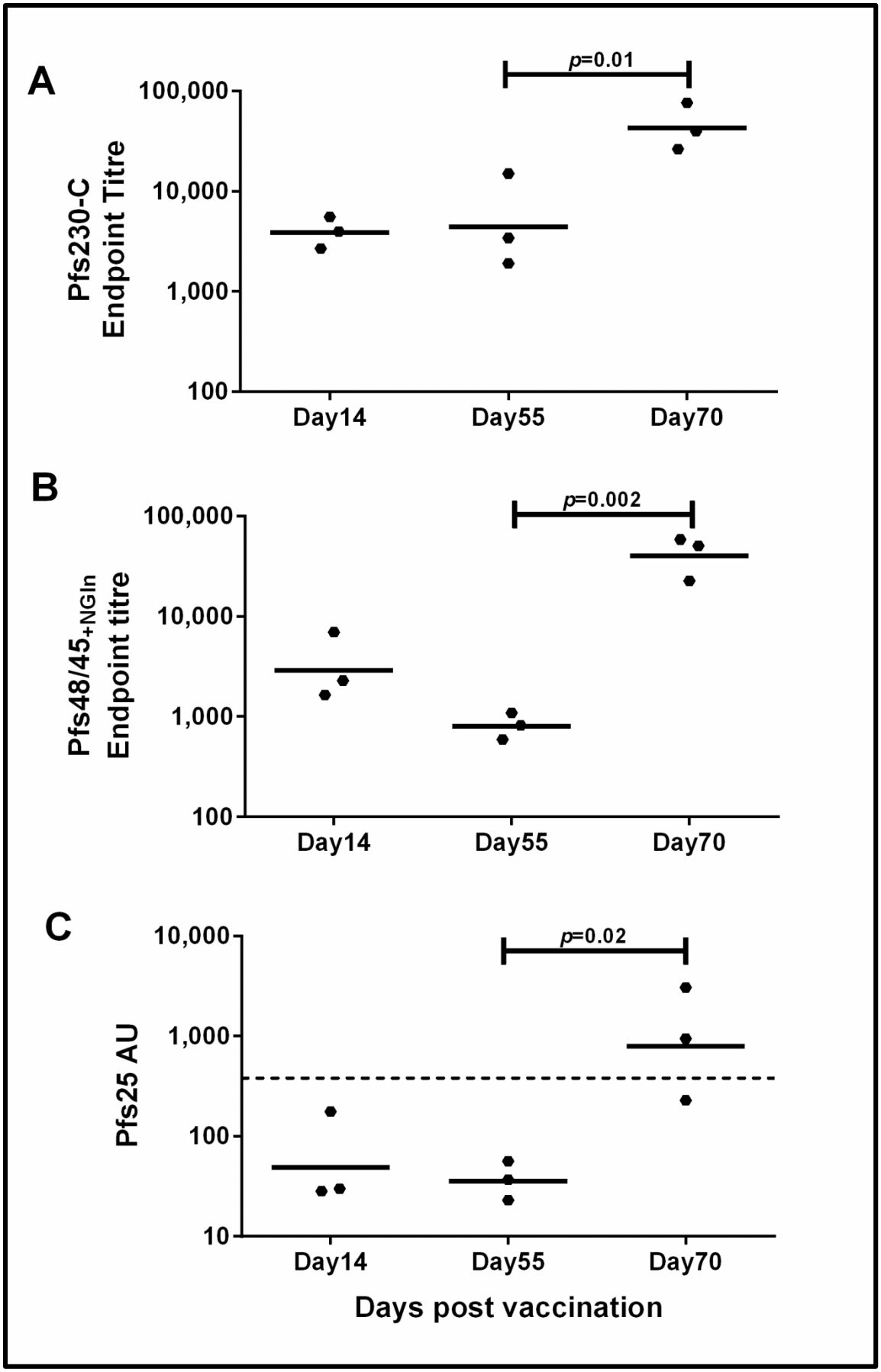

A
p=0.01
100,000
10,000
1,000
100
Pfs230-C
Endpoint Titre
Day14
Day55
Day70
B
p=0.002
100,000
10,000
1,000
100
Pfs48/45+NGIn
Endpoint titre
Day14
Day55
Day70
C
p=0.02
10,000
1,000
100
10
Pfs25 AU
Day14
Day55
Day70
Days post vaccination

Figure 5.8. Antibody responses induced in rabbits after ChAd63-MVA prime-boost.

New Zealand white rabbits (n=3/group) were vaccinated i.m. with ChAd63 ($5x10^8$IU, day0) and MVA ($5x10^7$PFU, day56) individually expressing (**A**) Pfs230-C; (**B**) Pfs48/45$_{+NGln}$; and (**C**) Pfs25. Total IgG responses were measured on days 14, 55 and 70 by endpoint (**A and B**) and standardised (**C**) ELISAs. The data was log-transformed and responses represented as geometric mean at each time point. Dotted line in (**C**) represents rabbit-specific Pfs25 IC_{50} antibody units. Significance shown and considered at $p<0.05$.

Table 5.2. Antibody titres of pooled rabbit sera used to purify total IgG for SMFA

Antigen	Antibody titre
Pfs230-C	46, 083 Endpoint titre
Pfs48/45$_{+NGln}$	59, 077 Endpoint titre
Pfs25	575AU

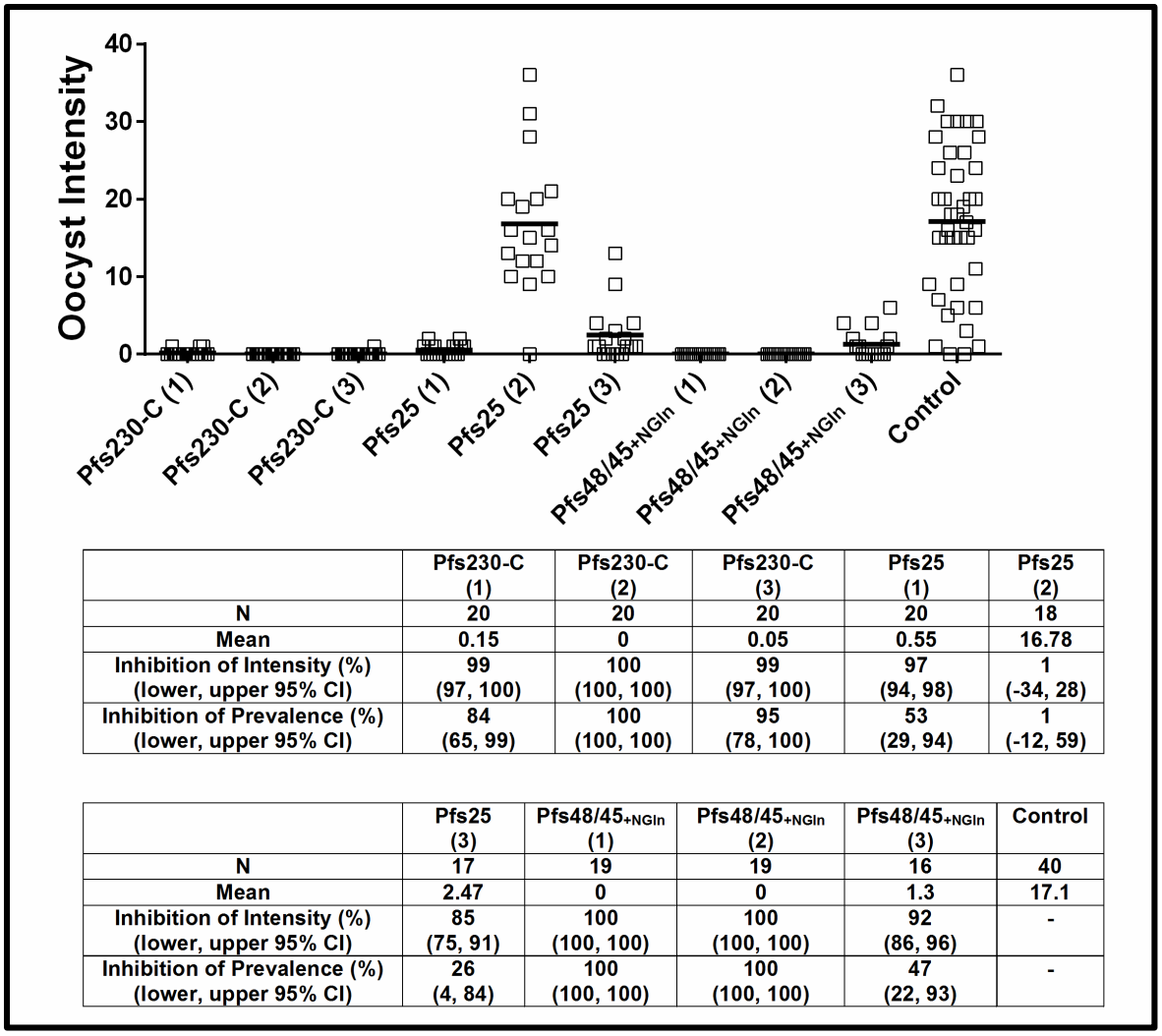
Antibody titres determined from an aliquot of sera from pooled vaccinated rabbits (n=3).

5.2.3.2. Assessment of *P. falciparum* NF54 infectivity to *A. stephensi*

To test whether the vaccine-induced antibody responses were able to inhibit the infectivity of *P. falciparum* NF54 to *A. stephensi*, day70 anti-sera from each individual vaccinated animal/group was used to purify total IgG (adjusted to a stock concentration of 20mg/ml). In addition, total IgG was also purified from pooled anti-sera from each group including viral-vector control vaccinated animals. Antibody titres for the pooled sera were determined by ELISA (**Table 5.2**).

5.2.3.2.1. Effect of individual responses on infectivity

The effect of vaccine-induced responses on parasite infectivity for each individual animal was determined in SMFA at a concentration of 3.75mg/ml of purified total IgG (**Figure 5.9**). The animal with the highest antibody response against Pfs230-C gave complete TBE (100%, $p<0.0001$, rabbit 2) whilst the other two animals with comparable antibody titres had similar effects at mediating efficacy. Two out of the three animals, with the highest comparable antibody titres, also mediated complete efficacy against Pfs48/45$_{+NGln}$ (100%, $p<0.0001$, rabbit 1 and 2). Efficacy against Pfs25 was also observed to be antibody titre-dependent with the highest responder inhibiting intensity (97%, $p<0.0001$) and prevalence (53%, $p<0.0001$). Antibody titres of 228AU had no effect on both parasite intensity and prevalence (rabbit 2, **Figure 5.9**). Overall, there was a strong positive correlation [determined by Spearman's rank test] between the antibody responses and the ability to inhibit intensity ($r_s=0.96$, $p=0.0003$) and prevalence ($r_s=0.94$, $p=0.0004$) (**Figure 5.10**). Interestingly, the amount of antibody required to inhibit prevalence was more than that required for intensity. From this experiment, the antibody titre had a significant effect on inhibition of parasite infectivity against all three antigens which were further confirmed by an independent assessment of Pfs48/45$_{+NGln}$ responses using anti-sera (Report from Nijmegen, **Appendix**).



	Pfs230-C (1)	Pfs230-C (2)	Pfs230-C (3)	Pfs25 (1)	Pfs25 (2)
N	20	20	20	20	18
Mean	0.15	0	0.05	0.55	16.78
Inhibition of Intensity (%) (lower, upper 95% CI)	99 (97, 100)	100 (100, 100)	99 (97, 100)	97 (94, 98)	1 (-34, 28)
Inhibition of Prevalence (%) (lower, upper 95% CI)	84 (65, 99)	100 (100, 100)	95 (78, 100)	53 (29, 94)	1 (-12, 59)

	Pfs25 (3)	Pfs48/45+NGln (1)	Pfs48/45+NGln (2)	Pfs48/45+NGln (3)	Control
N	17	19	19	16	40
Mean	2.47	0	0	1.3	17.1
Inhibition of Intensity (%) (lower, upper 95% CI)	85 (75, 91)	100 (100, 100)	100 (100, 100)	92 (86, 96)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	26 (4, 84)	100 (100, 100)	100 (100, 100)	47 (22, 93)	-

Figure 5.9. Anti-rabbit IgG inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*.

Total IgG purified from individual sera (day70) from vaccinated rabbits with each respective antigen or GFP (control) (**5.2.3.1**) was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* in SMFA. Total IgG was mixed at 3.75mg/ml (1:5.34 dilution). Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI. Squares represent oocyst numbers from each individual mosquito dissected.

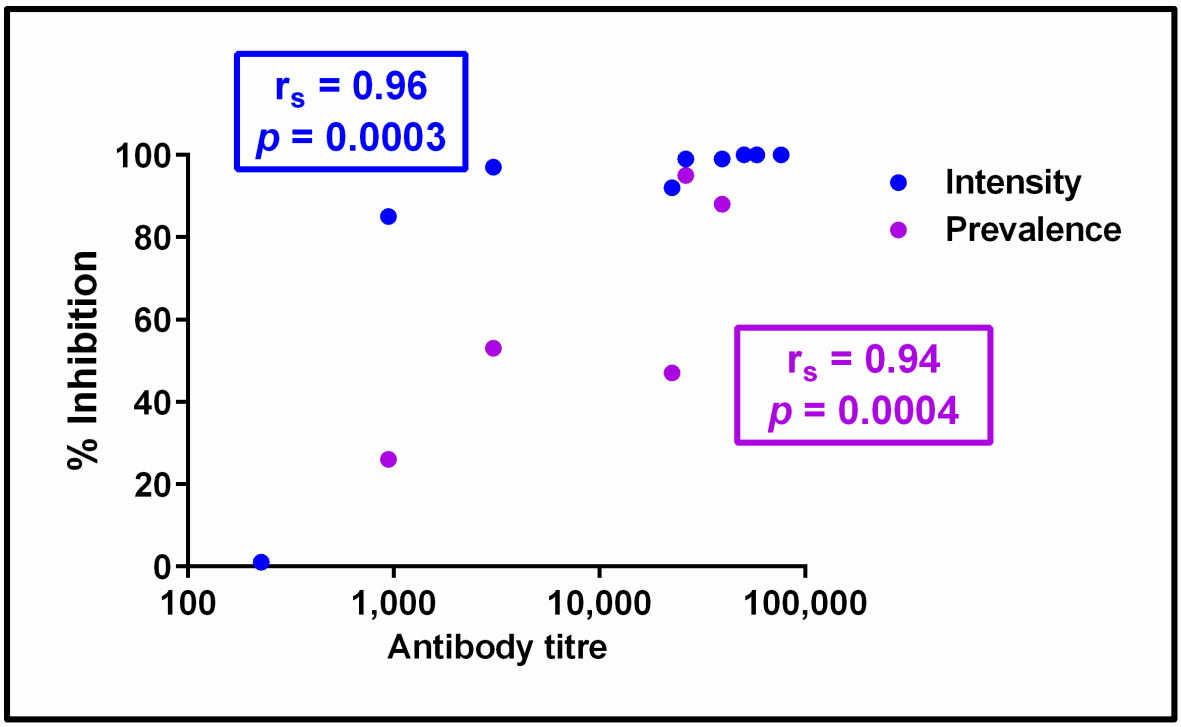


Figure 5.10. Relationship between antibody titre and parasite infectivity.

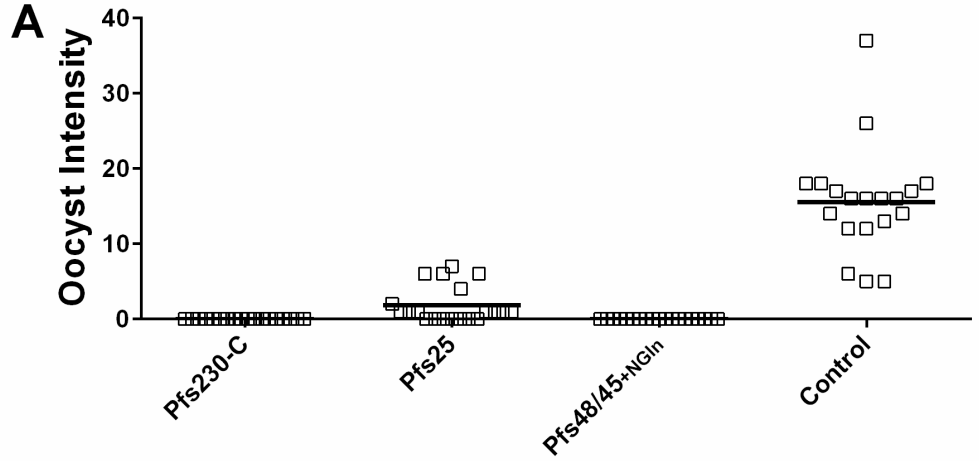
Results from individual rabbit SMFA experimental data was collated and matched to respective antibody titres for each individual sample. Antibody responses were log-transformed and a Spearman's rank test (r_s) used to determine the correlation between the inhibition of parasite intensity (blue dots) and prevalence (purple dots). The *y*-axis shows the percent (%) inhibition of parasite infectivity and the *x*-axis shows the log-transformed antibody titres. Significance is shown and was considered at $p<0.05$.

5.2.3.2.2. Effect of pooled responses on infectivity

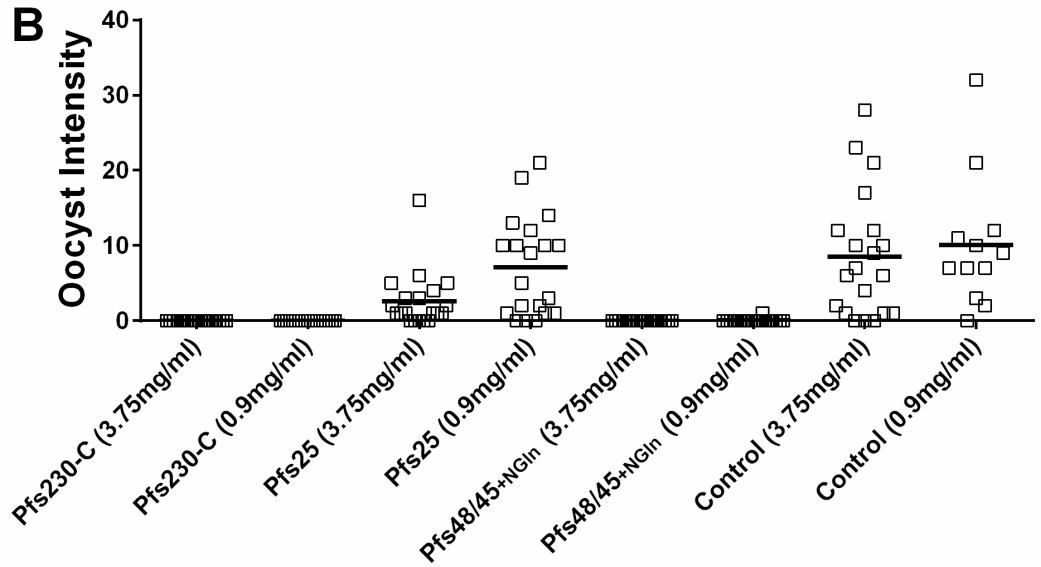
To assess the effect of vaccine-induced antibody responses on parasite infectivity, total IgG (3.75mg/ml) purified from pooled sera was tested in SMFA (**Figure 5.11**). There was complete TBE mediated against both Pfs230-C and Pfs48/45$_{+NGln}$ (**Figure 5.11A**, 100%, p<0.0001). In the case of Pfs25, there was insufficient complete blockade of parasite intensity (88%, p<0.0001) and prevalence (35%, p=0.001) (**Figure 5.11A**). The antibody titre of the pooled anti-Pfs25 group (**Table 5.2**) was greater than the IC_{50} titre [572].

Furthermore, to determine whether there was a concentration-dependent effect on efficacy, the pooled total IgG was tested at two concentrations (**Figure 5.11B**). Ditto, anti-Pfs230-C and anti-Pfs48/45 antibodies mediated complete efficacy at 3.75mg/ml (100%, p<0.0001 respectively). Moreover, at a lower concentration of anti-Pfs230-C IgG (0.9mg/ml), complete efficacy was observed (100%, p<0.0001); whilst that of anti-Pfs48/45$_{-NGln}$ IgG [at 0.9mg/ml] was reduced to 99% and 95% (p<0.0001) for intensity and prevalence respectively. However, antibodies against Pfs25 had an effect only at inhibiting intensity (69%, p=0.003), a lower magnitude compared to previously observed (**Figure 5.11A**) with no statistically significant efficacy at the lower IgG concentration tested.

In summary, there was >80% efficacy observed against Pfs230-C, Pfs25, and Pfs48/45$_{+NGln}$ that was attributed to be antibody titre-dependent from individual vaccine-induced responses. Although there was concentration-dependent efficacy observed in the pooled responses against Pfs25 and Pfs48/45$_{+NGln}$ there was no evidence of this effect against Pfs230-C. These findings for Pfs48/45$_{+NGln}$ were further supported by results from Nijmegen (**Appendix**). Therefore the observed hierarchy in efficacy in rabbits was Pfs230-C $\geq$ Pfs48/45$_{+NGln}$ > Pfs25.



	Pfs230-C	Pfs25	Pfs48/45$_{+NGln}$	Control
N	20	20	19	19
Mean	0	1.9	0	15.6
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	88 (80, 92)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (0.8, 1)	35 (0, 1)	100 (0.8, 1)	-



	Pfs230-C (3.75mg/ml)	Pfs25 (3.75mg/ml)	Pfs48/45+NGln (3.75mg/ml)	Control (3.75mg/ml)
N	20	20	20	20
Mean	0	2.6	0	8.5
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	69 (36, 85)	100 (100, 100)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	12 (-8, 50)	100 (100, 100)	-

	Pfs230-C (0.9mg/ml)	Pfs25 (0.9mg/ml)	Pfs48/45+NGln (0.9mg/ml)	Control (0.9mg/ml)
N	19	20	20	12
Mean	0	7.15	0.05	10.1
Inhibition of Intensity (%) (lower, upper 95% CI)	100 (100, 100)	27 (-41, 62)	99 (96, 99)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	100 (100, 100)	7 (-10, 71)	95 (56, 99)	-

Figure 5.11. Effect of IgG from pooled rabbit anti-sera on *P. falciparum* NF54 infectivity.

Total IgG purified from pooled sera (day70) from vaccinated rabbits with each respective antigen or GFP (control) (**5.2.3.1.**) was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* in SMFA. Total IgG was tested at (**A**) 3.75mg/ml and (**B**) concentrations shown in parenthesis. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as the arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI. Squares represent oocyst numbers from each individual mosquito dissected.

5.2.4. Pfs230-C and Pfs25 fusion vaccines

Overall, antibody responses to Pfs230-C and Pfs25 exhibited greater transmission-blocking efficacy as shown above and in **Chapter 4** in both SMFA and DMFA, even though Pfs25 vaccine-induced antibody responses were affected when co-administered with Pfs230-C. Therefore, fusion vaccines incorporating both Pfs230-C and Pfs25 expressed in the same viral-vector were generated to test whether, in this combination, vaccine-induced antibodies using fusion inserts would be able to inhibit parasite development better than mixed or co-administered viral-vectors.

5.2.4.1. Design and generation of fusion constructs

Fusion constructs were designed and generated using either a 2A or GP linker sequence as there is evidence for the use of either to induce protective responses [541] (**Figure 5.12**). This allowed the assessment of whether comparable responses would be obtained when Pfs230-C and Pfs25 were co-expressed with either linker sequence. For the design of the 2A constructs, a number of considerations were taken into account with regards to: (a) choice of 2A sequence; (b) choice of secretory signal peptides; (c) incorporation of a glycine-serine-glycine (GSG) linker; and (d) order of transgenes [573].

The choice of F2A linker sequence was informed from studies by Donnelly et al. (2001), who have shown a modified F2A consisting of the 19αα of F2A with a 5αα N-terminal extension had ~96% cleavage efficiency [555]. Yan et al (2010) have demonstrated that a second signal peptide (SP) on the downstream transcript is required for secretion in mammalian expression systems [574]. The F2A constructs were also designed to include separate signal peptides. F2A has also been used to express the heterodimers of Interleukin12 (IL-12) using separate SPs [575]. De Felipe et al. (2003) have additionally shown that addition of separate SPs

downstream of the F2A sequence is required for continued translation of the downstream proteins within the ribosome [560]. Therefore, the constructs were designed with the signal sequences for tPA (**Chapter 3**) and bovine IL-12p40 [575]. The addition of the Il-12p40 SP upstream of the second transcript also allows for secretion of the expressed protein and removal of the remaining proline after F2A cleavage. Furthermore, a GSG linker between the upstream transcript and the F2A sequence was incorporated to allow maximum cleavage efficiency as described and recommended [573, 576]. Pfs25 was placed upstream of the F2A sequence whilst Pfs230-C was downstream (**Figure 5.12A**). For the GP linked polypeptide, the same order of transgenes was used with tPA used as the SP (**Figure 5.12B**).

The protein sequences for Pfs25 and Pfs230-C were accessed from the NCBI database and bioinformatics determined as described previously **Chapter 3** and [372]. All the endogenous SP sequences were substituted for tPA and/or IL-12p40 (**Figure 5.12**). For Pfs25, the GPI anchor was removed and the sequence 22 – 193αα incorporated. In addition a Kozak sequence was added before the ATG start site to allow optimal initiation of translation as previously described [444]. A stop codon was inserted downstream of Pfs230-C for both F2A and GP constructs. Predicted N-glycosylation sites were substituted as described in **Chapter 3** (Pfs25 has 4 N-glycosylation sites at positions 112, 165, 187 and 202 but position 202 is within the GPI anchor [296]). Sequences were optimised for human codon usage by GeneArt® and restriction cloning sites corresponding to those utilised for cloning into ChAd63 and MVA incorporated upstream and downstream the antigen sequence (**Figure 5.12**). ChAd63 and MVA viral-vectors were generated as described in **Chapter 3**.

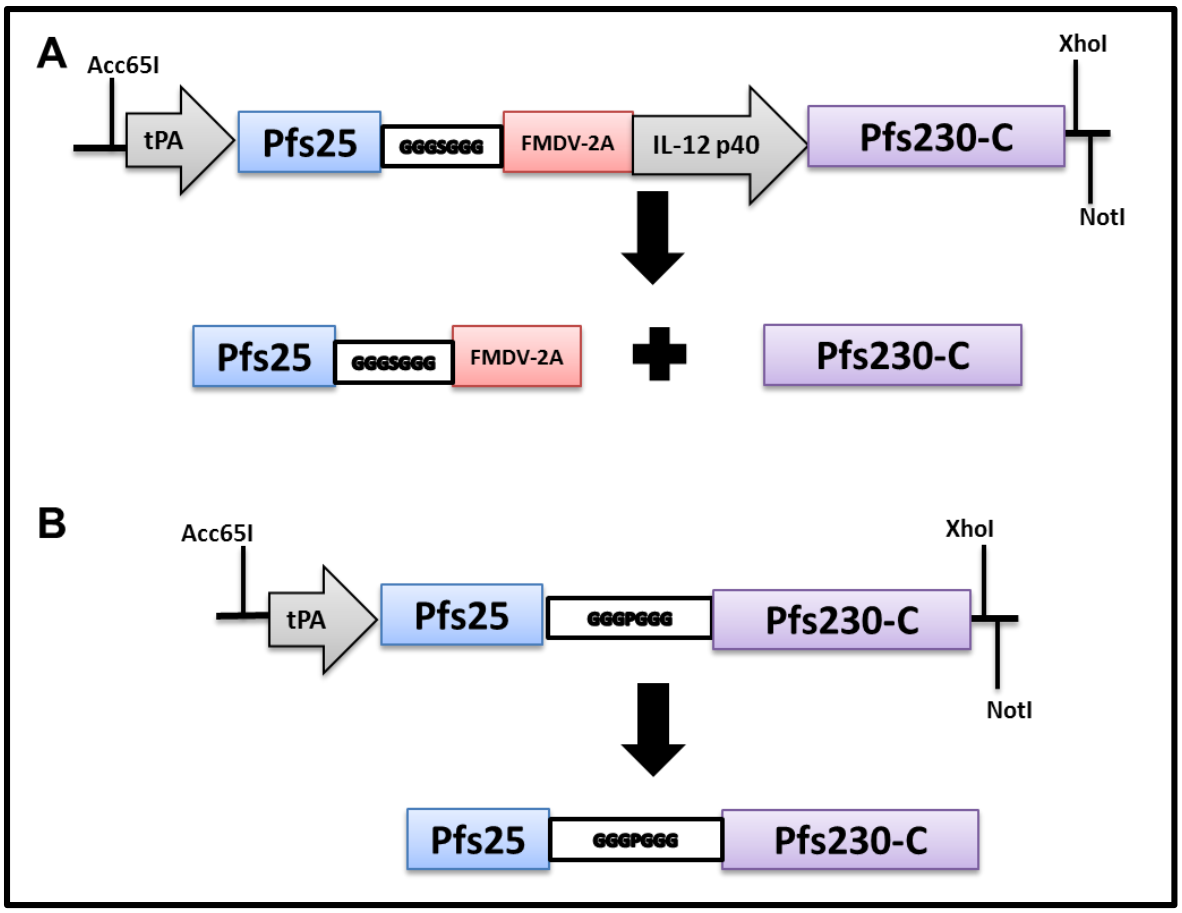


Figure 5.12. Schematic illustration of the antigen fusion constructs.

Pfs230-C and Pfs25 protein sequence were obtained from NCBI protein database from which endogenous SPs, GPIs, TMDs and N-glycosylation sites were omitted. The endogenous SPs were replaced with either tPA or IL-12p40 leader sequences. A Kozak sequence and start codon were placed upstream of the leader sequence, a stop codon downstream of the antigen open reading frame, and respective restriction sites incorporated. The sequences were then codon optimised for human usage. Represented are the fusion constructs and their respective products for (**A**) Pfs25-Pfs230-C FMDV-2A fusion construct; and (**B**) Pfs25-Pfs230-C glycine-proline fusion construct. FMDV-2A: foot-and-mouth disease virus 2A sequence; GGGPGGG: glycine-proline linker sequence; GGGSGGG: glycine-serine linker.

5.2.4.2. Expression of fusion constructs in mammalian cells

To verify cleavage in the F2A construct and determine expression in mammalian cells, pENTR™4LPTOS plasmid DNA expressing the F2A and GP fusion constructs were used to transfect HEK293 cells. Supernatant and cell lysates were harvested after 24hours and mixed with non-reducing and reducing SDS sample loading buffer. Additionally, transfections were performed with pENTR4-LPTOS expressing either Pfs230-C or Pfs25 for comparison from which only supernatant was used. The samples were run on polyacrylamide gels, transferred onto nitrocellulose membranes and immunoblots generated using day 70 pooled sera against either Pfs25-2A-Pfs230-C or Pfs25-GP-Pfs230-C (**Figure 5.13**).

Pfs25 was observed to be cleaved from Pfs25-2A-Pfs230-C when anti-Pfs25-2A-Pfs230-C anti-sera was used to obtain immunoblots with both Pfs25 and Pfs230-C being recognised (**Figure 5.13A)**. However, the observed cleaved product for Pfs25 was at a higher molecular weight of 25kDa than expected at 22.4kDa (**Figure 4.4C**), with bands more evident in the reduced form in both supernatant and lysate (lanes 5–8, **Figure 5.13A**). The observation of the higher molecular weight species could be explained by the presence of F2A at the C-terminus (Pfs25-2A predicted at 25kDa) as previously alluded to and described for proteins upstream of the F2A sequence [576, 577]. On the other hand, Pfs25 expressed in pENTR4-LPTOS-Pfs25 was faintly recognised at the predicted molecular weight of 22.4kDa (**Figure 5.13A**, lanes 1 and 2). Pfs230-C bands migrated closer to the predicted size of the uncleaved product (107kDa) in supernatant (**Figure 5.13A**, lanes 5 – 8). This observation could be as a result of inefficient cleavage during translation even though it is evident that the product is secreted into the supernatant. Furthermore, two bands were observed in the lysate under reducing conditions (lane 8), one migrating at ~100kDa and another concurrently with that of pENTR4-LPTOS-Pfs230-C (lane 3 and 4) which could further explain the inefficient cleavage. Additionally, anti-Pfs25-GP-Pfs230-C anti-sera recognised a large molecular

weight species at ~100kDa (predicted size of 105kDa) which was secreted in supernatant (**Figure 5.13B**, lanes 5–8). Both Pfs25 and Pfs230-C were recognised at their respective predicted sizes (lanes 1–4). Taken together, Pfs25 and Pfs230-C are both expressed and secreted in the F2A construct and the GP fusion product is also expressed and secreted with anti-sera being able to recognise the singly expressed protein products.

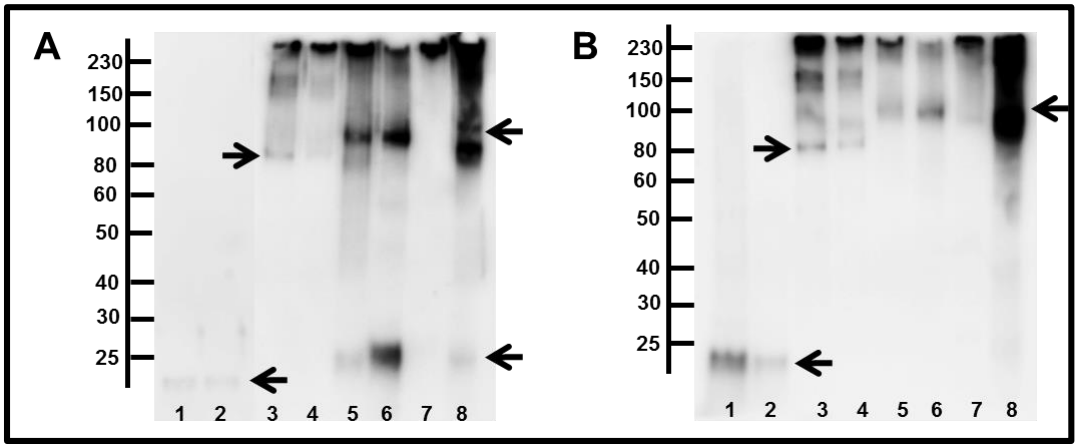


Figure 5.13. Expression profile of fusion constructs in HEK239s.

pENTR4-LPTOS plasmid DNA expressing (**A**) Pfs25-2A-Pfs230-C and (**B**) Pfs25-GP-Pfs230-C was used to transfect HEK293s for 24hours. Supernatant and cell lysate were harvested and mixed with either non-reducing or reducing SDS loading sample buffer. Samples were run on 12% polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using day70 pooled sera. Pfs25 and Pfs230-C supernatants were used as controls for expression. Lanes: 1 and 2 – Pfs25 supernatant in non-reducing and reducing buffer; 3 and 4 – Pfs230-C supernatant in non-reducing and reducing buffer; 5 and 6 – Pfs25-2A-Pfs230-C or Pfs25-GP-Pfs230-C supernatant in non-reducing and reducing buffer; 7 and 8 – Pfs25-2A-Pfs230-C or Pfs25-GP-Pfs230-C lysate in non-reducing and reducing buffer. Arrowheads indicate the relative migration mobility expected.

5.2.4.3. Vaccine-induced responses to fusion constructs

To determine whether expressing Pfs25 and Pfs230-C either as Pfs25-2A-Pfs230-C or Pfs25-GP-Pfs230-C would have an impact on vaccine-induced antibody responses, age-matched BALB/c mice were prime-boost vaccinated with ChAd63-MVA expressing the individual respective fusion vaccines. In addition, two groups were vaccinated with ChAd63-MVA individually expressing either Pfs25 or Pfs230-C for comparison. IgG responses were determined on days 14, 55 and 70 by standardised ELISA (**Figure 5.14**). Boostability of responses within groups was ascertained by Wilcoxon test whilst differences between groups across time points by Kruskal-Wallis with Dunn's multiple comparisons post-test.

As observed previously, ChAd63 priming responses were boosted in the single vaccination groups at varying magnitudes; Pfs230-C (**Figure 5.14A**, 26-fold, p=0.002) and Pfs25 (**Figure 5.14B**, 15-fold, p=0.002). For the fusion vaccines, anti-Pfs230-C responses induced by Pfs25-2A-Pfs230-C and Pfs25-GP-Pfs230-C were boosted by 17-fold (p=0.008) and 28-fold (p=0.002) respectively (**Figure 5.14A**). Those against Pfs25 were also boosted by 71-fold (p=0.001) and 41-fold (p=0.002) in the respective groups (**Figure 5.14B**). Responses against Pfs230-C were comparable between Pfs25-2A-Pfs230-C and Pfs25-GP-Pfs230-C at all time points with no statistically significant differences (**Figure 5.14A,** p>0.05). This was also true of anti-Pfs25 responses (**Figure 5.14B**). This was an interesting finding as interference was observed when viral-vectors were co-administered (**5.2.2.1**). However, anti-Pfs230-C responses were higher at day14 post-prime in the Pfs230-C only group compared to the 2-fold lower antibody titre in the Pfs25-2A-Pfs230-C and Pfs25-GP-Pfs230-C groups (p<0.05 in both cases). On the other hand, at day55, there was a trend for responses in both Pfs25-2A-Pfs230-C and Pfs25-GP-Pfs230-C groups being lower, though no statistically significant difference was detected. Nonetheless, at day70, antibody titres were comparable between

Pfs230-C and Pfs25-GP-Pfs230-C groups whilst Pfs25-2A-Pfs230-C responses were 2-fold lower (p<0.05). In the case of anti-Pfs25 responses, there were no statistically significant differences detected at all time points across the groups.

To determine antigen-specific ASC responses and the relationship with antibody responses, ELISPOT assays were performed using spleens from all vaccinated mice **(Figure 5.15)**. Antigen-specific ASCs were detected in all the groups for both antigens (**Figure 5.15**). For Pfs230-C, ASC responses were comparable between the groups receiving either Pfs230-C on its own or in a GP linked fusion with Pfs25 (**Figure 5.15A**). However, the median ASC responses were 10-fold lower when Pfs230-C was expressed as a F2A fusion with Pfs25 (Pfs230-C vs. Pfs25-2A-Pfs230-C, p<0.01). When analysed individually, there was no statistically significant correlation observed between the antibody and ASC responses with the association in the Pfs25-GP-Pfs230-C group trending in a negative direction. However, when the responses were pooled, there was a significant positive correlation between the antibody titre and ASC response (**Figure 5.15B**, r_s=0.57, p=0.02). In regards to Pfs25, there was no statistically significant difference (p>0.05) across all the groups though median ASC responses in the Pfs25-GP-Pfs230-C group appeared to be higher (**Figure 5.15C**). When the groups were analysed individually for correlations between antibody and ASC responses, there was no statistically significant relationship observed. When the responses were pooled, ditto, there was a positive association between antibody and ASC responses (**Figure 5.15D**, r_s=0.57, p=0.02).

Overall, vaccine-induced responses to both antigens were comparable whether expressed as F2A or GP fusion vaccines. In comparison, there was an effect on Pfs230-C antibody and ASC responses in the F2A fusion vaccine compared to Pfs230-C alone, though the responses were comparable between the fusion vaccines (i.e. F2A and GP).

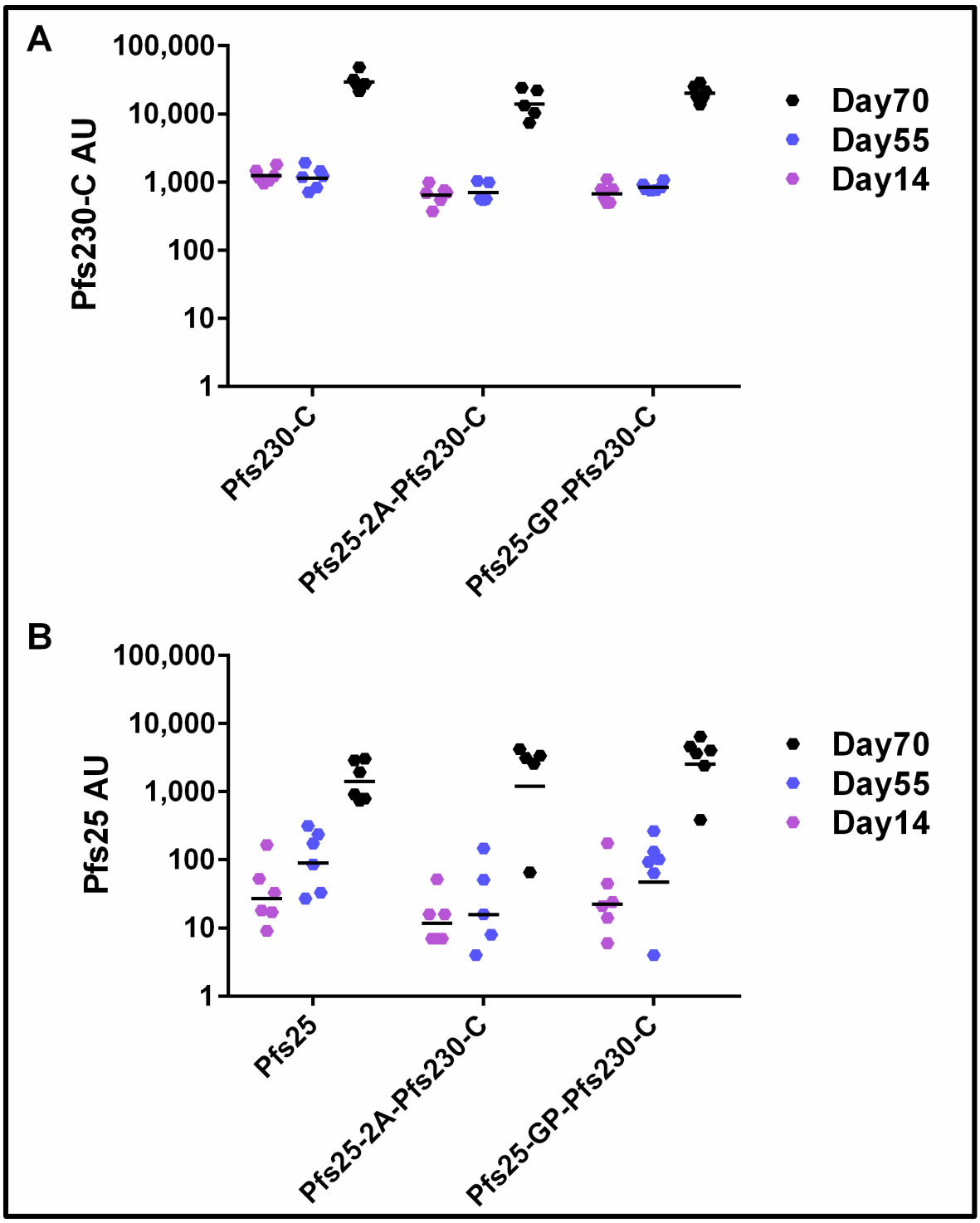
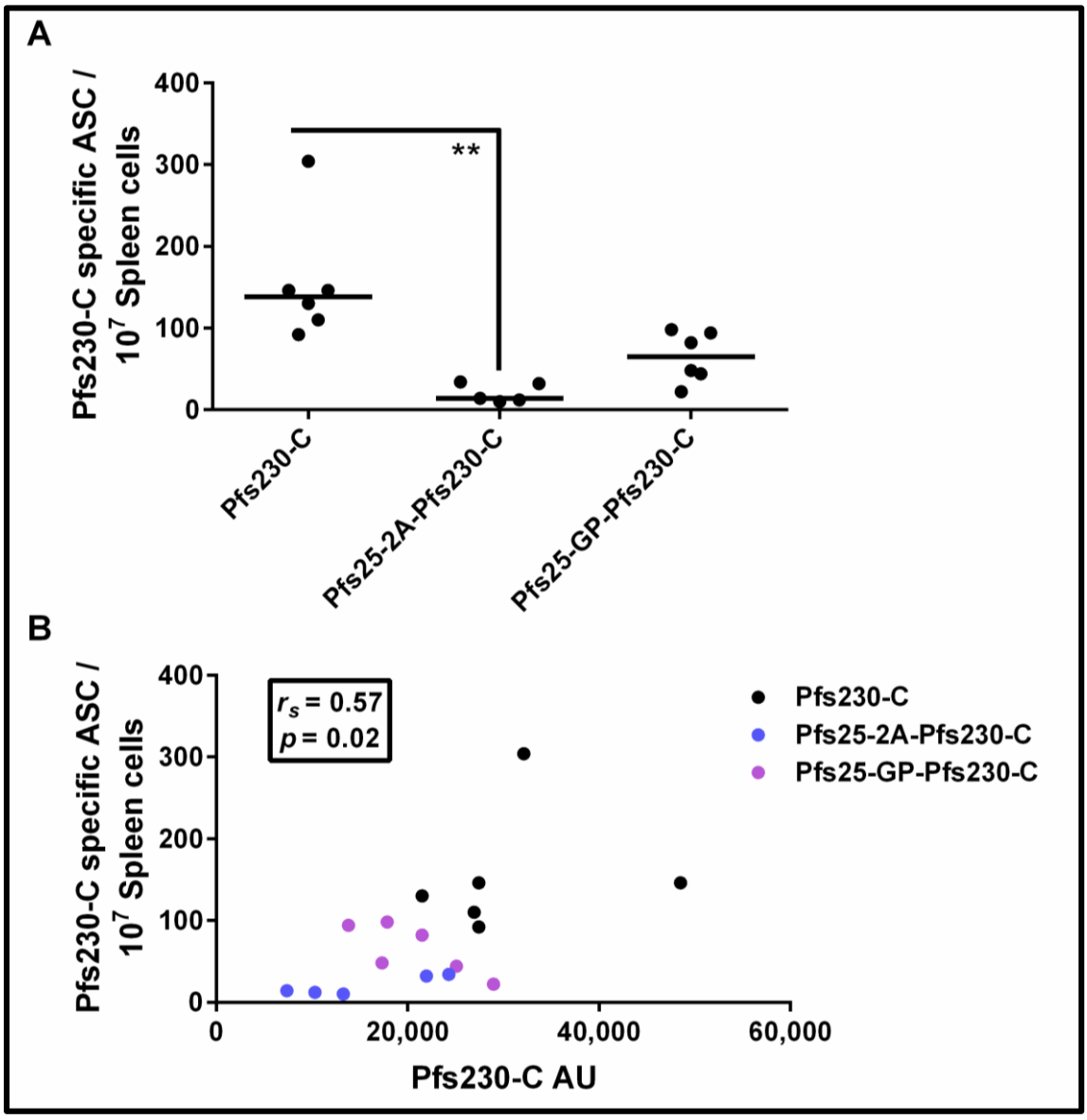

A
Pfs230-C AU
100,000
10,000
1,000
100
10
1
Pfs230-C
Pfs25-2A-Pfs230-C
Pfs25-GP-Pfs230-C
Day70
Day55
Day14
B
Pfs25 AU
100,000
10,000
1,000
100
10
1
Pfs25
Pfs25-2A-Pfs230-C
Pfs25-GP-Pfs230-C
Day70
Day55
Day14

Figure 5.14. IgG responses to fusion vaccines after ChAd63-MVA prime-boost.

BALB/c mice (n=5-6/group) were vaccinated i.m. with ChAd63 ($1x10^8$IU, day0) and boosted with respective MVA ($1x10^7$PFU, day56) expressing either Pfs230-C, Pfs25, Pfs25-2A-Pfs230-C or Pfs25-GP-Pfs230-C. Whole IgG responses were measured on days 14, 55 and 70 by standardised ELISA using (**A**) Pfs230-C and (**B**) Pfs25 recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points per group. Dots represent responses from individual mice.



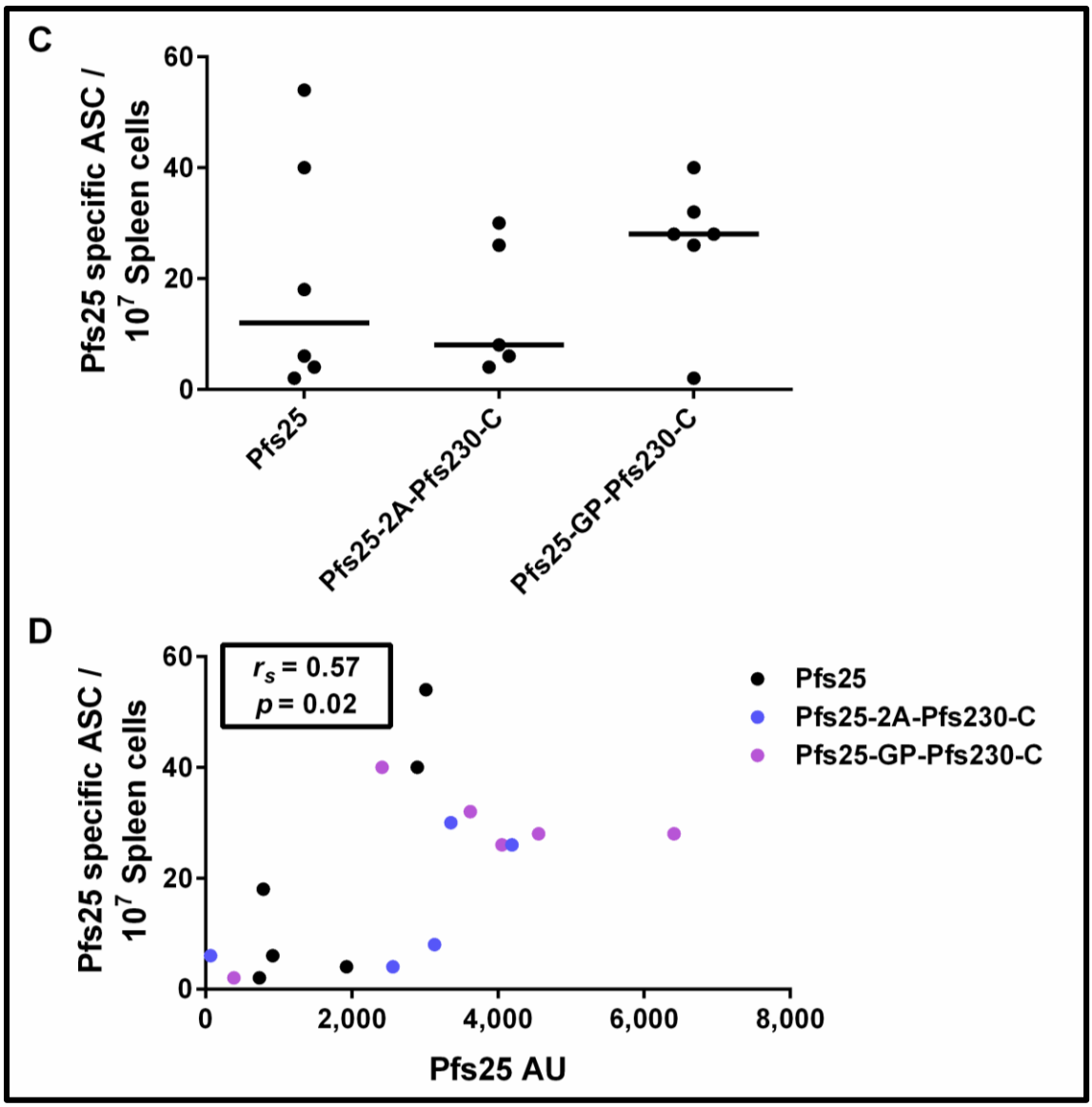


Figure 5.15. Antibody-secreting cell responses to fusion vaccines.

BALB/c mice (n=5-6/group) were vaccinated as described in **Figure 5.12.** At day70, spleens from each individual mouse were used to isolate splenocytes. ASCs specific for Pfs230-C (**A and B**) and Pfs25 (**C and D**) were measured by ELISPOT. (**A and C**) shows antigen-specific ASC responses/10^7 splenocytes; and (**B and D**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as medians and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. Asterisks represent statistical significance. ASC: antibody-secreting cell.

5.2.4.4. Assessment of *P. falciparum* NF54 infectivity to *A. stephensi*

To test whether there was additive, synergistic, and/or comparable TBA against *P. falciparum* NF54 in SMFA, day 70 sera from all vaccinated animals/group was pooled. In addition, sera from animals vaccinated with ChAd63-MVA expressing GFP was used as control. Total IgG (4mg/ml) was purified and IgG responses for the pooled sera determined by standardised ELISAs for each antigen (**Table 5.3**). To determine any evidence of additivity, synergism or comparability, the same concentration (750μg/ml, 1:5.34 dilution) of purified total IgG was tested in two independent SMFA experiments (**Figure 5.16**).

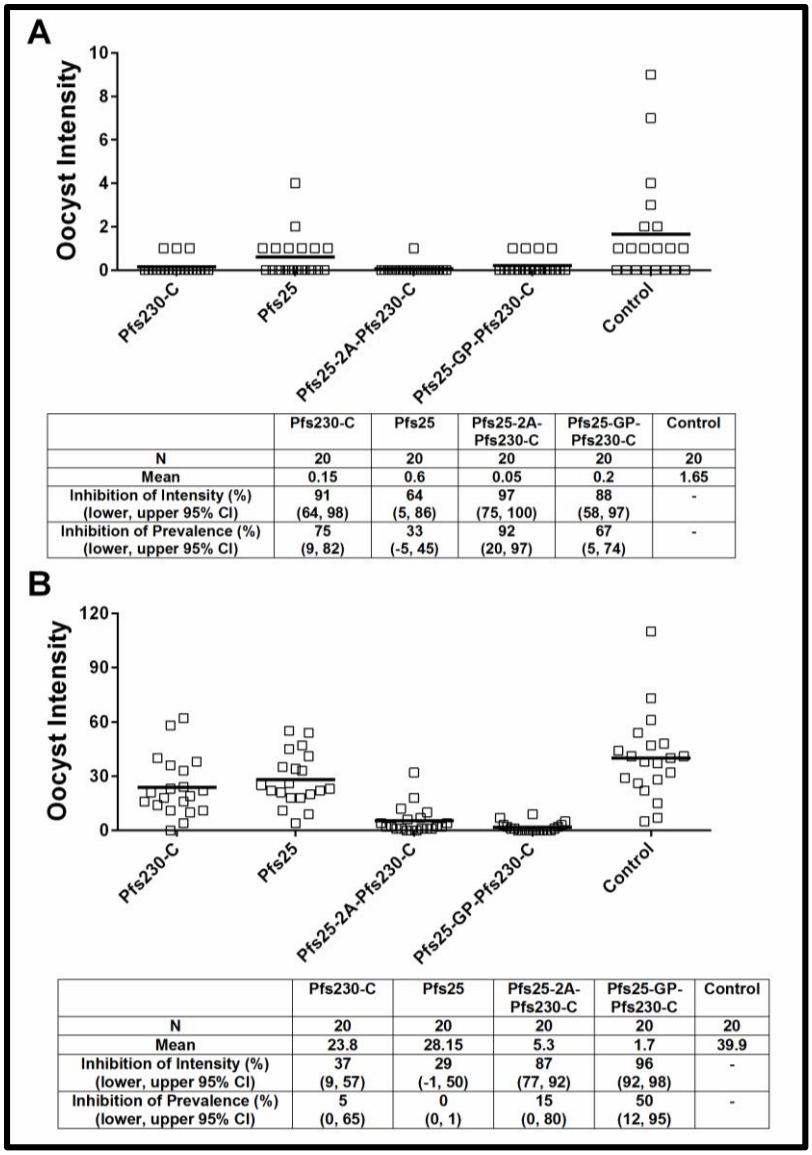
Overall, there was comparable inhibition of parasite intensity and prevalence mediated by antibodies against Pfs25-2A-Pfs230-C and Pfs25-GP-Pfs230-C in both experiments (**Figure 5.16A** and **B**) regardless of the parasite exposure (mean control oocyst 1.65 and 39.9 respectively). In the first experiment, i.e. lower parasite exposure (**Figure 5.16A**), there was no evidence of any additive or synergistic effect in the ability to inhibit parasite intensity and prevalence by anti-Pfs25-2A-Pfs230-C IgG (97% and 92% respectively, $p<0.0001$) or anti-Pfs25-GP-Pfs230-C IgG (88%, $p<0.0001$ and 67%, $p=0.009$ respectively). This was due to the observation that inhibition against Pfs230-C was 91% ($p<0.0001$) and 75% ($p=0.003$) whilst that against Pfs25 was only statistically significant against parasite intensity (64%, $p=0.04$). However, in the second experiment, a synergistic effect at inhibiting parasite intensity and prevalence for both anti-Pfs25-2A-Pfs230-C IgG (87%, $p<0.0001$ and 15%, $p=0.04$) and anti-Pfs25-GP-Pfs230-C IgG (96% and 50%, $p<0.0001$) was observed (**Figure 5.16B**). This was more so as anti-Pfs25 IgG efficacy wasn't statistically significant whilst statistical significance against Pfs230-C was only observed at inhibiting intensity (37%, $p=0.02$) (**Figure 5.16B**).

Therefore, antibodies raised against Pfs230-C and Pfs25 had synergistic parasite inhibitory effects confirming what was observed with mixing IgG in SMFA (**Figure 5.1D**). Of note is that the antibody titre in the F2A group was almost 2-fold lower than that of GP or Pfs230-C groups. Thus the comparable ability to inhibit parasite infectivity further attests to the effectiveness of inducing a broad antibody response.

Table 5.3. Antibody titres for pooled sera used to purify total IgG in SMFA

Group	Pfs230-C titre	Pfs25 titre
Pfs230-C	33,556AU	-
Pfs25	-	1,339AU
Pfs25-2A-Pfs230-C	16,978AU	3,183AU
Pfs25-GP-Pfs230-C	32,112AU	4,155AU

Antibody titres determined from an aliquot of sera from pooled vaccinated mice (n=5/6).



	Pfs230-C	Pfs25	Pfs25-2A-Pfs230-C	Pfs25-GP-Pfs230-C	Control
N	20	20	20	20	20
Mean	0.15	0.6	0.05	0.2	1.65
Inhibition of Intensity (%) (lower, upper 95% CI)	91 (64, 98)	64 (5, 86)	97 (75, 100)	88 (58, 97)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	75 (9, 82)	33 (-5, 45)	92 (20, 97)	67 (5, 74)	-

	Pfs230-C	Pfs25	Pfs25-2A-Pfs230-C	Pfs25-GP-Pfs230-C	Control
N	20	20	20	20	20
Mean	23.8	28.15	5.3	1.7	39.9
Inhibition of Intensity (%) (lower, upper 95% CI)	37 (9, 57)	29 (-1, 50)	87 (77, 92)	96 (92, 98)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	5 (0, 65)	0 (0, 1)	15 (0, 80)	50 (12, 95)	-

Figure 5.16. Assessment of inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*.

Total IgG was purified (4mg/ml) from sera (day70) pooled from mice vaccinated with each respective antigen or GFP (control) (**5.2.4.2**). The total IgG was diluted at 750µg/ml in PBS, mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* in SMFA. Midguts were dissected 7-8days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as the arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI. Two independent experiments were performed (**A)** and (**B**). Squares represent oocyst numbers from each individual mosquito dissected.

5.2.5. Pfs230-C and Pfs25 protein-in-adjuvant responses

Considering that the majority of regimen involving TBV candidate antigens have been with protein-in-adjuvant formulations, the two most promising targets, Pfs230-C and Pfs25, were evaluated in the traditional three-shot protein-in-adjuvant regimen (PPP). Recombinant Pfs230-C protein produced by wheat-germ cell-free expression system [451] and recombinant Pfs25 produced in yeast [339] were used for vaccinations. AbISCO®-100 which is a phospholipid, cholesterol and saponin complex (containing a mixture of fractions purified from Quil A) adjuvant was used [578, 579]. This adjuvant has been tested in pre-clinical [270, 579-584] and clinical studies [585] and shown to induce a balanced, broad Th1/Th2 antibody response. High antibody titres have been induced using this adjuvant irrespective of the antigen used [580, 581] and in comparison to clinically tested and approved adjuvants [583, 584]. There is, however, no published evidence of the use of this adjuvant with TBV candidate antigens.

5.2.5.1. Protein-in-adjuvant vaccine-induced responses

The initial experiment was to determine whether Pfs230-C and Pfs25 would be immunogenic when formulated with AbISCO®-100. Thus age-matched BALB/c mice were vaccinated with 1.5μg of either protein in the recommended 12μg/dose of adjuvant. Vaccinations were administered four weeks apart and antibody responses assayed by standardised ELISA two weeks post every vaccination. After the first shot, only a few mice in the Pfs230-C group hadn't seroconverted whilst none of the mice in the Pfs25 group seroconverted (**Figure 5.17**). After the second shot, all the animals had seroconverted with detectable antibody responses. However for Pfs25, only one animal had titres above the IC_{50} titre (600AU in mice) but after the third vaccination, mean titres were 16-fold higher than the IC_{50} titre. Furthermore, responses were boosted (Wilcoxon matched-pairs test, p=0.008) after every vaccination at

varying magnitudes. Pfs230-C antibody responses were 47-fold and 13-fold higher (**Figure 5.17A**) whilst Pfs25 responses were 66-fold and 98-fold higher after the second and third vaccinations respectively (**Figure 5.17B**).

Additionally, induction of IgG isotypes was assayed two weeks after the last vaccination. Sera was used to detect IgG1, IgG2a, IgG2b and IgG3 by endpoint ELISAs. There was a mixed isotype profile induced with a bias towards an IgG1 dominant profile (**Figure 5.18**). All four isotypes were induced by vaccination against Pfs230-C even though not all the animals had a detectable IgG3 response (**Figure 5.18A**). In regards to Pfs25, only IgG1, IgG2a and IgG2b were induced (**Figure 5.18B**). Furthermore, ASC responses were determined by ELISPOT assays using spleens at the final time point and association with antibody responses determined by Pearson's correlation test (r) **(Figure 5.19)**. Antigen-specific ASCs were detected against Pfs230-C and Pfs25 (**Figure 5.19A**). However, there was no correlation between the antibody and ASC responses to either antigen (**Figure 5.19B**).

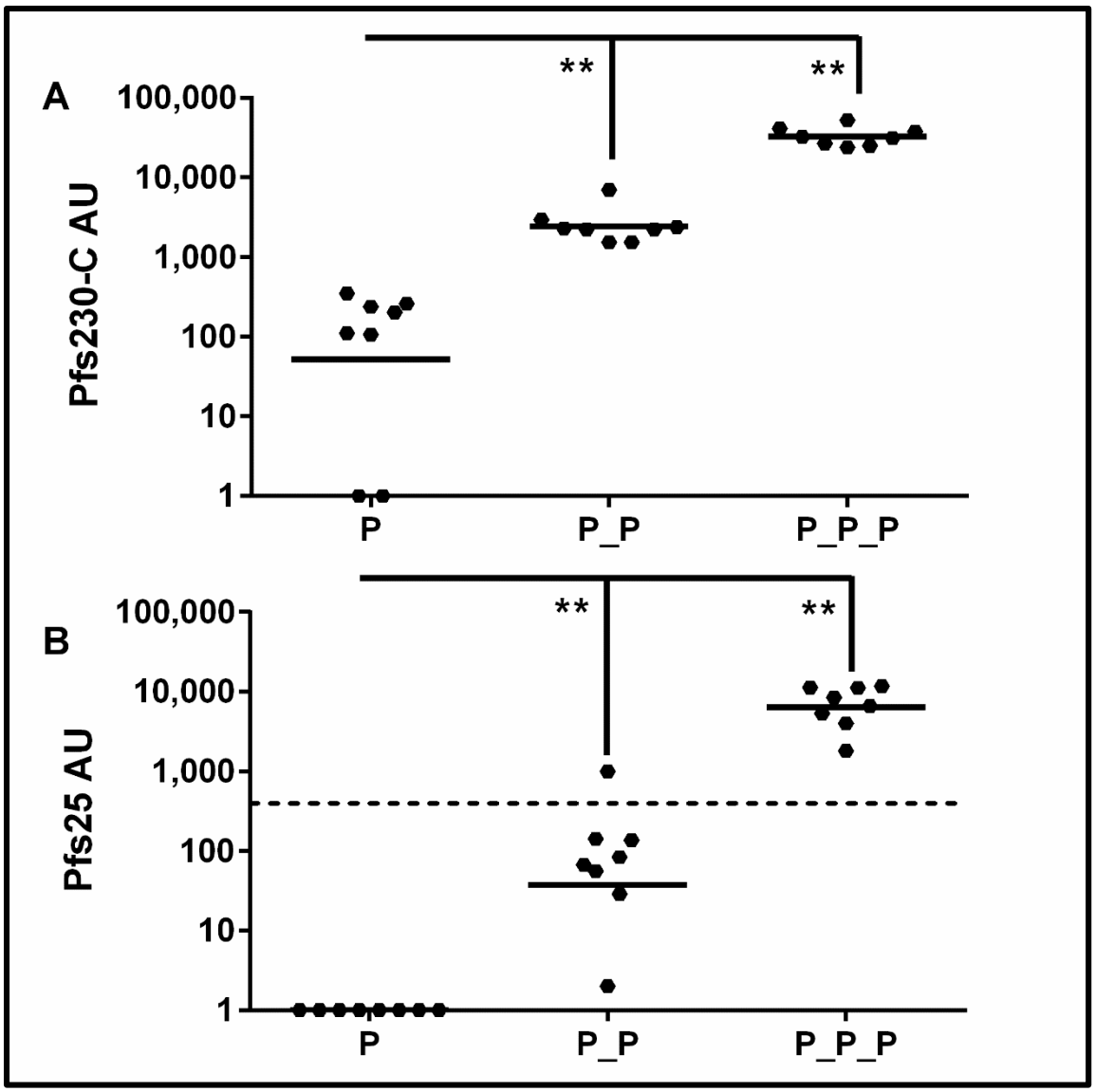


Figure 5.17. IgG responses to Pfs230-C and Pfs25 after protein-in-adjuvant vaccination.
BALB/c mice (n=8/group) were vaccinated i.m. with 1.5μg of either Pfs230-C or Pfs25 recombinant protein in AbISCO®-100 administered in three vaccinations four weeks apart. Total IgG responses were measured every two weeks after the first (P), second (P_P) and third (P_P_P) shots by standardised ELISA using (**A**) Pfs230-C and (**B**) Pfs25 recombinant protein. The data was log-transformed and presented as geometric mean with dots representing responses from individual mice. Dotted line in (**B**) represents mouse-specific Pfs25 IC_{50} antibody units. Significance shown and considered at $p<0.05$. Asterisks represent statistical significance.

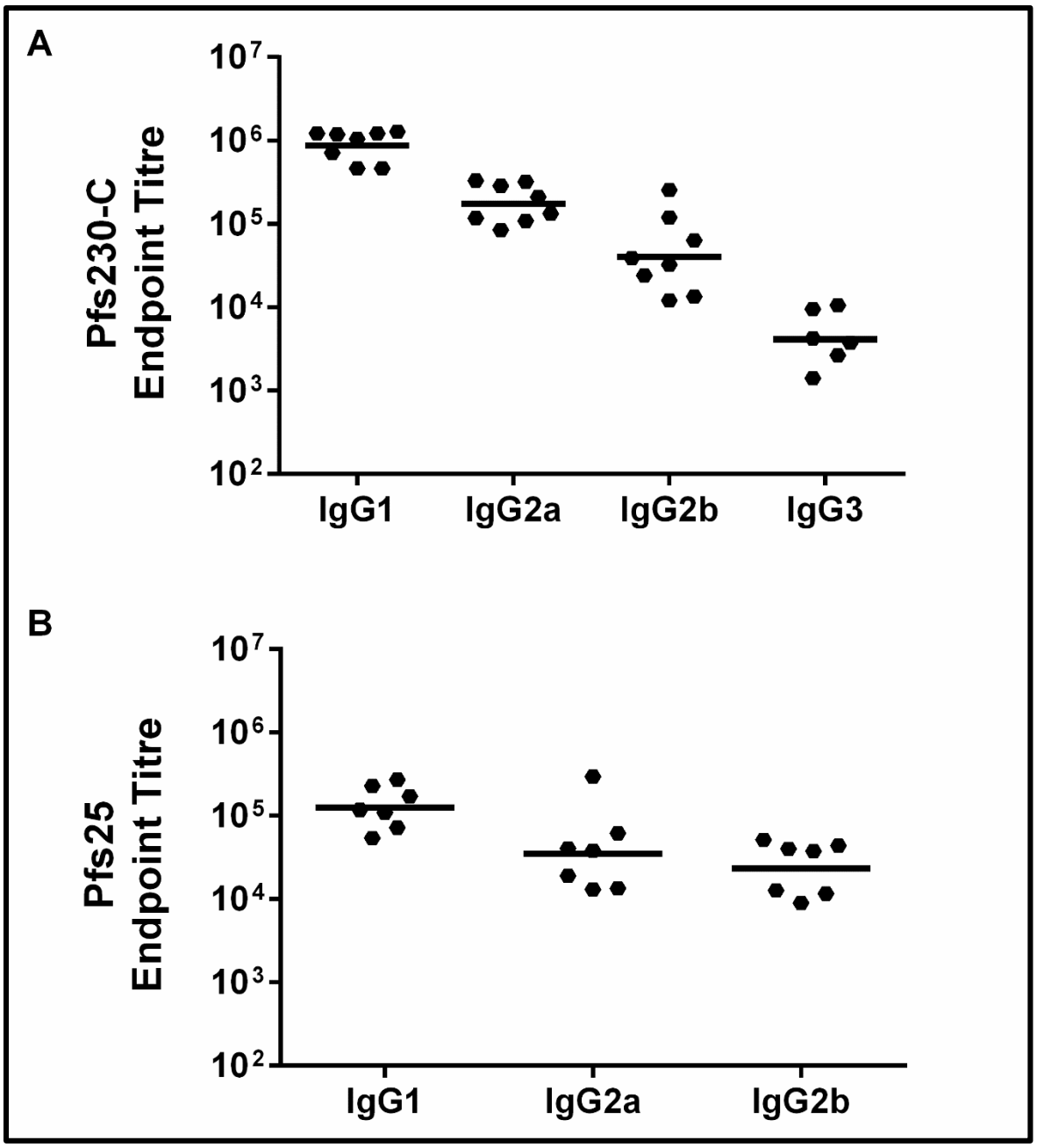


Figure 5.18. Induction of IgG isotypes following protein-in-adjuvant vaccination.

Sera from BALB/c mice (n=8/group) previously vaccinated (**5.2.5.1**) two weeks post final boost were used to assay IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) by endpoint ELISAs using recombinant protein against (**A**) Pfs230-C and (**B**) Pfs25. The data was log-transformed and presented as geometric mean with dots representing responses from individual mice.

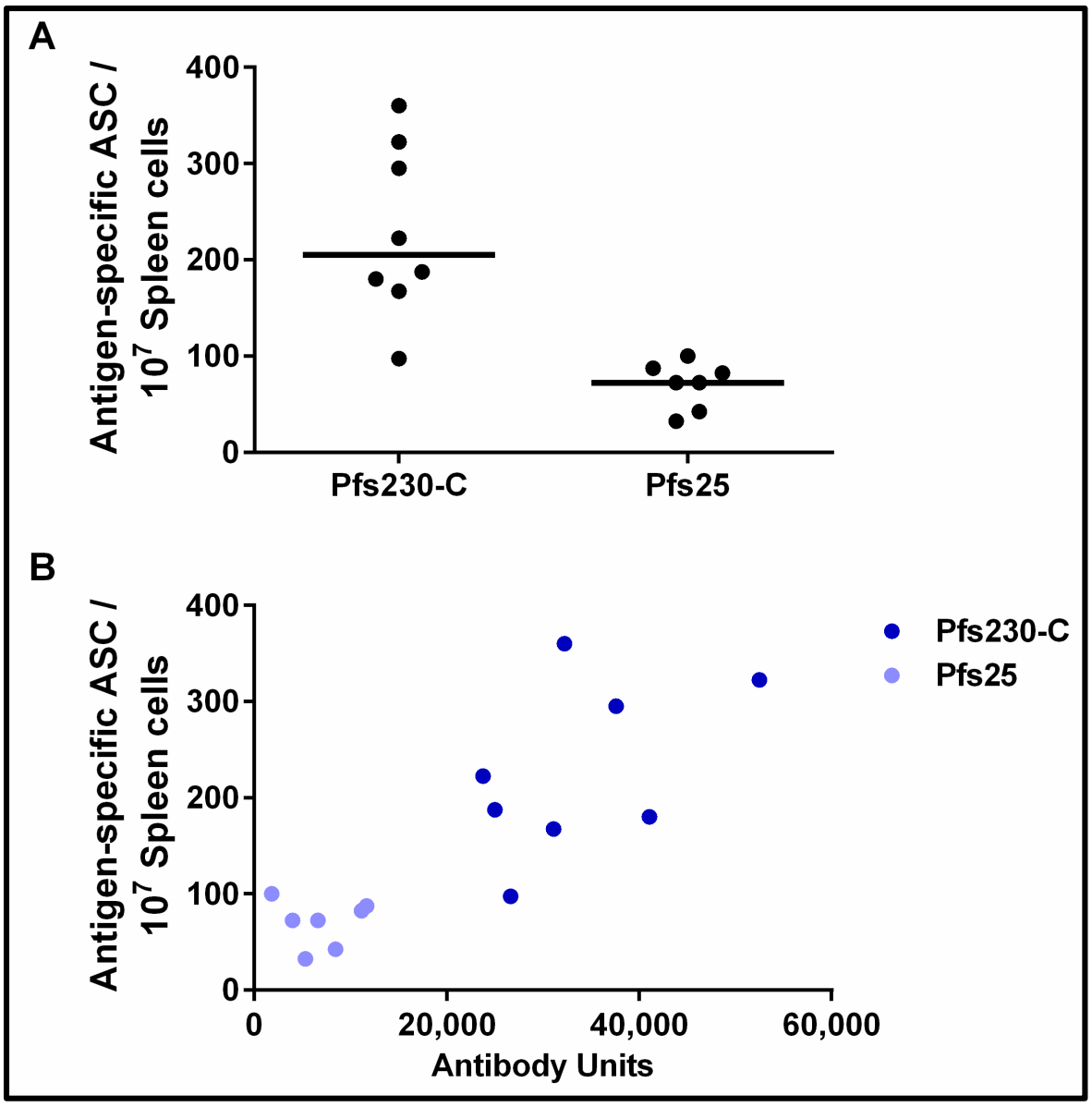


Figure 5.19. ASC responses to Pfs230-C and Pfs25 protein-in-adjuvant vaccination.

BALB/c mice (n=8/group) previously vaccinated as described in **Figure 5.16.** Two weeks post final boost, spleens from each individual mouse/group were used to isolate splenocytes. ASCs specific for Pfs230-C and Pfs25 were measured by ELISPOT. (**A**) Shows antigen-specific ASC responses/10^7 splenocytes; and (**B**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as medians and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. ASC: antibody-secreting cell.

5.2.5.2. Pfs230-C and Pfs25 protein-in-adjuvant co-administration

Vaccine-induced responses against Pfs230-C and Pfs25 ChAd63-MVA co-administration showed antigen interference (**5.2.2.1**). To determine antigen interference in PPP of antibody and ASC responses, co-administration experiments were conducted. Age-matched BALB/c mice were vaccinated with Pfs230-C and Pfs25 combinations either administered in separate sites or mixed in the same syringe. Control combinations were with respective antigen and ovalbumin (OVA). Comparisons were made between method of vaccine administration (separate sites vs. same syringe) and effect of antigen (antigen vs. control).

Antibody responses were assessed two weeks after every vaccination by standardised ELISA. Anti-Pfs230-C and anti-Pfs25 antibody responses were boosted in all the groups after the first, second and third shots at varying magnitudes (**Figure 5.20**, $p<0.0001$). Unlike ChAd63-MVA induced responses, there were no statistically significant differences observed in anti-Pfs230-C responses after the first shot (**Figure 5.20A**). However, a 2-fold difference was observed after the second shot when Pfs230 was co-administered with OVA in the same syringe in comparison to administration with Pfs25 (Pfs230-C+OVA vs. Pfs230-C+Pfs25, **Figure 5.20A**, $p<0.0001$), where responses in the former were lower than the latter. Nonetheless, these responses were comparable after the third shot with no statistically significant differences across all the groups. In regards to anti-Pfs25 PPP responses (**5.2.5.1**), there were no or low detectable antibody responses irrespective of the combination or method of co-administration after the first vaccination (**Figure 5.20B**). Even so, responses induced by subsequent vaccinations were detectable in all vaccinated animals irrespective of group. Furthermore, there were no statistically significant differences observed regardless of antigen or method of co-administration at all time points ($p>0.05$). Thus taken together, there was no evidence of antigen interference against either Pfs230-C or Pfs25.

Antigen-specific ASC responses were measured from spleens by ELISPOT two weeks post-boost **(Figure 5.21)**. Antigen-specific ASCs were detected in all groups for both antigens with a few non-responders. In spite of this, there were no statistically significant detectable differences across groups for both antigens as observed for antibody responses at this peak time point (**Figure 5.21A** and **C**). However, there was a trend to lower median responses in groups receiving OVA co-administered with either antigen in the same syringe (Pfs230-C+OVA and Pfs25+OVA), [consistent with trends observed in antibody responses] though not statistically significant. Interestingly, there was no correlation observed between antibody and ASC responses irrespective of group and even when all responses were pooled against Pfs230-C (**Figure 5.21B**, r=0.06, *p*=0.5). On the other hand, against Pfs25, there was correlation across all groups between antibody and ASC responses (**Figure 5.21D**, r_s=0.42, *p*=0.02).

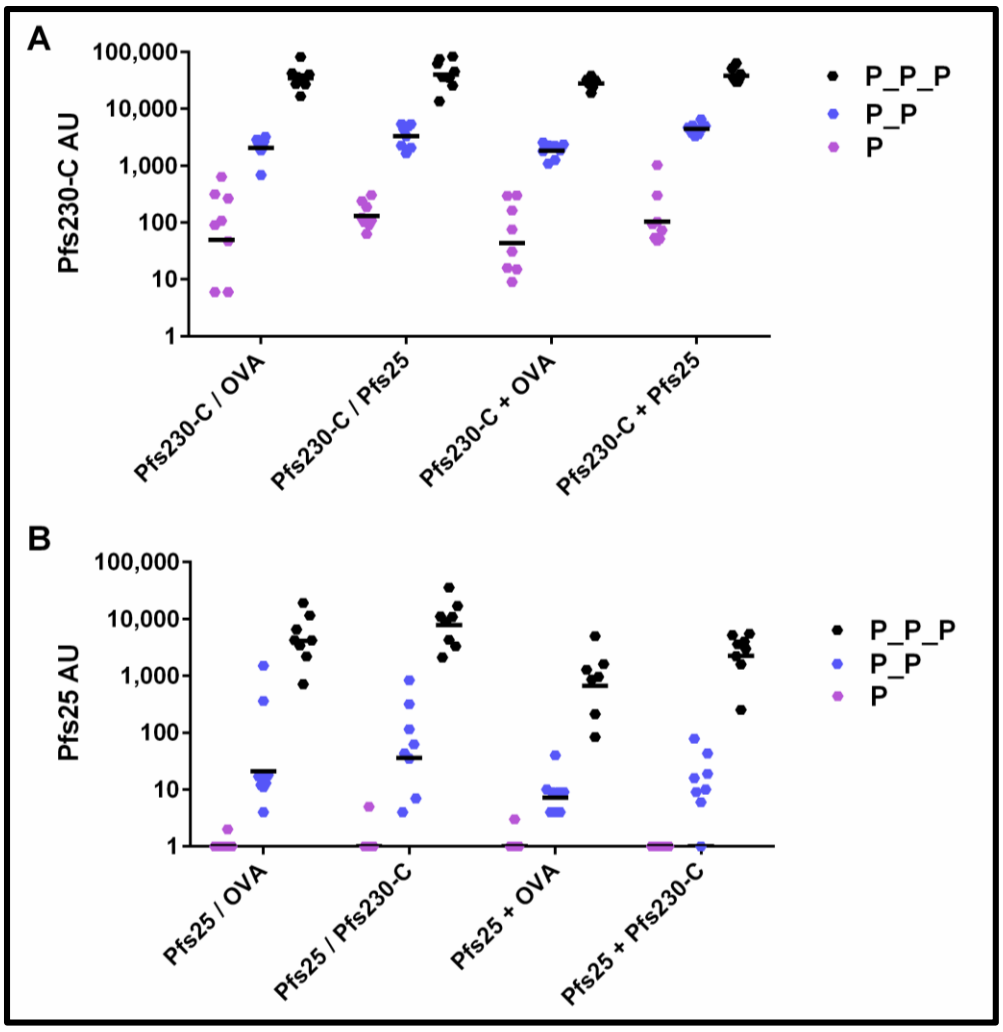
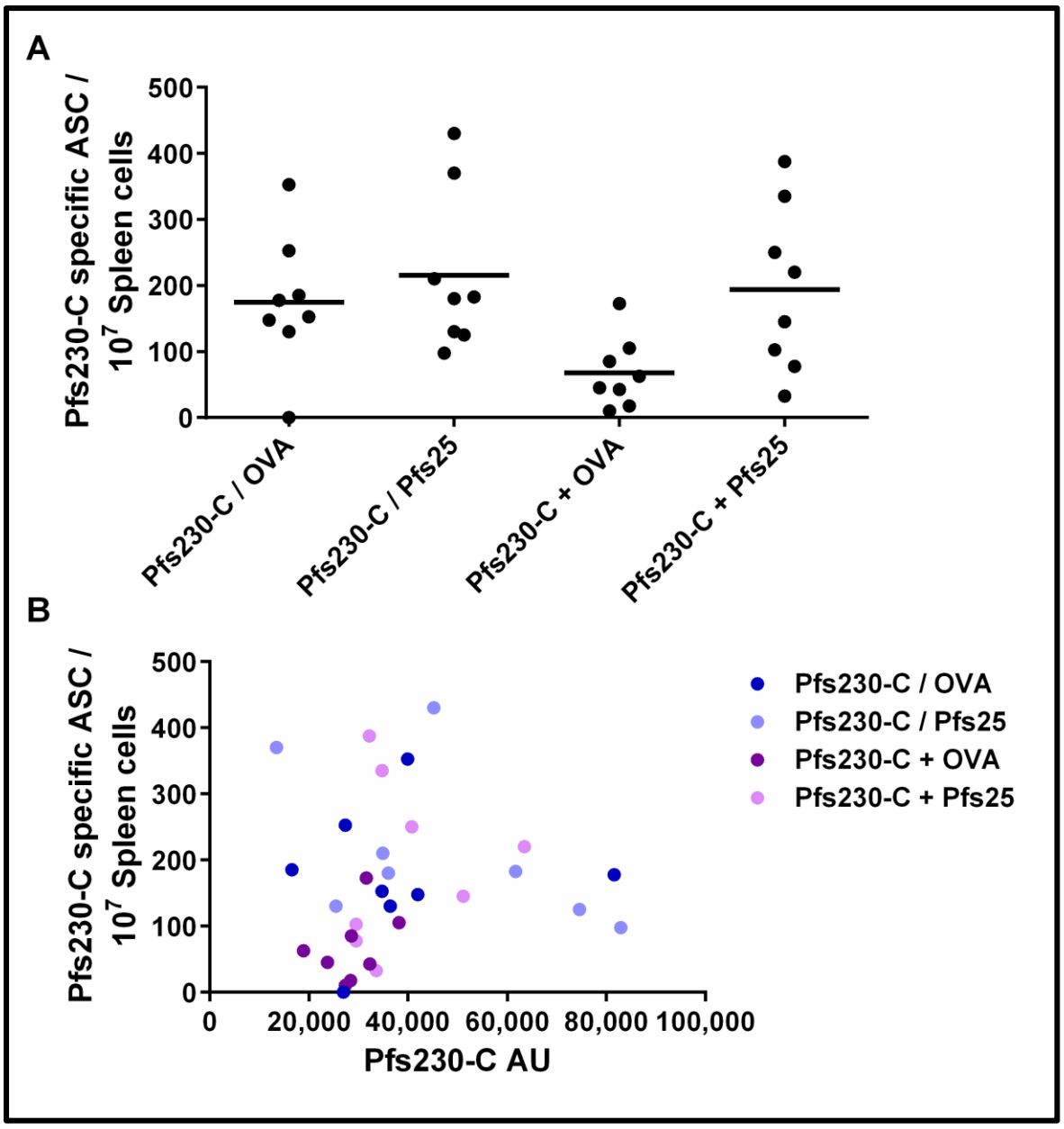


Figure 5.20. Pfs230-C and Pfs25 IgG responses to protein-in-adjuvant co-administration.

BALB/c mice (n=8/group) were vaccinated i.m. with 1.5μg of either Pfs230-C, Pfs25 or OVA recombinant protein in AbISCO®-100 co-administered in separate sites (denoted by /) or mixed in the same syringe (denoted by +). IgG responses were measured every two weeks after the first (P), second (P_P) and third (P_P_P) shots four weeks apart by standardised ELISA using (**A**) Pfs230-C and (**B**) Pfs25 recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points per group. Dots represent responses from individual mice.


A
Pfs230-C specific ASC / 10^7 Spleen cells
500
400
300
200
100
0
Pfs230-C / OVA
Pfs230-C / Pfs25
Pfs230-C + OVA
Pfs230-C + Pfs25
B
Pfs230-C specific ASC / 10^7 Spleen cells
500
400
300
200
100
0
0
20,000
40,000
60,000
80,000
100,000
Pfs230-C AU
Pfs230-C / OVA
Pfs230-C / Pfs25
Pfs230-C + OVA
Pfs230-C + Pfs25

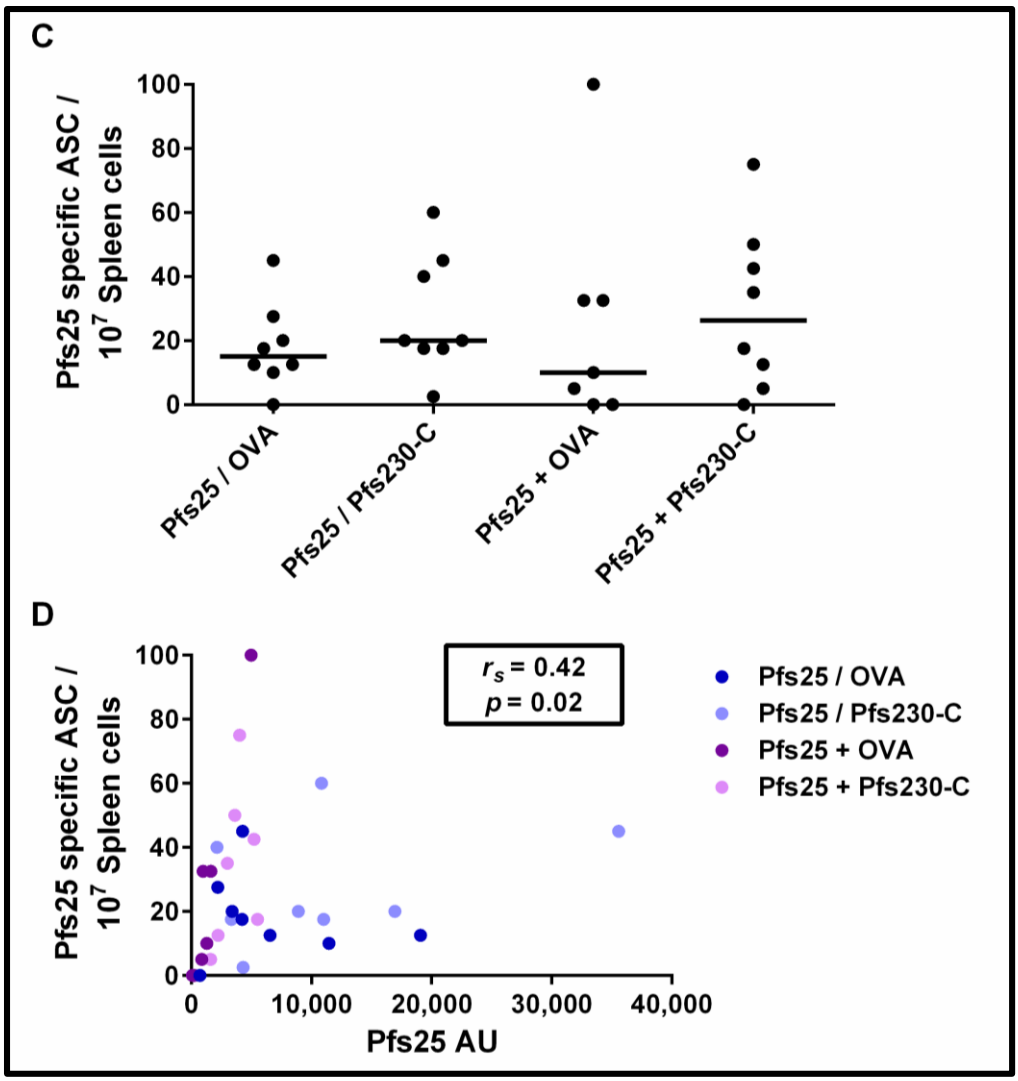


Figure 5.21. Pfs230-C and Pfs25 ASC responses to protein-in-adjuvant co-administration.

BALB/c mice (n=8/group) were vaccinated as described in **Figure 5.22**. Two weeks post final boost, spleens from each individual mouse/group were used to isolate splenocytes from which ASCs specific for Pfs230-C (**A and B**) and Pfs25 (**C and D**) were measured by ELISPOT. (**A and C**) shows antigen-specific ASC responses/group; and (**B and D**) shows correlation of antigen-specific ASC responses with antibody titres. ASC responses are represented as medians and circles represent individual responses from each animal. Significance shown and considered at $p<0.05$. ASC: antibody-secreting cell; OVA: ovalbumin.

5.3. Discussion

This chapter describes the evaluation of antigen combinations using the most efficacious antigens described in **Chapter 4**. This work has demonstrated that a number of approaches and strategies can be utilised to down-select candidate antigens to be considered for further testing. Considering the first approach used for down-selection, it was difficult to determine and tease apart the collective contribution towards efficacy within SMFA. On the other hand, when the vaccines were evaluated for immune interference, there were differential effects observed on vaccine-induced responses depending on the antigen combination and method of co-administration. The only antigen combination that had no evidence of immune interference was that of Pfs25 and Pfs48/45$_{+NGln}$. However, when Pfs230-C and Pfs25 were co-expressed as fusion vaccines, antibody responses induced using two fusion strategies resulted in comparable synergistic TBA.

Evaluating whether additivity or synergy occurred by mixing IgG showed variability in SMFA between individual experiments. This variability between feeds has been reported where no single feed gave identical efficacy [328]. This could be due to the fact that each individual feed has an independent parasite exposure (represented by the mean control oocyst numbers) [378, 586]. Furthermore, there is a limitation in trying to determine additivity or synergism as each IgG has its own independent dose-dependent inhibitory effect. This is so as anti-Pfs48/45$_{+NGln}$ IgG [in all experiments analysed] had efficacy only up to 164μg/ml whilst anti-Pfs230-C and anti-Pfs25 IgG had efficacies even at concentrations of 43 and 10.75μg/ml respectively. Thus it would be ideal to determine the IC$_{50}$ of each independent IgG and then determine the possibility of additivity or synergistic inhibitory effects. However, this would have to be done over several experiments and derived as previously published by Cheru et al. (2010) [572]. An alternative approach would be to test anti-Pfs25

antibodies (most potent antibodies) at a fixed concentration against varying concentrations of anti-Pfs230-C and/or anti-Pfs48/45$_{+NGln}$ IgG. This has been employed by Williams et al. (2012) investigating additivity or synergy by mixing vaccine-induced blood-stage antibodies within an assay [587]. However, these approaches are experimentally laborious and as observed [against Pfs25] and highlighted would result in different observations unless qualified as reported by Miura et al. (2013) [586]. On the other hand, it has been reported that mixing Pfs25 and Pfs28 in a single vaccination didn't induce synergistic TB effects [529] compared to mixing of anti-sera in SMFA [328]. This has also been demonstrated against Pvs25 and Pvs28 [588]. However, it was clear that the most potent, anti-Pfs230-C and anti-Pfs25 IgG had synergistic effects when mixed in SMFA tested at supposed IC_{50} concentrations and consistently inhibited parasite infectivity regardless of the parasite exposure.

In order to determine potential antigen synergies, an additional strategy was evaluated by vaccine co-administration. In this instance, immune interference was observed depending on the antigen combination utilised. The most promising candidate antigens, Pfs230-C and Pfs25, showed immune interference with regards to the mode of administration in respect to antibody responses. This fits in with the hypothesis that delivering antigen in separate sites would result in less antigenic interference or competition as the antigens would potentially drain to different compartments as opposed to mixing in the same syringe. However, in this study, it was observed that this interference appeared to be specific to ChAd63-MVA in comparison with protein-in-adjuvant co-administration for Pfs230-C and Pfs25 antigen combinations. This could be due to the possibility of viral-vectors inducing a by-stander immune response against a possibly immunodominant antigen that interferes with responses to the other co-administered antigen [589-591]. The other possibility is that the different

regimes would be delivering antigens to different draining sites thus different mechanisms would be involved in inducing the response [592, 593]. Hence it would be interesting to further characterise the differences in the induction of antibody responses between these two regimens to determine the overall pattern of the ensuing response.

Miyata et al. (2011) on the other hand, alluded to a preliminary experiment mixing Pfs25 and Pvs25 antigens expressed in HuAd5 had no effect on the induction of antibody responses [340]. Thus antigen interference could be considered antigen-dependent as observed in this study. Moreover co-administration of Pfs230-C and Pfs48/45$_{+NGln}$ resulted in the reversal of what was observed with the co-administration of Pfs230-C and Pfs25. In the Pfs230-C and Pfs48/45$_{+NGln}$ combination, antibody responses were affected by administering in separate sites as opposed to mixed in the same syringe. Additionally, the combination of Pfs25 and Pfs48/45$_{+NGln}$ didn't seem to be affected in either methods of administration. There is limited published evidence on the impact on antibody responses with respect to comparisons of methods of co-administration with a number of studies focusing on mixing in the same syringe and administered in the same site. Sedegah et al. (2004) comparatively assessed antibody responses induced by DNA vaccination in the same site or separate sites [545]. Antibody responses to CSP were shown to be suppressed when administered in the same site as a mixture with 8 different antigens in comparison to separate sites [545]. On the other hand, Forbes et al. (2010) have shown that mixing HuAd5-MVA CSP and MSP in the same syringe had no impact on antibody responses to either antigen but immune interference was observed with cell-mediated immunity [549]. This effect on T-cell responses wasn't observed when the MVA boost was administered in separate sites [549]. However, a more recent study mixing CSP and AMA1 in a DNA-Adenovirus prime-boost regimen has shown sterile protection mediated by cellular immunity with no interference reported [269]. This is in

comparison to efforts when both antigens were administered mixed either as DNA or Adenovirus homologous vaccinations [401, 594]. Additionally, other studies in influenza have shown that co-administration of different antigens improves and induces a broad range of responses [595, 596]. Therefore, co-administration might be advantageous however it still remains to be determined whether there is an impact on TBE as a result of immune interference. Thus this approach might not reflect what might be observed when target antigens are co-expressed in the same virus.

A recurring theme is the need to induce high enough antibody titres to block parasite development within the mosquito. This was evident in rabbits. Thus testing vaccine-induced efficacy in more than one species should also inform down-selection of candidate antigens. However, Cheru et al. (2010) have shown, at least for Pfs25, that there is a species-effect in the amount of antibody required to induce inhibitory effects [572]. Therefore, it should be imperative to comparatively assess the efficacy induced against the three candidate antigens [tested in this chapter] in more than one species. Vaccine-induced responses to Pfs25 have been reported to be poor and variable in humans [285] with efficacy being antibody titre-dependent [191]. Variability has been demonstrated here not only for Pfs25 but also for Pfs230-C and Pfs48/45$_{+NGln}$. In the rabbit model, where testing of individual vaccine-induced responses was possible, efficacy was largely dependent on antibody titre. This was evident against Pfs25 even with respect to pooled responses. This was also true for Pfs48/45$_{+NGln}$, where antibody titre influenced not only the level of efficacy mediated but also the recognition of native parasite antigen. More so, in the mouse model, especially for Pfs25, inhibition of parasite infectivity was also dependent on the titre of the pool. Therefore, it is paramount that vaccine delivery platforms induce high antibody titres regardless of the animal model to have a significant effect on parasite infectivity. Thus evaluation of viral-

vectored responses of the most efficacious strategy in non-human primates would have to be tested.

This study has also described responses against Pfs230-C and Pfs25 in a protein-in-adjuvant regimen. Both recombinant antigens were made in expression systems shown to produce correctly folded, immunogenic proteins with potent TBA [597, 598]. The IgG isotype profile induced in this protein-in-adjuvant regimen against both antigens was consistent with published PPP findings [324]. This observation has the potential to affect antibody-mediated inhibitory effects against Pfs230-C as inhibition has been shown to be isotype-dependent [173], and could explain the lack of incomplete inhibition mediated against Pfs230-C recently observed [324].

In order to induce antibody responses that would have additive and/or synergistic effects on parasite infectivity, fusion vaccines consisting of Pfs25 and Pfs230-C were generated. These two candidate antigens induced the most potent TB antibodies with induced antibodies targeting different forms of the parasite. Antibodies against Pfs230-C have been shown to act by inhibiting fertilisation of gametes whilst those against Pfs25 inhibit ookinete formation [373]. A number of malaria antigens have been expressed as fusion vaccines including Pfs25 and Pfs28 [529, 530]. However, this study provides the first evidence of the fusion of TBV candidate antigens expressed on different sexual-stages, down-selected for their ability to induce potent TB antibodies. The advantage of incorporating more than one antigen enables the induction of broad responses that have inhibitory effects. This has been widely demonstrated for combinations involving blood-stage candidate antigens mainly MSP1 fused to either AMA1 or MSP8 [249, 524-526, 599, 600]. More so, there is evidence in HIV, influenza and tuberculosis that fusion vaccines are a better approach [418, 419, 535, 601, 602]. The other advantage is that this would result in one ChAd63 and one MVA being

manufactured for clinical testing as opposed to two ChAd63s and two MVAs expressing the respective antigens.

In this chapter, comparison of two fusion strategies to ensure that the best approach was advanced for further testing and development has been demonstrated. It was encouraging to observe comparable antibody responses being induced between the two fusion strategies which had synergistic effects at inhibiting parasite infectivity in one out of two experiments. Ideally, it is important to test against different parasite exposures to get a better understanding of the impact of antibody responses. Ponnudurai et al. (1986) showed that lower parasite exposures resulted in antibodies having enhanced inhibitory activity possibly due to an increased antibody:parasite ratio [376]. Thus it would be difficult to determine the combined effects of the antibodies. This is more so as synergistic effects of Pfs230-C and Pfs25 were only evident against a 'higher' parasite exposure in comparison to a 'lower' exposure. This is further confirmed with studies in **Chapter 4**, where anti-AnAPN1$_{fl}$ IgG was found to have efficacy against field *P. falciparum* field isolates in one out of three independent experiments performed which was also true for Pfs48/45 variants (**Figure 4.12**). However, when the two experiments were analysed together, the evidence of synergistic effects was very evident against both Pfs230-C and Pfs25 (data not shown).

Of note, Pfs230-C responses induced by F2A were lower than those induced by ChAd63-MVA-Pfs230-C possibly as a result of incomplete cleavage. Lack of complete cleavage could be improved with longer F2A N-terminal extensions as described by Donnelly et al. (2001) [555] and Funston et al. (2008) [603]. However, these extensions would remain at the C terminal end of Pfs25 and possibly induce an immune response which could interfere with the desired antigen-specific responses. Therefore, it would be pertinent to determine if (a) antibodies are induced to the F2A peptide (b) there is an effect on protein conformation by

evaluating ability to recognise native parasite antigen; (c) antibody responses decline over time due to the co-expression of the antigens; and (d) re-design of the construct to include a furin sequence would allow removal of the external C-terminus. Studies by Fang et al. (2005) have shown that the addition of a furin proteinase cleavage site upstream of the F2A sequence results in the removal of the F2A sequence [604].

Summary of overall findings:

- It was difficult to ascertain additivity and/or synergy in SMFA as the most potent antibodies resulted in complete inhibition of parasite infectivity with anti-Pfs230-C and anti-Pfs25 IgG being the most potent irrespective of parasite exposure.
- Immune interference was dependent on the mode of administration and the antigen combination utilised with differences observed against Pfs230-C and Pfs25 between viral-vectored and protein-in-adjuvant regimens.
- Efficacy was dependent on IgG concentration and antibody titre which strongly correlated with inhibition of both parasite intensity and prevalence.
- Combination of Pfs230-C and Pfs25 using either F2A or GP resulted in comparable responses induced against both antigens that strongly correlated with ASC responses and had comparable synergistic effects against parasite infectivity.

6. Transmission-blocking *P. berghei* Model

6.1. Introduction

The understanding of the pathogenesis of *P. falciparum* infections has in part been aided by the use of animal models. Animal models with their counterpart permissive *Plasmodium* species have enabled the pre-clinical discovery and testing of vaccine candidate antigens. TBI was first described using the avian malaria parasite-mosquito model of *P. gallinaceum* – *Aedes aegypti* [144, 145, 166, 167, 605, 606]. The use of this avian parasite enabled identification of antigens on gametocytes and/or gametes responsible for TBA [169, 607-609]. This led to the identification of orthologs in *P. falciparum* [322, 373, 486, 487, 610, 611]. Discovery and identification of rodent *Plasmodium* species [612] has further necessitated identification of targets and mechanisms of TBI. In particular, rodent *Plasmodium* parasites have proved very useful in the testing of potential candidate vaccine efficacy especially *P. berghei*. More so, the generation of transgenic parasites expressing not only *P. falciparum* but also *P. vivax* antigens have enabled the evaluation of vaccine candidate efficacy [370, 372, 613-615]. This hasn't only been useful in testing TBV candidate antigens but also pre-erythrocytic and blood-stage parasite antigens [616-618].

The use of rodent *Plasmodium* species has also further led to the elucidation of mechanisms involved in mediating transmission blockade to susceptible mosquito species [146, 152, 153, 619, 620] and importantly, the identification of candidate antigen *P. falciparum* orthologs [621, 622]. Sequence alignments [308, 313, 623] and gene knock-outs [293, 294, 334] have aided identification of putative *P. berghei* TBV candidate antigens. However, the most characterised *P. berghei* sexual-stage antigen is the ortholog of Pfs28, Pbs21 (henceforth referred to as Pbs28) [325, 620, 624, 625]. Antibodies raised against Pbs28 expressed either as native antigen [626], in *E. coli* [627], or baculovirus [628, 629] have resulted in inhibition

of parasite development with vaccine-induced antibodies against *E. coli* expressed recombinant antigen being less potent.

Analysis of P230 protein structure of *Plasmodium* species revealed that the protein can be subdivided into two distinct regions that account for species-specific variability as well as protein conservation [313]. Doi et al, (2011) were able to show that Pfs230 was more closely related to Pbs230 than Pvs230 and arose from a recently diverged common ancestor [313]. Genomic studies reveal that Pbs230 and Pfs230 are highly conserved [622] with both playing a vital role in male fertility [294]. However, disruption of Pbs230 results in a phenotype distinct from that observed in Pfs230 knock-out parasites [294, 318]. There is no published evidence of induction of antibodies to Pbs230 or their potential TBA. In spite of this, there is potential that upon expression, Pbs230 specific vaccine-induced antibodies would be able to inhibit parasite development.

As Pfs25 and Pfs28 are ookinete-specific proteins in *P. falciparum*, Pbs25 and Pbs28 are the respective *P. berghei* orthologs with redundant but distinct roles in ookinete and oocyst development [333, 334]. Double knockout of Pbs25 and Pbs28 genes has shown that parasites have reduced ookinetes and oocysts compared to single knockouts [334]. In comparison, Pbs28 and Pfs28 are more closely related than Pbs25 and Pfs25, however they all have risen from one common ancestor [308]. Moreover, antibodies raised against native Pbs25 have been able to inhibit parasite development in *A. stephensi* [630] and thus it remains to be shown whether recombinant expression could mediate the same effect.

Identification of Pfs48/45 ortholog in *P. berghei* revealed that Pbs48/45 is involved in fertilisation of gametes and development of zygotes [293, 294]. van Dijk et al. (2001) demonstrated that P48/45 proteins were conserved with a role in male fertility in both human *P. falciparum* and rodent *P. berghei* parasites [293]. A DNA-based vaccine expressing

Pbs48/45 didn't induce any detectable antigen-specific responses [521]. However, Haddad et al. (2006) were able to show that infection was able to boost antibody responses after a single DNA prime [521]. Considering that the role of Pbs48/45 in fertilisation is essential, it is yet to be established whether antigen-specific antibodies raised against this antigen have an effect on parasite development.

Pbs230 and Pbs48/45 are both essential for fertilisation [294]. Pbs25 is vital for development of the zygote to ookinete and oocyst stages and mediates ookinete transversal of the mosquito midgut [334, 335]. Thus antibodies raised against these antigens should block development of *P. berghei* oocysts. This mechanism of action can potentially be investigated by determining *in vitro* ookinete conversion rates [323, 361, 363, 365] and *in vivo* oocyst development in the mosquito midgut [323, 361, 365]. The *in vitro* ookinete development assay and SMFA, respectively, could thus be employed to measure the potential blockade of these two processes. Therefore, in the *P. berghei* model, TBA of antibodies could be investigated and verified using these approaches.

More recently, investigation of the potential blockade of vaccine-induced antibodies has employed use of either purified antigen-specific IgG [572] or total IgG [572, 586] (**Chapter 4 and 5**). This is to circumvent effect of other components of either serum or plasma that have been shown to mediate TBA [150, 151, 164]. Use of both serum and IgG from the same immunogen has however not been investigated for potential differential effects on parasite development. Nor have components of serum, apart from antibodies and complement, been fully identified that inhibit parasite development with indistinguishable roles for cytokines such as tumour necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) [156, 157, 162].

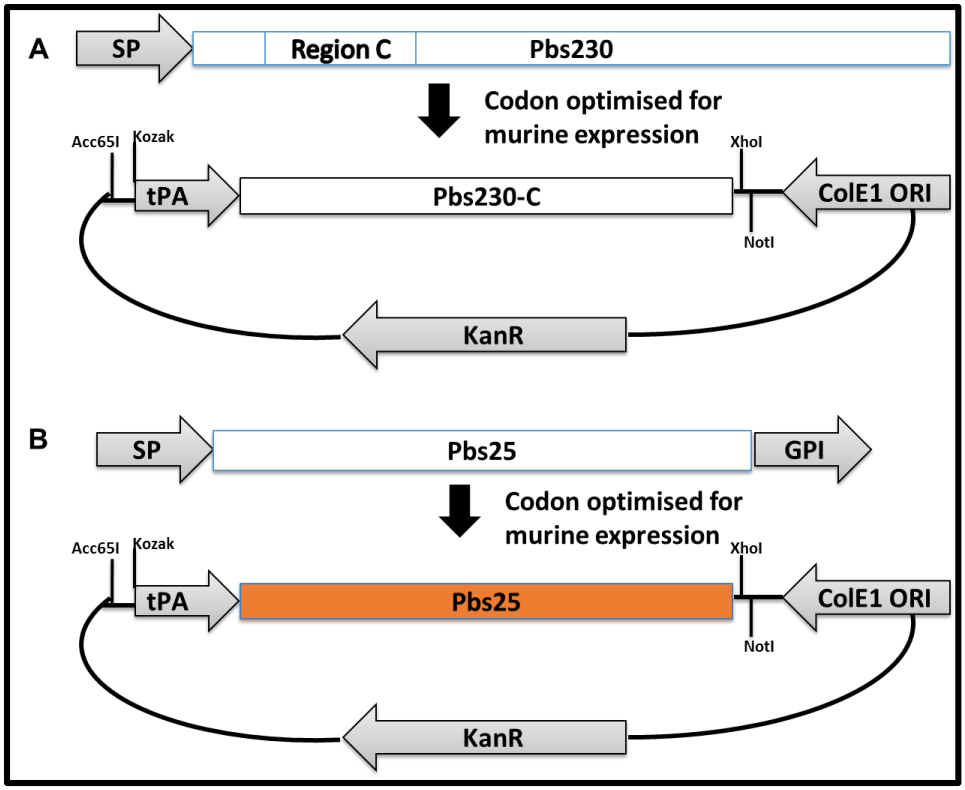
The similarity of sexual-stage antigens between *P. berghei* and *P. falciparum* led to the hypothesis that *P. berghei* would be a good model to simulate efficacy of *P. falciparum* TBV candidate antigens. Thus, complementary region C of Pfs230-C in *P. berghei*, Pbs230-C, Pbs25 and Pbs48/45 were generated in ChAd63 and MVA viral-vectors. For Pbs48/45, two variants were designed to test whether N-glycosylation site substitution had an impact on immunogenicity and parasite infectivity as observed in *P. falciparum*. As well as determining efficacy in SMFA, comparative quantification of efficacy was undertaken in an ookinete development assay as a possible predictor of SMFA efficacy outcomes.

6.2. Results

6.2.1. Design and generation of P. berghei vaccines

6.2.1.1. Antigen design

Protein antigen sequences were accessed from either the NCBI database or PlasmoDB Genomics Resource and bioinformatics determined for all antigens (**Table 2.2**). Antigens were designed as previously achieved for *P. falciparum* antigens (**Chapter 3**) (**Figure 6.1**). To test whether N-glycosylation site substitution had an effect on immunogenicity and efficacy, predicted N-glycosylation sites in Pbs48/45 were either substituted or left intact. The sequences were then codon optimised for murine usage by GeneArt®. Restriction cloning sites corresponding to those utilised for cloning into ChAd63 and MVA were incorporated upstream and downstream antigen sequences (**Figure 6.1**).


A
SP
Region C
Pbs230
Codon optimised for
murine expression
Acc65I
Kozak
XhoI
tPA
Pbs230-C
ColE1 ORI
NotI
KanR
B
SP
Pbs25
GPI
Codon optimised for
murine expression
Acc65I
Kozak
XhoI
tPA
Pbs25
ColE1 ORI
NotI
KanR

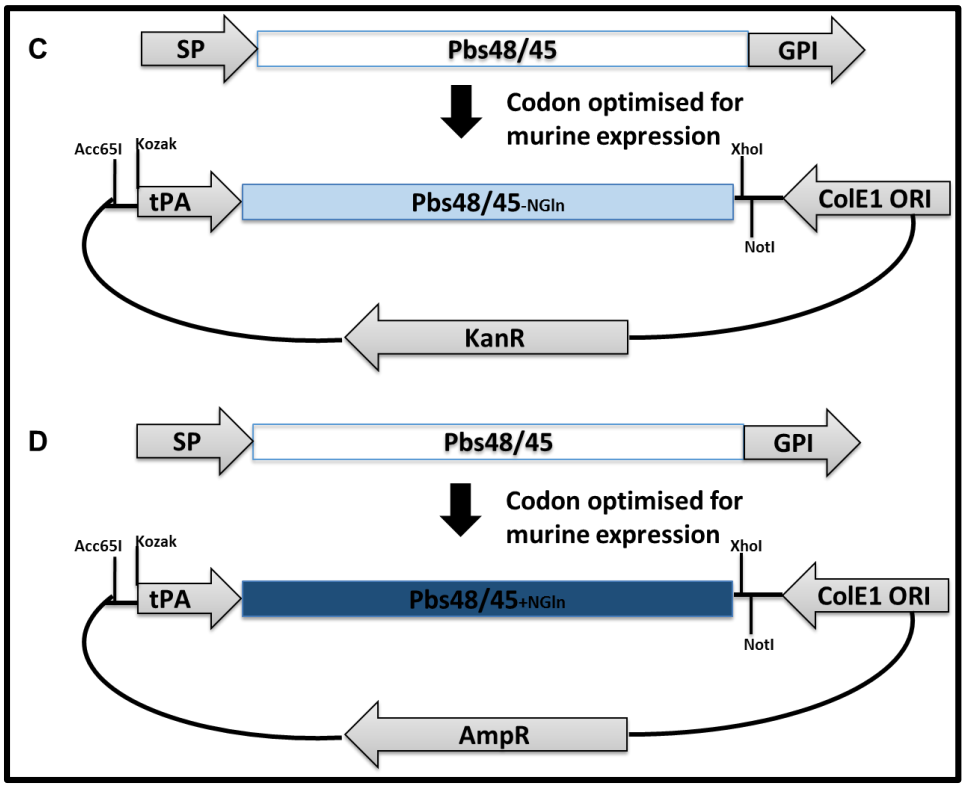

C
SP
Pbs48/45
GPI
Codon optimised for
murine expression
Acc65I
Kozak
XhoI
tPA
Pbs48/45-NGln
ColE1 ORI
NotI
KanR
D
SP
Pbs48/45
GPI
Codon optimised for
murine expression
Acc65I
Kozak
XhoI
tPA
Pbs48/45+NGln
ColE1 ORI
NotI
AmpR

Figure 6.1. Schematic illustration of *P. berghei* antigen design and construction.

Antigen sequences were obtained from NCBI protein database or PlasmoDB Genomics Resource and bioinformatics determined for the presence of SPs; GPI anchors; TMDs and N-glycosylation sites. Endogenous SPs, GPIs, and TMDs were omitted for (**A**) Pfs230-C; (**B**) Pbs25; (**C**) Pbs48/45$_{\text{-NGln}}$; and (**D**) Pbs48/45$_{\text{+NGln}}$. N-glycosylation sites were substituted for all except Pfs48/45$_{\text{+NGln}}$. Endogenous SPs were replaced with tPA, and a Kozak sequence and start codon placed upstream of this. In addition, a stop codon downstream of the antigen open reading frame, and respective restriction sites were incorporated. Sequences were codon optimised for murine usage into respective plasmids expressing either kanamycin (KanR) or ampicillin (AmpR) resistance genes with an origin of replication (ColE1 ORI). For Pbs230-C (**A**), only the region corresponding to 233-791αα was incorporated (Region C).

6.2.1.1.1. Pbs230-C

Pbs230 like Pfs230 is expressed on gametocytes and gametes [294, 622] that is processed after emergence from pRBCs, however, the cleavage site and predicted size differs between the two orthologs [294, 313]. It contains fourteen CRDs characteristic of Pfs230 thus for antigen design, the fragment corresponding to Pfs230-C was determined from previously published amino acid sequence alignments [313]. Analysis of these two sequences revealed the putative site of cleavage and predicted region C (**Figure 6.2**). Therefore, the vaccine construct was designed to incorporate 233 – 791αα based on *P. berghei* ANKA strain (PlasmoDB ID: PBANKA_030610 (**Table 2.2**)). There exists an SP (1-22αα), like Pfs230 no predicted GPI anchor, no TMD and sixteen predicted N-glycosylation sites with seven of these being present in region C. All seven N-glycosylation sites were substituted (positions 237, 256, 313, 420, 536, 556, and 743 (**Figure 6.1A**)).

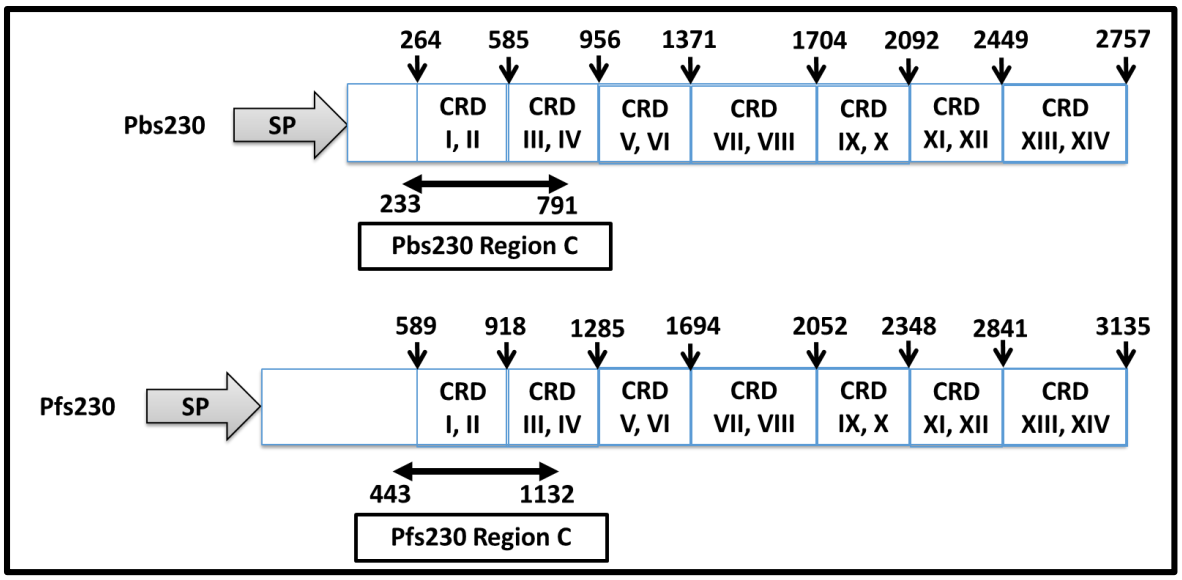


Figure 6.2. Schematic illustration of the primary structure of Pbs230 and Pfs230.

Amino acid sequences for Pbs230 and Pfs230 were aligned [313] to determine the complementary regions between the two antigens. Represented are signal peptides (SP), cysteine-rich domains (CRD), and region C of Pbs230 and Pfs230. The positions of CRD I – XIV for Pbs230 is from 264-2757αα in relation to Pfs230 (589-3135αα) as previously described [291, 313] with the arrowheads indicating the start of the amino acid positions demarcating each domain. The region corresponding to Pbs230 and Pfs230 region C spans CRD I – IV, 233-791αα and 443-1132αα respectively.

6.2.1.1.2. Pbs25

Pbs25 is expressed on the surface of the macrogamete through to the oocyst stage [630] and consists of 217αα like Pfs25 [296, 623]. The vaccine construct was designed based on *P. berghei* ANKA strain sequence (NCBI REFSEQ BAA19925.1 (**Table 2.2**)). This sequence contains a SP [623] and GPI anchor [630]. This sequence was substituted with tPA and the GPI anchor removed from positions 194-217αα (**Figure 6.1B**). In comparison, Pfs25 has four potential N-glycosylation sites (positions 112, 165, 187 and 202) [296] whilst Pbs25

only two predicted at positions 55 and 202. The latter was in the GPI anchor and hence not part of the vaccine construct whilst the former was substituted.

6.2.1.1.3. Pbs48/45

The gametocyte and gamete protein Pbs48/45 has been shown to be expressed as a doublet consisting of 455αα with 50% protein sequence identity to Pfs48/45 [293]. Characterisation of this *P. berghei* ortholog revealed that like Pfs48/45, it has a SP (1-31αα), GPI anchor (432-454αα) and no TMD. Unlike Pfs48/45 with seven N-glycosylation sites, Pbs48/45 had only two at positions 135 and 194. Vaccine design was based on *P. berghei* ANKA strain (NCBI REFSEQ AAG59815.1 (**Table 2.2**)). To test whether N-glycosylation site substitution had an impact on immunogenicity and efficacy as observed for Pfs48/45 (**Chapter 4**), two versions of Pbs48/45 were also designed (**Figure 6.1C and D**). Constructs had either N-glycosylation sites substituted (Pbs48/45$_{-NGln}$, (**Figure 6.1C**)) or intact (Pbs48/45$_{+NGln}$, (**Figure 6.1D**)).

6.2.1.2. Generation of recombinant viral-vectors

Replication-deficient recombinant ChAd63 vaccines expressing each individual vaccine construct were generated by restriction and Gateway cloning as previously described in **Chapter 3** (section **3.2.2.1**). For the generation of recombinant MVA vaccines, tPA-Insert plasmids (**Figure 6.1**) were generated as described in section **3.2.3.1**. To confirm identity and purity of generated recombinant viral-vectors, PCR reactions were performed using ID primers and flank-to-flank primers (**Table 6.1**). The presence and purity of antigens in ChAd63 was confirmed with the respective PCR products being amplified with no observed contamination (**Figure 6.3A**). Presence and purity in MVA viruses was also confirmed with only target sequences being amplified (**Figure 6.3B**). For each recombinant virus, pre-viral plasmid amplification products, used as positive control, confirmed that presence of antigen

sequences. To further confirm and delineate between the two Pbs48/45 constructs, Pbs48/45-$_{NGln}$ viral-vectors were sequenced and determined to be true-to-type.

Table 6.1. List of primers used to determine identity and purity of viral-vectors.

Primer pair	Sequence	PCR Purpose
J1212 J1198	GCAACAGCTTCTCCTCGTTC CGAGATCGAGTCCATCAACA	ID for ChAd63-Pbs230-C
J645 J1189	GCCGTCCACCGTGGATCCCATAACATTC GTGCATCATCAACGAGGACAGCGTGAACGAC	ID for ChAd63-Pbs25
J1212 J1199	GCAACAGCTTCTCCTCGTTC CCAAATCTACAAGCCCCTGA	ID for ChAd63- Pbs48/45$_{-NGln}$
J1212 J1250	GCAACAGCTTCTCCTCGTTC ACGAGGCCAACATCTACACC	ID for ChAd63- Pbs48/45$_{+NGln}$
J1209 J1210	TCAGAGGTAACTCCCGTTGC CAGCGAGCTCTAGCATTTAGG	Flank-to-flank for ChAd63
J1205 J1198	CCATCGAGTGCGGCTACTAT CGAGATCGAGTCCATCAACA	ID for MVA- Pbs230-C
J1205 J1189	CCATCGAGTGCGGCTACTAT GTGCATCATCAACGAGGACAGCGTGAACGAC	ID for MVA- Pbs25
J1205 J1199	CCATCGAGTGCGGCTACTAT CCAAATCTACAAGCCCCTGA	ID for MVA- Pbs48/45$_{-NGln}$
J1205 J1250	CCATCGAGTGCGGCTACTAT ACGAGGCCAACATCTACACC	ID for MVA- Pbs48/45$_{+NGln}$
J1204 J1205	TGTGGGTAATGTTCTCGATGTC CCATCGAGTGCGGCTACTAT	Flank-to-flank for MVA

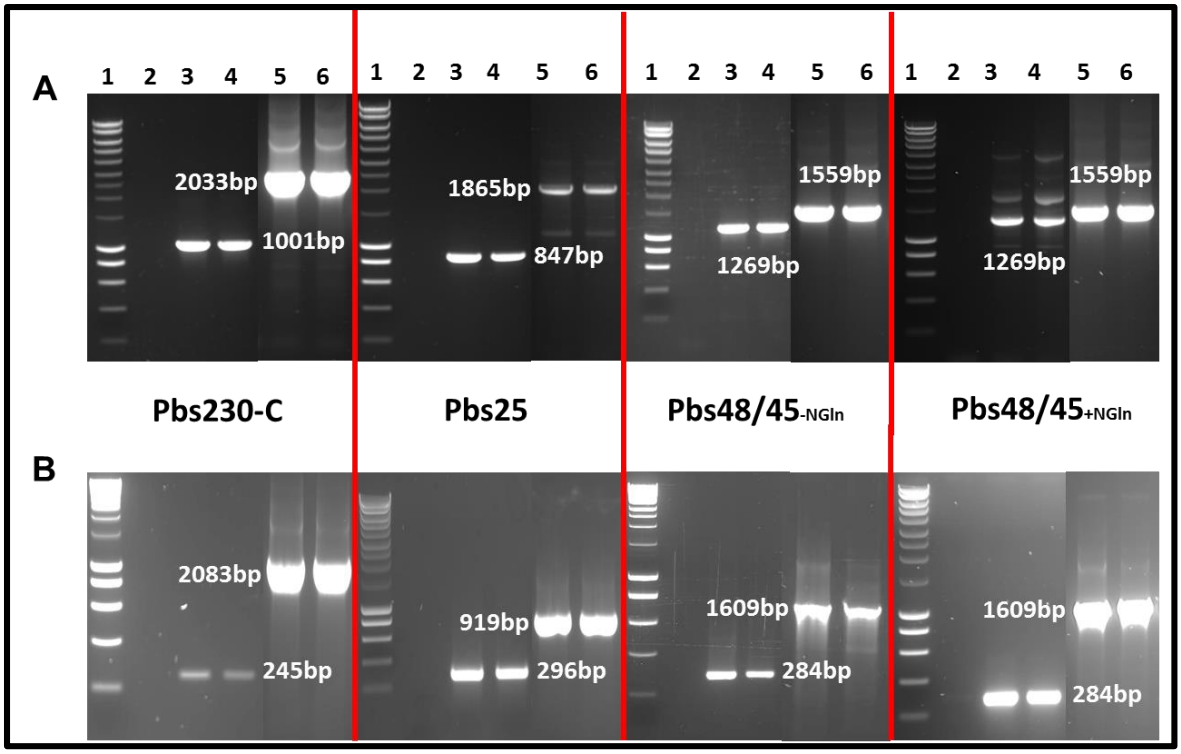


Figure 6.3. PCR confirmation of recombinant viral-vectored viruses.

PCR reactions were performed using purified viral DNA and products were run on agarose gels. Respective pre-viral plasmid DNA and water were used as positive and negative controls respectively. Depicted are purified recombinant (**A**) ChAd63 and (**B**) MVA virus for each antigen. Expected product sizes are shown alongside each respective corresponding band. Lanes: 1 – Smart DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA; 5 – pre-viral plasmid DNA; and 6 – viral DNA. Lanes 3 and 4 show identity, and, 5 and 6 purity PCR products. Red line demarcates PCR products for respective antigens.

6.2.2. Immunogenicity of P. berghei antigens

6.2.2.1. Generation of recombinant proteins

To assess the immunogenicity of *P. berghei* antigens expressed in ChAd63-MVA, recombinant protein was produced using HEK293 suspension cells. This expression system has been used to express *Plasmodium* antigens either for screening of vaccine-induced responses [275] or to study receptor-ligand interactions [449, 450, 631]. Codon optimised antigen sequences (**Figure 6.1**) were cloned by In-Fusion cloning with insert-specific primers (**Table 6.2**) into a pENTR™4LPTOS plasmid containing a tPA, biotin acceptor peptide (BAP) in tandem with six histidine (His_6) residues and CMV_LP to drive protein expression (**Figure 6.4**). The BAP was incorporated in order to allow enzymatic biotinylation of the expressed protein by co-transfection with an *E. coli* biotin ligase, BirA, as described by Bushell et al. 2008 [449]. This enabled detection of antigen-specific responses on streptavidin-coated plates without the need to purify the protein. The resulting plasmid DNA was used to transfect HEK293E suspension cells which have been shown to produce high yields of proteins [448].

The presence of the antigens was assessed by immunoblotting supernatants either straight after harvesting (**Figure 6.5A**) or after dialysing (**Figure 6.5B**) using anti-His antibody. Only Pbs25 was detectable both before and after dialysing at 25kDa, which has been shown to be expressed at 28kDa [630]. Bands ~40kDa were observed in all supernatants which didn't correspond to the size of any of the proteins that were being expressed. These could possibly have been detected due to reactivity with anti-His antibody though this hasn't been described before in this expression system [448].

To further determine whether all the antigens had been expressed, pooled antigen-specific sera were used to ascertain reactivity with dialysed supernatants at various dilutions in an ELISA. The supernatants showed reactivity to respective antigen-specific sera with anti-Pbs25 having the highest OD_{405} values irrespective of the dilution tested (**Figure 6.6**). For anti-Pbs230-C, anti-Pbs48/45$_{-NGln}$, and anti-Pbs48/45$_{+NGln}$, positive responses were only detected at the top dilution. These results were reminiscent of what had been observed in the immunoblotting analysis (**Figure 6.5**) with only Pbs25 being visible and thus abundantly expressed whilst the other antigens were expressed but albeit at much lower yields. It has been observed that even though the expression system is robust, not all proteins are expressed at high yields [448].

Table 6.2. List of primers used for protein expression cloning.

Plasmid	Forward primer	Reverse primer
Pbs230-C	ATCGACCAGATCCAAGAGACA TACG	TTTGGAGATGATCAGTTCCACG
Pbs25	ATCAGCCCCAACACCCAGTGC	GATGCACTTGTTCTCTTCGATG CTCAGC
Pbs48/45*	ATCGACCAGATCCAAGAGACA TACG	TTTGGAGATGATCAGTTCCACG

TTCAGACGGGGTACC and GCCGCTAGCGGATCC 15bp sequences were added to the 5'end of forward and reverse primers respectively. *Primer pairs and PCR conditions were used for both the Pbs48/45 variants, Pbs48/45$_{-NGln}$ and Pbs48/45$_{+NGln}$.

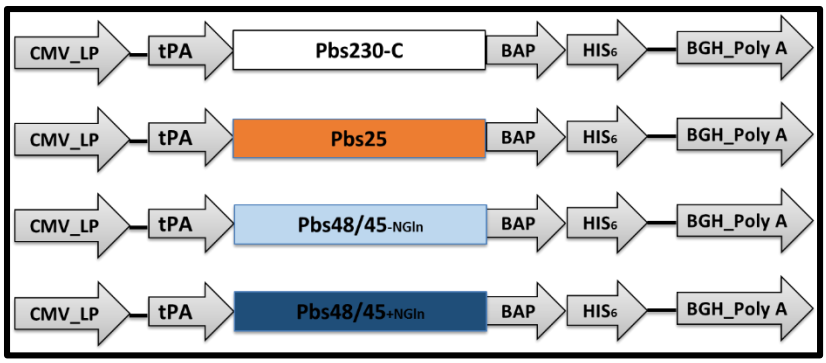


Figure 6.4. Schematic of protein expression constructs.

Codon optimised sequences were PCR amplified from respective plasmids (**Figure 6.1**) using primers (**Table 6.2**) with 15bp complimentary to pENTR™4LPTOS containing tPA, BAP, six histidine residues (His_6) and BGH_Poly A. pENTR™4LPTOS was linearised by restriction digest (Acc65I and BamHI) and used to perform an in-fusion reaction with respective PCR products. CMV_LP: cytomegalovirus long promoter; tPA: tissue plasminogen signal peptide; BAP: biotin acceptor peptide; and BGH_Poly A: bovine growth hormone polyadenylation sequence.

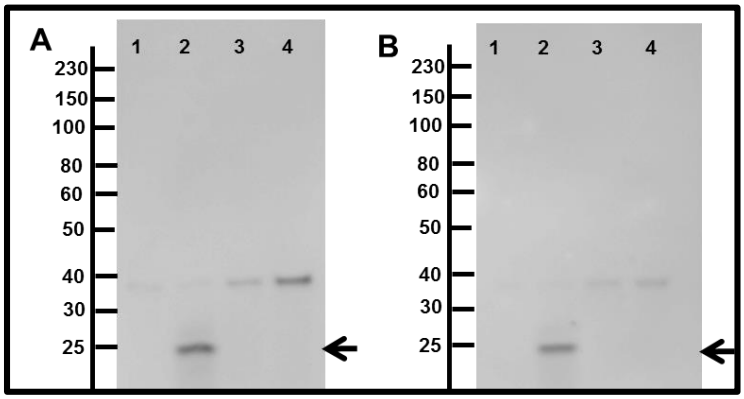


Figure 6.5. Production of recombinant *P. berghei* proteins.

Supernatants from suspension HEK293E cells were harvested and either mixed immediately (**A**); or dialysed and mixed (**B**); with reducing SDS buffer. Samples were run on 12% polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using anti-Histidine antibody. Lanes: 1 – Pbs230-C; 2 – Pbs25; 3 – $Pbs48/45_{-NGln}$; and, 4 – $Pbs48/45_{+NGln}$. Arrowheads denote the relative mobility expected.

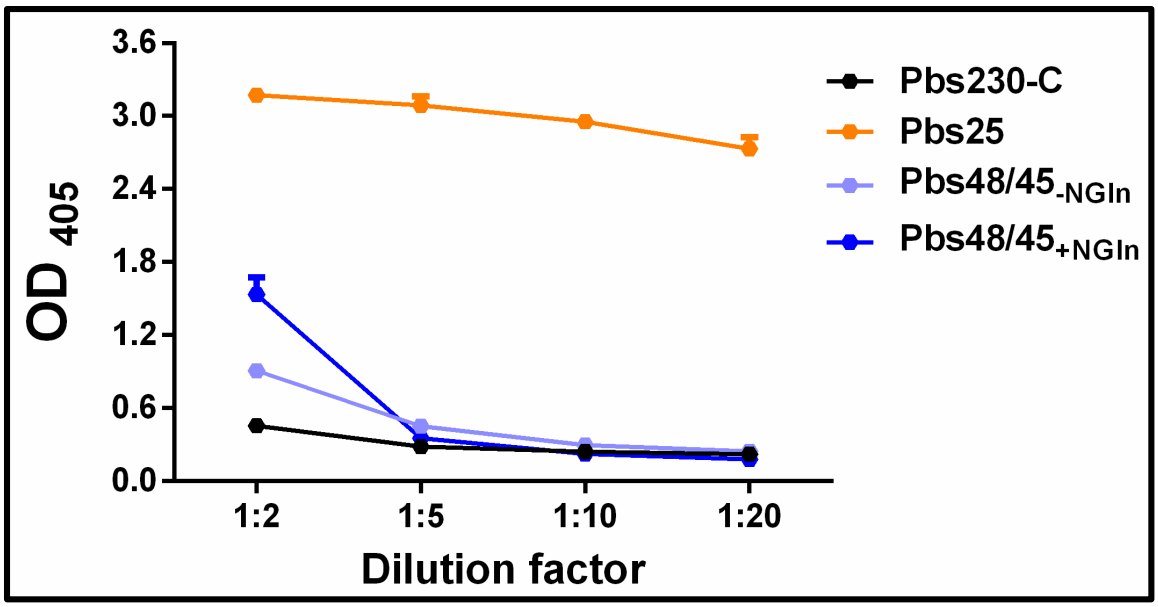


Figure 6.6. Reactivity of antigen-specific sera to HEK293E expressed protein.

Dialysed protein supernatant was used to coat streptavidin-coated plates in a dilution series in PBS. Pooled sera from vaccinated mice (n=5-6/group) were used to detect whole IgG responses by ELISA. Represented are mean ± SEM of four replicate OD_{405} absorbance readings.

6.2.2.2. IgG responses after ChAd63-MVA prime-boost vaccination

To assess antibody responses induced to the respective antigens, BALB/c mice were prime-boost vaccinated with ChAd63-MVA individually expressing Pbs230-C, Pbs25, Pbs48/45-NGln and Pbs48/45+NGln from which sera was used to assess individual antibody responses. IgG responses were assayed by endpoint ELISA using streptavidin coated plates. In this instance, boosting of ChAd63 responses was 13weeks post-prime (day91) due to unavailability of MVA-Pbs48/45+NGln vaccine. To detect antigen-specific total IgG responses, protein supernatants were diluted according to the optimal reactivity (**Figure 6.6**). Responses were determined for all individual samples (**Figure 6.7**) including pooled samples (**Table 6.3**). To detect statistically significant differences in boosting of vaccine-induced IgG responses, Mann-Whitney test was performed comparing days91 and 105.

Antigen-specific responses for all antigens were prime-boosted at varying magnitudes despite the lag in time between priming and boosting. Pbs230-C IgG responses were boosted by 4-fold (p=0.002) (**Figure 6.7A**) whilst responses to Pbs25 were 8-fold higher (p=0.002) (**Figure 6.7B**). Responses to Pbs48/45-NGln and Pbs48/45+NGln were 5-fold and 13-fold higher respectively (p=0.01 and p=0.002) (**Figure 6.7C**). There was no difference between post-boost responses to Pbs48/45-NGln and Pbs48/45+NGln (p>0.05), however there was a trend of responses to the latter being higher than the former (**Figure 6.7C**).

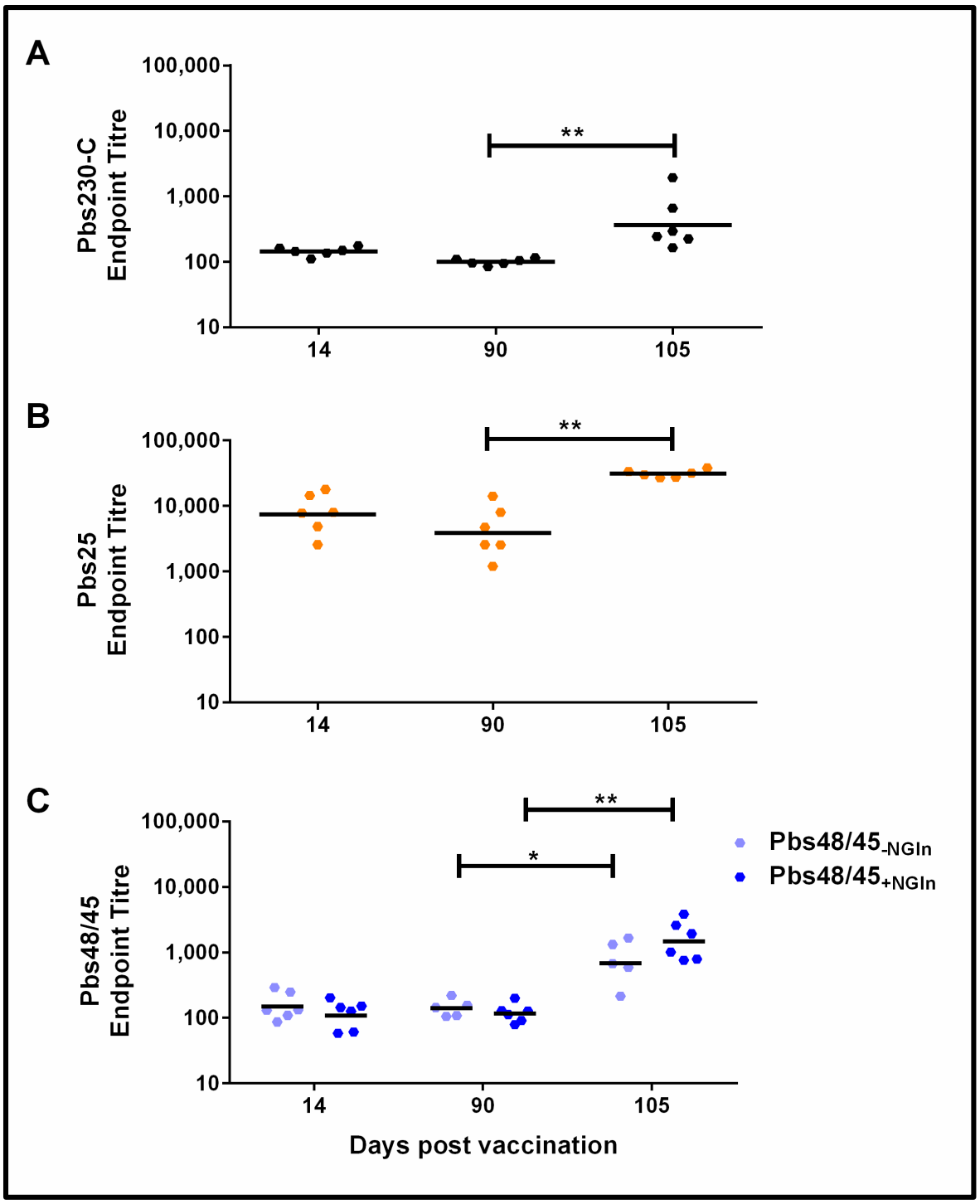

A
Pbs230-C Endpoint Titre
100,000
10,000
1,000
100
10
**
14
90
105
B
Pbs25 Endpoint Titre
100,000
10,000
1,000
100
10
**
14
90
105
C
Pbs48/45 Endpoint Titre
100,000
10,000
1,000
100
10
**
*
14
90
105
Days post vaccination
Pbs48/45-NGIn
Pbs48/45+NGIn

Figure 6.7. Vaccine-induced IgG responses after ChAd63-MVA prime-boost.

BALB/c mice (n=5-6/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with the respective MVA ($1x10^7$pfu, day91) expressing (**A**) Pbs230-C; (**B**) Pbs25; and (**C**) Pbs48/45$_{-NGln}$ and Pbs48/45$_{+NGln}$. Whole IgG responses were assayed on days 14, 90 and 105 by endpoint ELISA using recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points. Dots represent responses from individual mice. Significance shown and considered at $p<0.05$. Asterisks represent statistical significance.

Table 6.3. Antigen-specific antibody titres for pooled sera

Antigen	Antibody titre
Pbs230-C	703
Pbs25	32,161
Pbs48/45$_{-NGln}$	789
Pbs48/45$_{+NGln}$	2,529

Antibody titres were determined by endpoint ELISA from an aliquot of sera pooled from vaccinated mice (n=5-6/group).

6.2.2.3. Reactivity of vaccine-induced antibodies to native antigen

To determine whether antibodies raised against *P. berghei* antigens were able to recognise native antigen, anti-sera reactivity was assessed by immunoblotting using purified gametocytes (anti-Pbs230-C, anti-Pbs48/45$_{-NGln}$, and anti-Pbs48/45$_{+NGln}$) and ookinetes (anti-Pbs25) (**Figure 6.8**). Purified gametocytes or ookinetes were mixed in both non-reducing or reducing SDS buffer and run on gels. Probing with Pbs230-C anti-sera revealed a band at 230kDa and faint bands at approximately 100, 60, 40, and 20kDa (**Figure 6.8A**) regardless of the sample buffer. The molecular weight of native Pbs230-C is predicted to be 64kDa [632]. In the case of anti-Pbs25 sera, recognition of a 25kDa species was observed (**Figure 6.8B**) confirming reactivity with native antigen previously described to migrate between 26 and 28kDa [629, 630]. On the other hand, a faint band was observed migrating at 50kDa which could be a dimer.

Anti-sera raised against Pbs48/48$_{-NGln}$ showed reactivity to gametocytes with two distinct bands observed in both non-reducing and reducing conditions at 230 and 100kDa (**Figure 6.8C**). Faint bands were observed at 40, 60, and 15kDa reminiscent of the migration pattern observed with Pbs230-C anti-sera. Pbs48/45$_{+NGln}$ anti-sera on the other hand, revealed a bands at 230kDa ~100kDa (**Figure 6.8D**). The reactivity patterns observed for either Pbs48/45$_{-NGln}$ or Pbs48/45$_{+NGln}$ are inconsistent with what has been previously described. van Dijk et al. (2001) have shown that native gametocyte Pbs48/45 migrates as a doublet under non-reducing conditions at 53kDa and as a single species at 55kDa in reducing conditions [293].

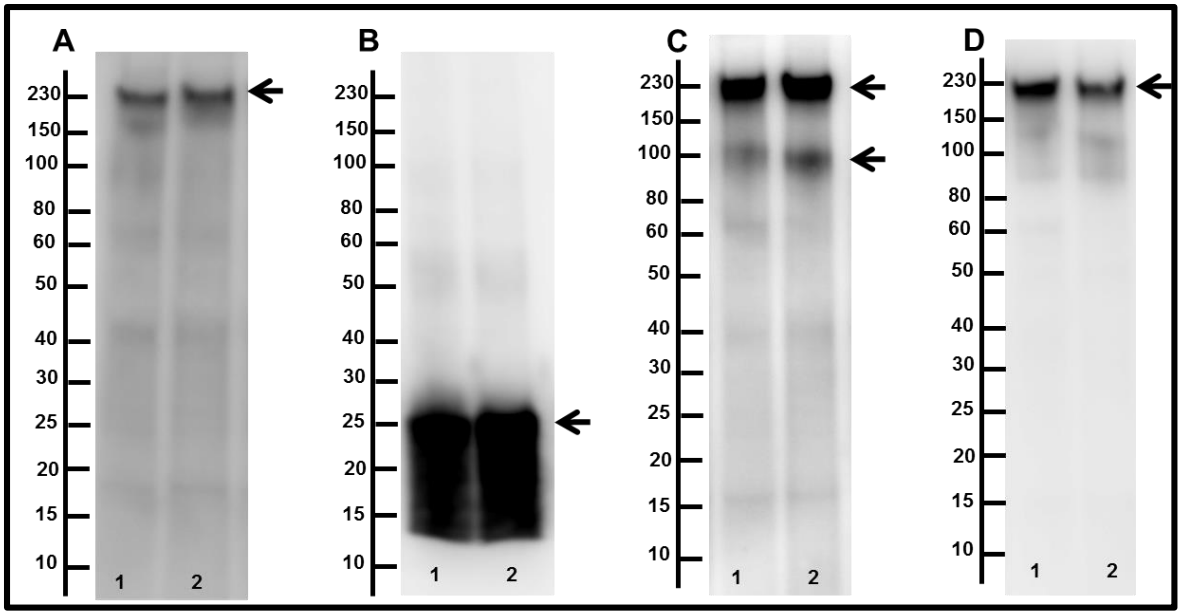


Figure 6.8. Reactivity of vaccine-induced antibodies against parasite lysate.

P. berghei purified gametocytes (10μg/lane) or ookinetes ($5x10^6$/lane) were either mixed with non-reducing and reducing SDS buffer. Samples were run on 12% polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using (**A**) anti-Pbs230-C; (**B**) anti-Pbs25; (**C**) anti-Pbs48/45$_{-NGln}$; and (**D**) anti-Pbs48/45$_{+NGln}$ sera. Lanes: 1 – non-reducing; 2 – reducing. Arrowheads denote the relative migration mobility observed and expected. Figures **A**, **C** and **D** represent immunoblots from purified gametocytes and **B** from ookinetes.

6.2.3. Efficacy of P. berghei antigens

To establish whether antibodies raised by ChAd63-MVA were able to block parasite development in *A. stephensi* mosquitoes, *in vitro* and *in vivo* ookinete and membrane feeding assays, respectively, were used to assess efficacy. To ascertain whether there were any differential effects mediated by antibody compared to non-antibody, both anti-sera and total IgG were used to assess efficacy. To be able to establish this effect, the same pool of anti-sera used to purify total IgG was used to measure the effects of anti-sera and the same material was used in both *in vitro* and *in vivo* assays (**6.2.2.2, Table 6.3**).

6.2.3.1. Inhibition of *P. berghei* development mediated by total IgG

To establish *in vitro* activity, ookinete development assay was performed. Ookinetes have been shown to develop after 22hours in culture from pRBCs obtained from mice that have been PH-treated [363]. All experiments were performed in duplicates with each experiment being initiated from a different mouse. At initiation of culture, pRBCs were co-incubated with IgG diluted in ten serial dilutions from a top concentration of 400μg/ml. As controls, cycloheximde (CH) which has been shown to completely block development of ookinetes [363] and total IgG from viral-vector control vaccinated animals were used as positive and negative controls respectively. CTRp.GFP *P. berghei* clone 15cy1A [455] was used for infections as it has been shown to be a good indicator of gametocyte-to-ookinete development with reproducible results being obtained by GFP fluorescence [363].

To measure efficacy, after co-incubation, GFP expression at each individual concentration was measured. Inhibition of ookinete development was only mediated at 400μg/ml against Pbs25 (36%) (**Figure 6.9A**). Total IgG against Pbs230-C, Pbs48/45$_{-NGln}$, and Pbs48/45$_{+NGln}$ had no effect on ookinete development. To confirm the inhibition observed, the number of

ookinetes in each individual well/experiment was counted (**Figure 6.9B**). In agreement, only anti-Pbs25 IgG had a 2-fold reduction in ookinetes compared to control (326.5 compared to 767.5) at 400μg/ml.

To measure *in vivo* effects on parasite development SMFAs were performed. Considering that 400μg/ml of IgG *in vitro* either had minimal or no effect on parasite development, total IgG was tested at 500μg/ml. Ditto, only anti-Pbs25 IgG had an effect on inhibiting *P. berghei* infection intensity and prevalence at 85% (p=0.00001) and 45% (p=0.008) respectively (**Figure 6.10**). On the other hand, Pbs48/45$_{-NGln}$ compared to Pbs48/45$_{+NGln}$ had a non-statistically significant impact on parasite intensity (43%, p=0.14). Taken together, only anti-Pbs25 IgG had considerable efficacy both *in vitro* and *in vivo* against *P. berghei* development. Thus, efficacy *in vitro* was a reflection of that *in vivo* despite the concentration of IgG.

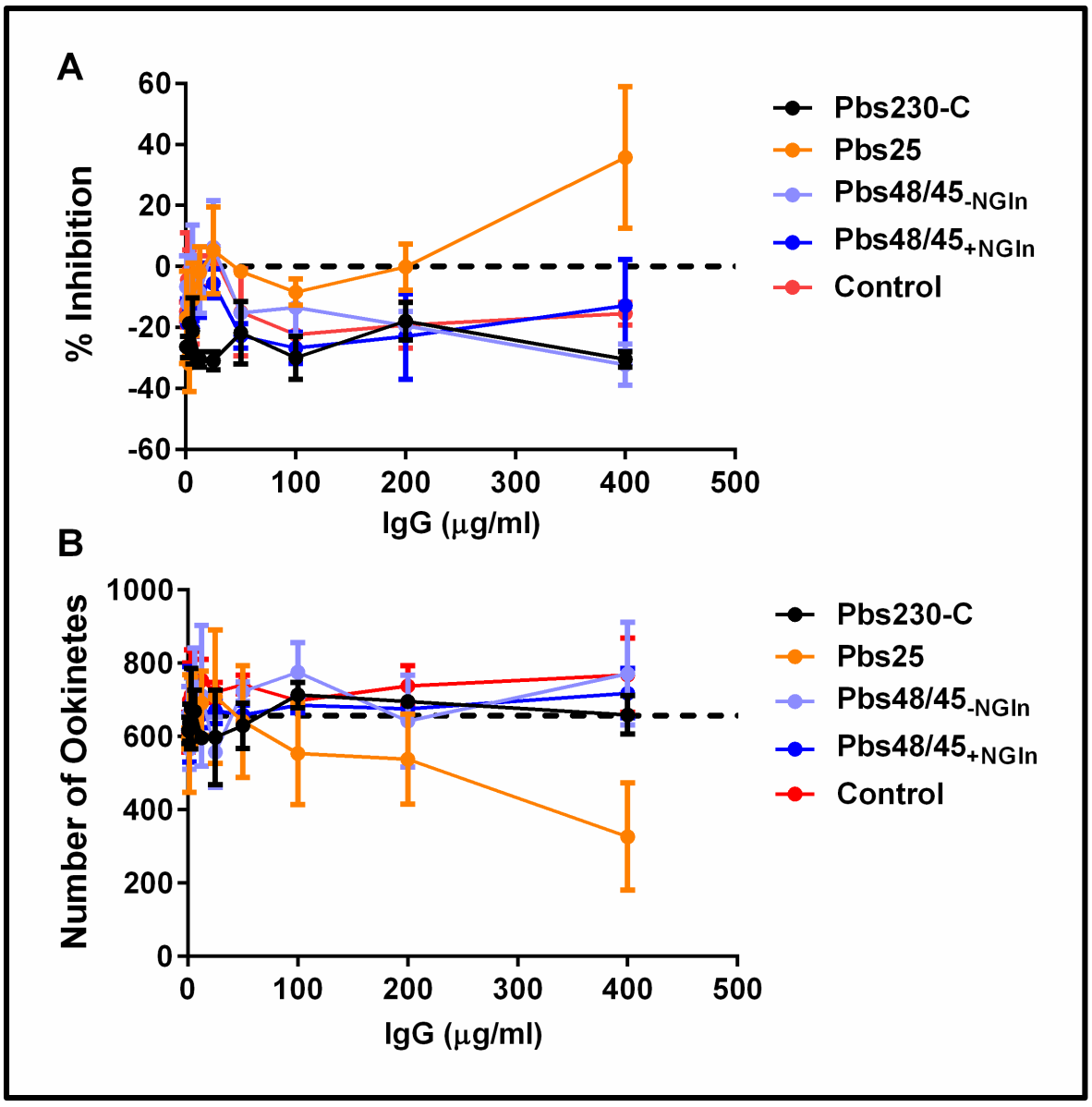
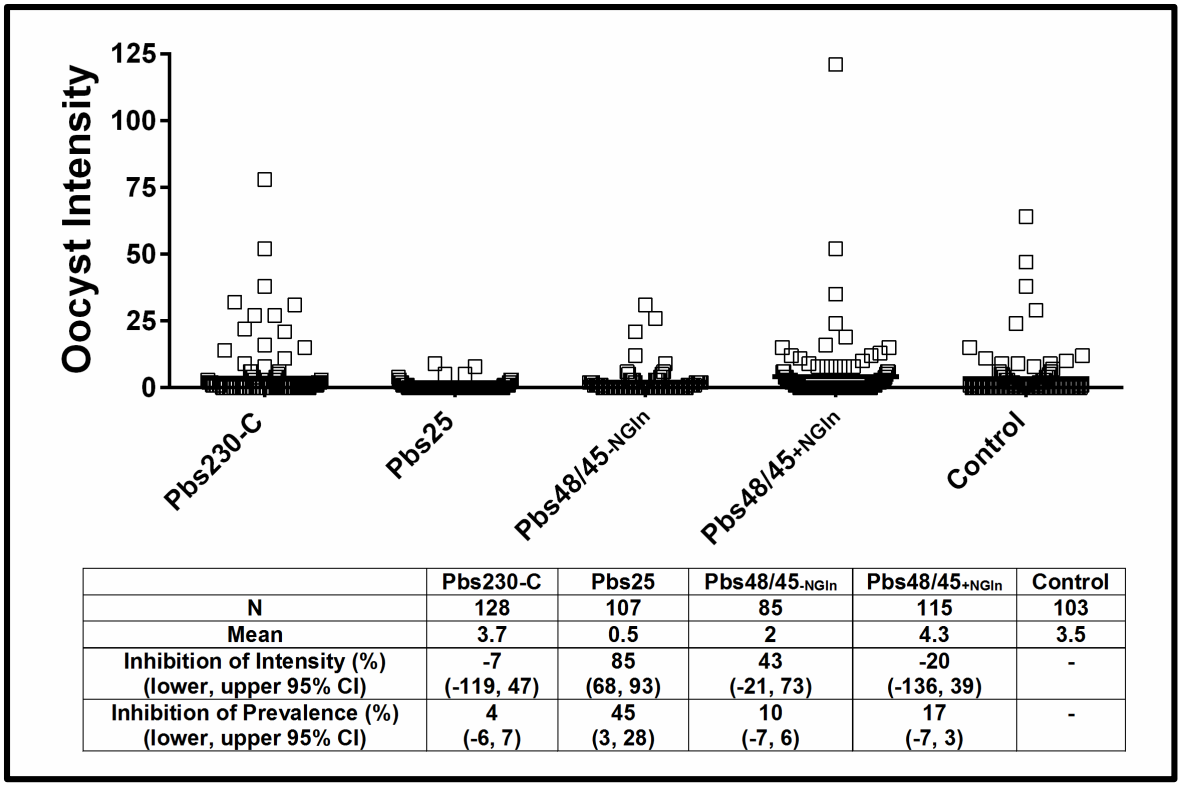


Figure 6.9. Effect of vaccine-induced IgG on *P. berghei* ookinete development.

Sera from mice vaccinated with ChAd63-MVA expressing each respective antigen or GFP (control) (**6.2.2.1**) was used to purify total IgG. *P. berghei* ookinete development was determined in IVOA in the presence of ten independent two-fold concentrations of purified total IgG. Parasitised RBCs were incubated with the IgG for 22hours at 19°C. (**A**) Percentage inhibition of ookinete development and (**B**) the number of ookinetes of two independent experiments collated and represented as mean ± SEM. Dashed line represents CH values.



	Pbs230-C	Pbs25	Pbs48/45$_{-NGIn}$	Pbs48/45$_{+NGIn}$	Control
N	128	107	85	115	103
Mean	3.7	0.5	2	4.3	3.5
Inhibition of Intensity (%) (lower, upper 95% CI)	-7 (-119, 47)	85 (68, 93)	43 (-21, 73)	-20 (-136, 39)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	4 (-6, 7)	45 (3, 28)	10 (-7, 6)	17 (-7, 3)	-

Figure 6.10. Effect of vaccine-induced IgG at inhibiting *P. berghei* infectivity.

Sera from mice vaccinated with ChAd63 and MVA expressing each respective antigen or GFP (control) (**6.2.2.1**) was used to purify total IgG. Total IgG was diluted to 500µg/ml in PBS, mixed with parasitised RBCs from mice infected with *P. berghei* and fed to *A. stephensi* mosquitoes in SMFA. Midguts were dissected 11-13days post-feeding, the number of oocysts and prevalence of infection determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected of two independent experiments. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI. Squares represent oocyst numbers from each individual mosquito dissected.

6.2.3.2. Inhibition of *P. berghei* development mediated by anti-sera

To establish whether anti-sera mediated effects on parasite inhibition, *in vitro* and *in vivo* development of parasites was determined. The *in vitro* ookinete assay was performed in three independent experiments. In this instance, anti-sera was diluted starting at 1:10 in ten serial dilutions and incubated with pRBCs for 22hours. As observed with IgG, only anti-Pbs25 IgG was able to inhibit ookinete development at a rate of 87% (3,216 titre, **Figure 6.11**). In addition, inhibition of ookinete development was antibody titre dependent with inhibition evident at antibody titres of 1,608 (68%) and 804 (37%). However, regardless of the antibody titre, anti-Pbs230-C, anti-Pbs48/45$_{-NGln}$, and anti-Pbs48/45$_{+NGln}$ sera had no impact on ookinete development.

To determine efficacy *in vivo*, anti-sera was tested at two dilutions in SMFA. Two independent experiments were performed, in the first, all were tested at both dilutions (**Figure 6.12**) whilst in the second, only three were tested at both dilutions due to insufficient amounts (**Figure 6.13**). Therefore these experiments are represented as separate experiments (**Figures 6.12** and **6.13**). Interestingly, efficacy was observed against Pbs25 and Pbs48/45$_{-NGln}$ at the highest concentration tested (3,216 and 78.9 titres respectively, **Figure 6.12A**). The former had 94% (p=0.01) and 52% (p=0.002) effect on intensity and prevalence respectively; whilst the latter had 68% (p=0.03) and 33% (p=0.04). There was no inhibition mediated against either Pbs230-C or Pbs48/45$_{+NGln}$. On the other hand, there was no efficacy observed against any antigen at lower antibody titres, however, anti-Pbs25 sera had 30% inhibiton of prevalence (643 antibody titres, p=0.03, **Figure 6.12B**). When tested further, anti-Pbs25 and anti-Pbs48/45$_{-NGln}$ sera only had an effect on intensity, 63% (p=0.03) and 61% (p=0.01) respectively (3,216 and 78.9 titres respectively) (**Figure 6.13A**). When tested at lower antibody titres, no inhibition was observed against any of the antigens (**Figure 6.13B**).

Efficacy against only Pbs25 *in vitro* was replicated *in vivo* with efficacy being dependent on the antibody titre. Interestingly, against Pbs48/45$_{-NGln}$ only with anti-sera was efficacy observed *in vivo* despite anti-Pbs48/45$_{-NGln}$ anti-sera having a lower antibody titre compared to Pbs48/45$_{+NGln}$ (**Table 6.3**).

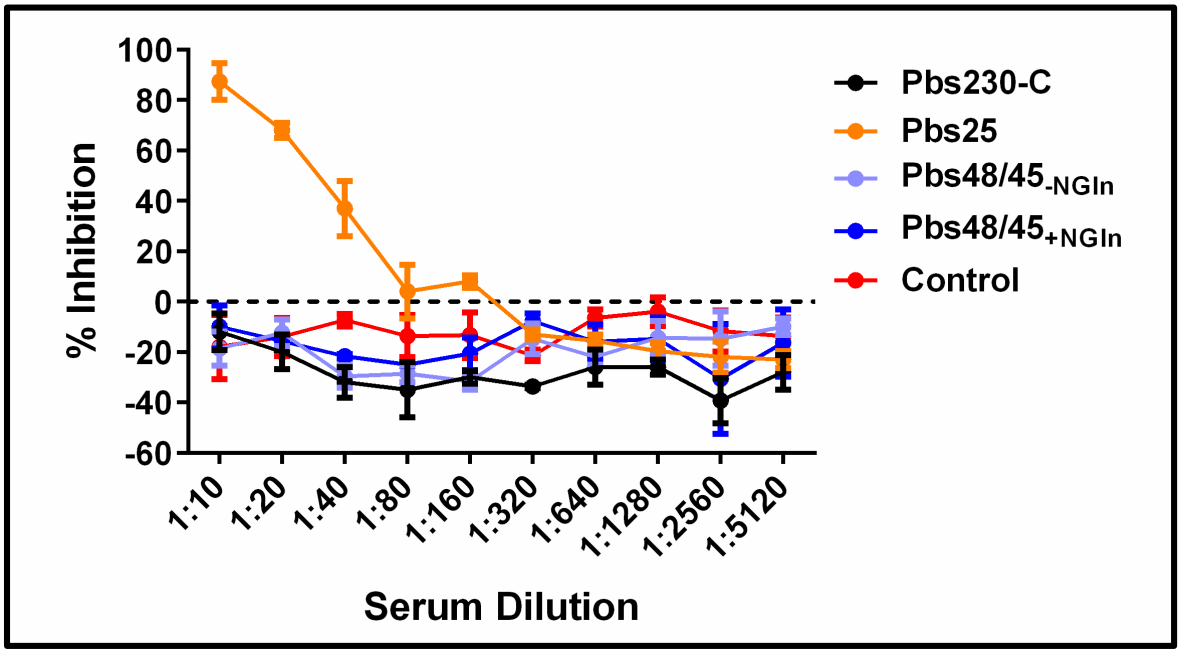
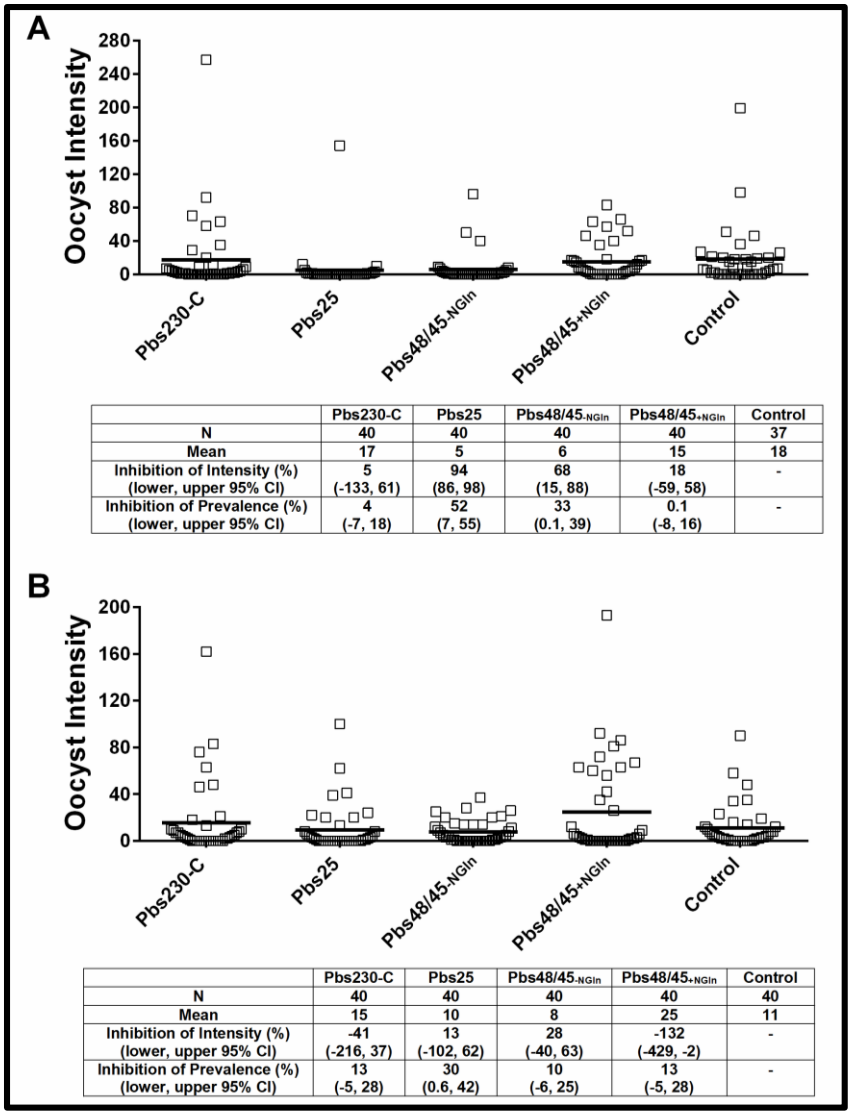


Figure 6.11. Effect of vaccine-induced anti-sera on *P. berghei* ookinete development.

Sera from mice vaccinated with ChAd63-MVA expressing each respective antigen or GFP (control) (**6.2.2.1**) was used to determine development *P. berghei* ookinetes in IVOA. *P. berghei* parasitised RBCs were incubated in the presence of ten independent two-fold dilutions of sera for 22hours at 19°C. Percentage inhibition of ookinete development was determined from three independent experiments and represented as mean ± SEM. Dashed line represents the positive control baseline values.

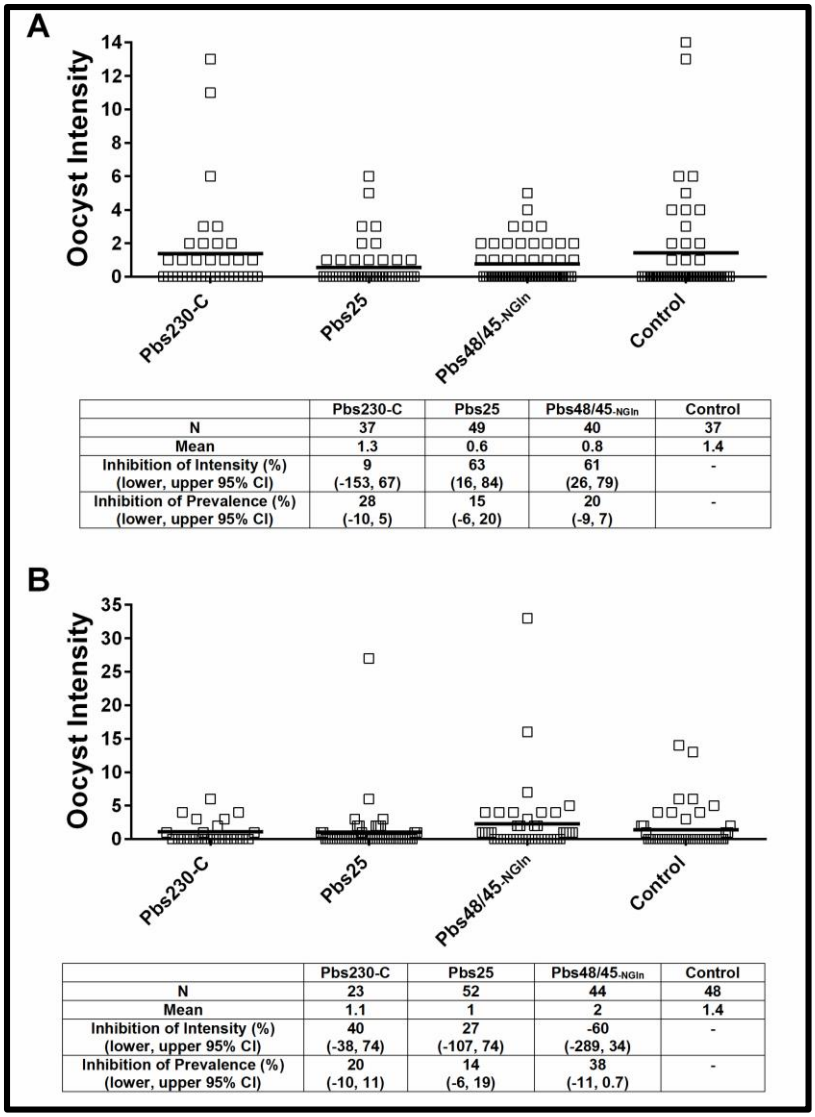


	Pbs230-C	Pbs25	Pbs48/45-NGln	Pbs48/45+NGln	Control
N	40	40	40	40	37
Mean	17	5	6	15	18
Inhibition of Intensity (%) (lower, upper 95% CI)	5 (-133, 61)	94 (86, 98)	68 (15, 88)	18 (-59, 58)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	4 (-7, 18)	52 (7, 55)	33 (0.1, 39)	0.1 (-8, 16)	-

	Pbs230-C	Pbs25	Pbs48/45-NGln	Pbs48/45+NGln	Control
N	40	40	40	40	40
Mean	15	10	8	25	11
Inhibition of Intensity (%) (lower, upper 95% CI)	-41 (-216, 37)	13 (-102, 62)	28 (-40, 63)	-132 (-429, -2)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	13 (-5, 28)	30 (0.6, 42)	10 (-6, 25)	13 (-5, 28)	-

Figure 6.12. Effect of vaccine-induced anti-sera at inhibiting *P. berghei* infectivity.

Sera from mice vaccinated with ChAd63 and MVA expressing each respective antigen or GFP (control) (**6.2.2.1**) was mixed in PBS with pRBCs from *P. berghei* infected mice and immediately fed to *A. stephensi* at (**A**) 1:10 and (**B**) 1:50 in SMFA. Midguts were dissected 11-13days post-feeding, the number of oocysts and prevalence of infection determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.



	Pbs230-C	Pbs25	Pbs48/45-NGIn	Control
N	37	49	40	37
Mean	1.3	0.6	0.8	1.4
Inhibition of Intensity (%) (lower, upper 95% CI)	9 (-153, 67)	63 (16, 84)	61 (26, 79)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	28 (-10, 5)	15 (-6, 20)	20 (-9, 7)	-

	Pbs230-C	Pbs25	Pbs48/45-NGIn	Control
N	23	52	44	48
Mean	1.1	1	2	1.4
Inhibition of Intensity (%) (lower, upper 95% CI)	40 (-38, 74)	27 (-107, 74)	-60 (-289, 34)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	20 (-10, 11)	14 (-6, 19)	38 (-11, 0.7)	-

Figure 6.13. Effect of vaccine-induced anti-sera at inhibiting *P. berghei* infectivity.

Sera from mice vaccinated with ChAd63 and MVA expressing each respective antigen or GFP (control) (**6.2.2.1**) was mixed in PBS with parasitised RBCs from *P. berghei* infected mice and immediately fed to *A. stephensi* at (**A**) 1:10 and (**B**) 1:50 in SMFA. Midguts were dissected 11-13days post-feeding, the number of oocysts and prevalence of infection determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.

6.3. Discussion

The work described in this chapter demonstrates the use of a *P. berghei* model to test TBV candidate antigens shown to be the most promising for clinical development (**Chapter 5**). This work was primarily to investigate whether efficacy observed against *P. falciparum* antigens could be replicated and thus validate the use of the model for antigen screening and selection. However, the overall findings indicate that efficacy against only Ps25 were replicated. The top three candidate antigens in *P. falciparum*, when expressed in *P. berghei*, demonstrated that only antibodies against Pbs25 were able to inhibit parasite development *in vitro* and *in vivo*. *P. berghei* has proved useful in identifying candidate antigens and their roles in parasite development with a number being identified to be important for antibody-mediated TBI [293, 294, 608, 609]. Thus, despite the similarities or conserved nature of the antigen sequences, there appears to be a divergence in how effective antibodies induced to these antigens are able to inhibit their interaction or function. More so for P48/45 which has been shown to have a conserved role in male fertilisation in both *P. falciparum* and *P. berghei* [293, 294]. Conversely, Pbs230, even though conserved between the two species [622], there is evidence that the two antigens are phenotypically distinct [294, 318]. Recent work by Goodman et al. (2013) also identified the lack of efficacy using model blood-stage *P. falciparum* antigens [633]. This follows on from other published work showing that antigens in *P. berghei* don't equate to vaccine-induced efficacy [514, 634]. Nonetheless, efficacy against the gametocyte and gamete antigens, Pbs230-C and Pbs48/45 has never been reported or demonstrated and therefore it would be useful to see whether the lack and limited efficacy observed is a fault of the expression system used to induce antibodies. Thus, there is need for more evidence to validate these results by possibly using other vaccine platforms to induce antibodies. Another way to validate the model, would be to express Pbs28, an antigen with well characterised antibody-mediated efficacy [625-629], and its *P. falciparum* ortholog,

Pfs28, in the same expression system and investigate whether efficacy can be replicated. On the other hand, a complementary region of PbsHAP2 has been expressed as a protein-in-adjuvant formulation, in *P. falciparum*, and has shown efficacy against *P. falciparum* infection in *A. stephensi* [324]. The same conserved region was shown by Blagborough et al. (2009) to inhibit *P. berghei* parasite development in *A. stephensi* by 81% [323].

Interestingly, effect of mutation of N-glycosylation sites in the Pbs48/45 vaccine construct was contrary to what was postulated. However, in comparison with Pfs48/45, the construct with mutations in N-glycosylation sites, Pbs48/45$_{\text{-NGln}}$, had lower antibody titre but antibodies raised were able to mediate inhibition using antisera. Nonetheless, with total IgG, there was lack of efficacy even though responses to the mutant appeared to mediate some efficacy although not statistically significant. One might argue that a higher IgG concentration might have been able to inhibit infectivity, with a statistically significant outcome. In *P. falciparum*, it was clear that the mutation of N-glycosylation sites had a significant impact on both the immunogenicity and the antibody-mediated efficacy (**Chapter 4**). Unlike Pfs48/45 with seven N-glycosylation sites [292], Pbs48/45 has only two putative sites. Even though this study hasn't shown any evidence of actual glycosylation of either the Pbs48/45 or Pfs48/45 constructs, there is need for a comprehensive analysis of the differences between *P. falciparum* and *P. berghei* to validate the impact of N-glycosylation in the rodent model. This will provide any evidence of differences between these two species in terms of N-glycosylation usage as there is limited evidence to speculate on the contrary. Weiss et al. (2010) on investigating the effect between *P. falciparum* and *P. berghei* N-glycosylation site mutation in the blood-stage candidate antigen, MSP1-42, showed that mutation of the four N-glycosylation sites in PbMSP1-42 didn't lead to better responses compared to the wild-type [514]. On the other hand, PfMSP1-42, like PyMSP1-42, has two N-glycosylation sites and

mutation of these led to better immune responses compared to the wild-type [512, 514]. Weiss et al. (2010) [514] concluded that substitution of N-glycosylation sites led to the enhancement of antibody responses to PfMSP1-42 expressed in a DNA vector with no observed effect on the PbMSP1-42 construct [514]. Thus there are antigenic differences in the rodent models used to simulate the possible impact of *P. falciparum* orthologs.

Ideally, antibodies raised against gametocyte, gamete and/or ookinete surface antigens should be able to inhibit development of gametocytes to ookinete and/or oocyst stages. Intriguingly, only antibodies raised against Pbs25 were able to block development of the parasite to both ookinete and oocyst stages. Pbs25 is expressed in macrogametes [630] which has also been reported in Pbs28 [635] and Pfs25 [636] and hence could explain the potential involvement in mediating inhibition of gametocyte-to-ookinete conversion. Other *P. berghei* antigens have been shown to also inhibit development of both ookinete and oocyst stages in IVOA and SMFA respectively [323, 363, 615, 625, 626, 637]. Antibodies against PbsHAP2, expressed on gametocytes and gametes, has been shown to inhibit ookinete development [323, 363] whilst those against the gamete and ookinete surface antigen, Pbs28, have also been reported [363, 615]. The lack of inhibition by antibodies against Pbs230-C and the two Pbs48/45 constructs in the ookinete assay was reflected by lack of efficacy in the *in vivo* assay. However, efficacy against Pbs48/45$_{\text{-NGln}}$ in the *in vivo* assay could be as a result of other serum factors which have been shown to be play a role in inhibiting parasite development [151]. This is more so as total IgG, free of serum factors, was unable to inhibit development of both ookinetes and oocysts. Even though these serum factors in this study remain unidentified, one can postulate that they might have played a role in mediating activity against Pbs48/45 either in conjunction with anti-Pbs48/45$_{\text{-NGln}}$ antibody or on their own. This was also observed for anti-Pbs25, where the level of inhibition using anti-sera was more than

that observed with anti-IgG. Therefore, the investigation of the effects of antibody-mediated effects in comparison to non-antibody mediated effects has been useful. However, each experiment was conducted with different mice, with different starting gametocytaemia and parasitaemia. It would thus be ideal to test the effects of using IgG and sera on the same level of gametocytaemia and parasitaemia. Even so, it would be difficult to replicate the results as each infection develops differently in each mouse. Of note in this study, is the consistency of the ability of anti-Pbs25 antibody to mediate inhibition of parasite development regardless of the assay or type of sample used. There is evidence that antibodies raised against P25 orthologs in animal models have TBA [608, 638, 639]. Tsuboi et al. (1997) have further shown that antibodies against P25 in *P. yoelii*, Pys25, are able to cross-react with Pbs25 antibodies [297]. The rodent model, if successful, would have been a useful way to study the combined effects of TBV candidate antigens, by either co-vaccination or mixing antibodies in the *in vitro* or *in vivo* assays. However, this wasn't possible due to limited and/or no efficacy exhibited by antibodies raised to Pbs230-C and either version of Pbs48/45.

Summary of findings:

- The use of the *P. berghei* rodent model resulted in generation of efficacy with only the P25 ortholog of *P. falciparum*.
- Mutation of N-glycosylation sites in *P. berghei* P48/45, didn't have the same effect as in *P. falciparum*.
- The *in vitro* ookinete assay can be used to predict efficacy *in vivo* with antibodies against a target that consistently blocks parasite development.
- Anti-sera have unidentified components that potentially aid inhibition of parasite development and thus IgG should be used as a means to determine antibody-mediated efficacy.

7. Optimisation of a Pan-malaria vaccine: AnAPN1 fragments – Immunogenicity and Efficacy

7.1. Introduction

The characterisation of mosquito-specific genes that have putative roles in mediating and regulating infection has led to their development as candidate TBVs. The attractiveness of these candidate genes is that they might have effects not only against *P. falciparum* but other *Plasmodium* species and other infections. The impact on parasite development isn't limited to midgut-specific genes. For instance, genes involved in mosquito immunity against bacterial infections have been implicated in *Plasmodium* development [640-644] whilst those involved in the integrity of the basal lamina have also been implicated [335, 645]. The antibacterial agent lysozyme was observed to be involved in facilitating the development of both rodent and human parasites in *A. gambiae* and *A. stephensi* [642]. Silencing of lysozyme c-1 gene expression led to the reduction of *P. berghei* parasite intensity and prevalence in *A. gambiae* [642] and in *A. dirus* [646], a mosquito vector known to be responsible for malaria transmission in Asia [647-649]. On the other hand, activation of the peptidoglycan recognition protein LC (PGRP-LC) mediated signalling pathway led to the mounting of an immune response to not just *P. berghei* but also field *P. falciparum* isolates [644]. Other immune-regulatory genes, when overexpressed have been shown to have an impact on parasite development and survival of the mosquito in a transgenic *A. stephensi* model [650]. In addition, other immune-regulating genes have been shown to be involved in oocyst development [651]. Antibodies raised against serine protease inhibitor 2 (serpin2) in ChAd63-MVA led to the inhibition of *P. berghei* infection whilst there was no observed impact on *P. falciparum* infectivity [652] . Williams et al. (2013), [652] were interestingly able to demonstrate that vaccine-induced antibody responses mediated against *A. gambiae* serpin2 were effective at reducing infectivity in *A. stephensi*. Thus the ability to target

multiple *Plasmodium* species in more than one mosquito species provides an added advantage and results in an attractive approach for TBV candidate antigen development.

An additional interesting aspect of the potential of mosquito-based antigens is their possible conserved nature. *Anopheles* ligands and/or receptors have been shown to be conserved across insect species and within anopheline species [348, 653, 654]. However, despite their conserved nature, the possibility of sequence homology with human orthologs might preclude them as potential vaccine candidates [349, 652]. Protein sequence alignment of rAnAPN1 revealed sequence homology of less than 30% with human CD13 [349]. CD13 is an alanyl (membrane) aminopeptidase (ANPEP) which is found in both mice and humans primarily located on the membranes of the small intestine and kidneys and distributed on various tissue plasma membranes [655-658]. CD13 is involved in various functions including digestion of peptides, receptor for viral infection and as a prognostic marker for cancer [659-663]. Therefore, the induction of antibodies against AnAPN1 has the potential to lead to immunopathology in the vertebrate host. This potentially raises the possibility of vaccine-associated adverse events more so in the human host if the antigen were to be developed as a vaccine for mass administration. Mathias et al. (2012) investigated whether rAnAPN1 vaccination in mice would lead to vaccine-associated immunopathologies and observed no evidence of these occurring [349].

In the work described so far, efficacy with antibodies raised against $AnAPN1_{fl}$ has only been observed against inhibiting parasite intensity from one gametocyte donor in *A. gambiae* whilst there was no efficacy against *P. falciparum* NF54 in *A. stephensi* (**Chapter 4**). Thus, it was imperative to optimise the design of this antigen to enable induction of antibodies with consistent TBA. Antibodies raised in mice against codon harmonised, *E. coli* expressed rAnAPN1 (sequence based on *A. gambiae* Keele strain) have been reported to inhibit *P.*

berghei infectivity to *A. stephensi* [349]. In addition, rAnAPN1 antibodies generated in rabbits have inhibited development of *P. falciparum* and *P. berghei* in *A. stephensi* and *A. gambiae* [348, 495]. However, antibodies from both these approaches haven't led to the complete inhibition of either infection intensity or prevalence.

The rationale for the work described in this chapter is based on the premise that optimising the full-length AnAPN1 antigen by generating fragments, including the N-terminal rAnAPN1 equivalent sequence, would (a) potentially reduce the sequence homology and reactivity to human CD13 hence aiding its development as a vaccine candidate; and more importantly, (b) enhance the ability to inhibit infectivity of *P. falciparum*. Thus, two constructs based on $AnAPN1_{fl}$ were designed and generated as recombinant ChAd63 and MVA viral-vectors and comparatively assessed for the ability of vaccine-induced IgG to inhibit *P. falciparum* infectivity to *A. stephensi* in SMFA.

7.2. Results

7.2.1. Anti-AnAPN1$_{fl}$ antibody efficacy against P. berghei

Despite the lack and/or limited efficacy observed with full-length AnAPN1 vaccine-induced antibodies against *P. falciparum* lab strain or field isolates, I sought to determine whether there would be inhibition of *P. berghei* infectivity. Anti-rAnAPN1 IgG at 100μg/ml has been reported to have an impact of 75% on *P. berghei* infection intensity in *A. gambiae* with similar results observed in *A. stephensi* [348]. This was thus included in the experiments as a comparator. In addition, the ability to inhibit *P. berghei* infectivity was determined in both *A. stephensi* (SDA500 strain) and *A. gambiae* (Yaoundé strain).

7.2.1.1. Inhibition of *P. berghei* infectivity in direct feeding assays

Direct feeding assays enable the investigation of the effect of parasite infectivity similar to natural mosquito infection acquisition using the rodent *Plasmodium* model allowing direct comparison with respective controls [361]. To determine whether AnAPN1$_{fl}$ had an effect on the infectivity of *P. berghei* in *A. stephensi* and *A. gambiae*, direct feeding assays were performed using mice prime-boost vaccinated with ChAd63 and MVA expressing AnAPN1$_{fl}$. As control, mice were vaccinated with ChAd63 and MVA expressing GFP. Two-weeks post the administration of MVA, the peak of the antibody response, individual mice were PH-treated, infected with parasitised RBCs, and on the day of the direct feed, checked for the presence of parasites and infectiousness by monitoring exflagellation events.

Each of the individual mice used to feed either *A. gambiae* or *A. stephensi* had antigen-specific IgG responses following prime-boost vaccination measured by standardised ELISA (**Figure 7.1**). Total IgG responses in the group of mice that were fed on *A. gambiae* were significantly boosted by 7-fold (p=0.008, day55 vs. day70 by Mann-Whitney test). For those used to infect *A. stephensi*, there was no statistical significance due to the sample size (n=2) but the responses were observed to be 10-fold higher two weeks post boost (p=0.3).

For the assessment of infectivity to mosquitoes, oocyst intensity and infection prevalence data for all the individual mice/group was collated and used to analyse the overall group effect on inhibiting infectivity (**Figure 7.2**). There was no effect mediated by anti-AnAPN1$_{fl}$ antibodies on inhibiting the infectivity of parasites in *A. stephensi* with a non-statistically significant 23% reduction in infectivity (p=0.2) and 6% reduction in prevalence (p=0.5) (**Figure 7.2A**). On the other hand, statistically significant reduction of infectivity to *A. gambiae* was demonstrated, reducing infection prevalence at 72% (p<0.0001) with a 26% non-statistically significant reduction in intensity (p=0.6) (**Figure 7.2B**).

To determine whether there was any evidence that the efficacy due to anti-AnAPN1$_{fl}$ antibodies varied between the two mosquito species, a log-likelihood ratio test was performed. There was no statistical evidence that inhibition of infection intensity or prevalence in *A. stephensi* varied from that in *A. gambiae* (p=0.13 and p=0.43 respectively). Therefore, there is insufficient evidence to suggest that efficacy observed was as a result of mosquito species effect. Nonetheless, this result doesn't take into account differences in infection intensity and amount of antigen-specific antibodies in each individual mouse. Therefore, a more robust comparison would be to estimate differences using the same infection intensity and antibody concentration.

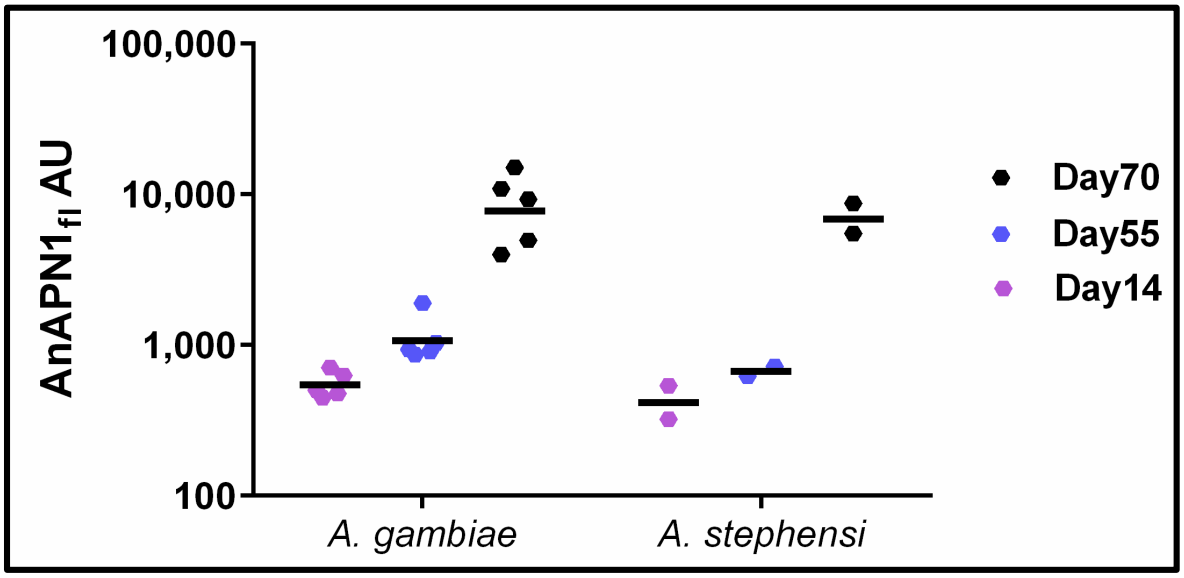
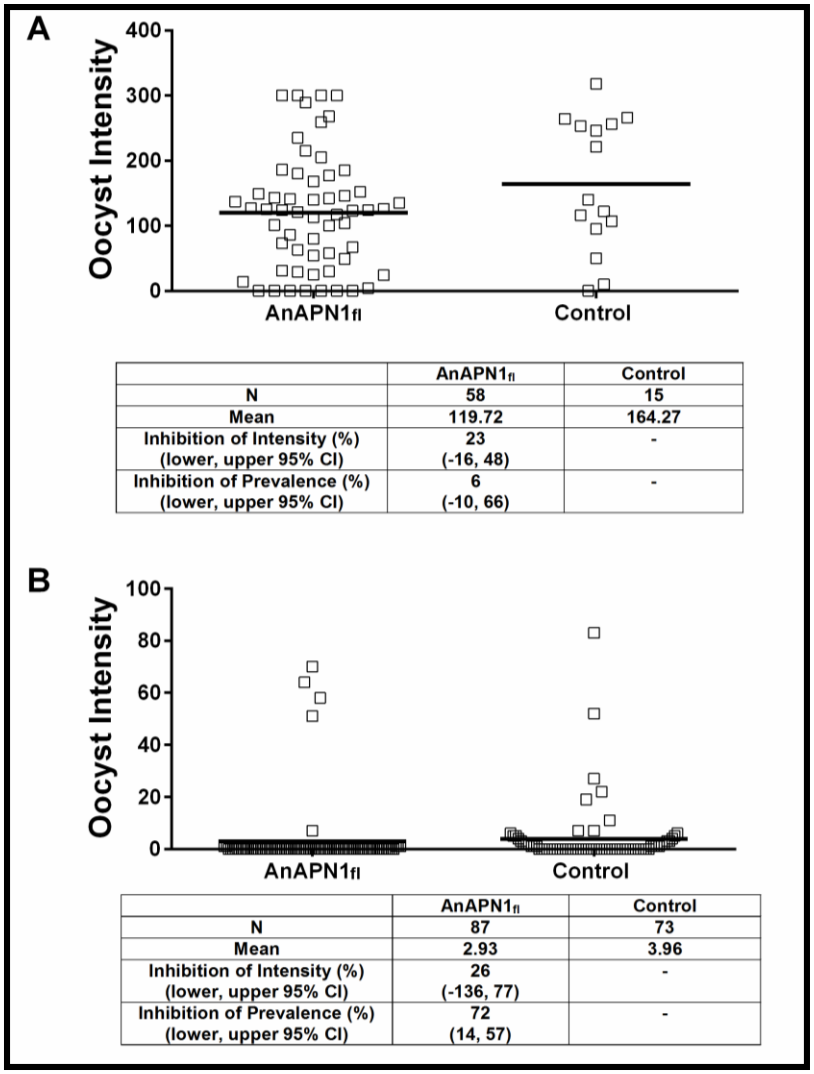


Figure 7.1. Vaccine-induced IgG responses after ChAd63-MVA prime-boost.

BALB/c mice (n=2-5/group) were vaccinated i.m. with ChAd63 (1x10^{8}IU, day0) and boosted with MVA (1x10^{7}PFU, day56) expressing AnAPN1$_{fl}$. Whole IgG responses were assayed on days 14, 55 and 70 by standardised ELISA using recombinant protein. The data was log-transformed and presented as geometric mean for mice used to feed *A. gambiae* and *A. stephensi* mosquitoes. Dots represent responses from individual mice.



	AnAPN1fl	Control
N	58	15
Mean	119.72	164.27
Inhibition of Intensity (%) (lower, upper 95% CI)	23 (-16, 48)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	6 (-10, 66)	-

	AnAPN1fl	Control
N	87	73
Mean	2.93	3.96
Inhibition of Intensity (%) (lower, upper 95% CI)	26 (-136, 77)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	72 (14, 57)	-

Figure 7.2. Effect of anti-AnAPN1$_{fl}$ antibodies against *P. berghei* infection in DFA.

BALB/c mice were vaccinated with ChAd63-MVA-AnAPN1$_{fl}$ or ChAd63-MVA-GFP (control), infected with parasitised *P. berghei* RBCs and fed directly to (**A**) *A. stephensi* or (**B**) *A. gambiae* in DFA. Midguts were dissected 11-13days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean of collated oocyst numbers from each individual mouse/group and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.

7.2.1.2. Inhibition of *P. berghei* infectivity in membrane feeding assays

To determine the specific anti-AnAPN1$_{fl}$ antibody contribution to efficacy, sera from individual animals previously vaccinated (**4.2.2.1**) was used in SMFA using *A. stephensi* and *A. gambiae* mosquitoes. As control, sera from mice vaccinated with ChAd63 and MVA expressing GFP was used. Two independent samples with different antigen-specific antibody concentrations were tested. These were defined to have low (7,630AU) and high (10,575AU) antigen-specific antibodies.

7.2.1.2.1. Effect due to a low antibody concentration

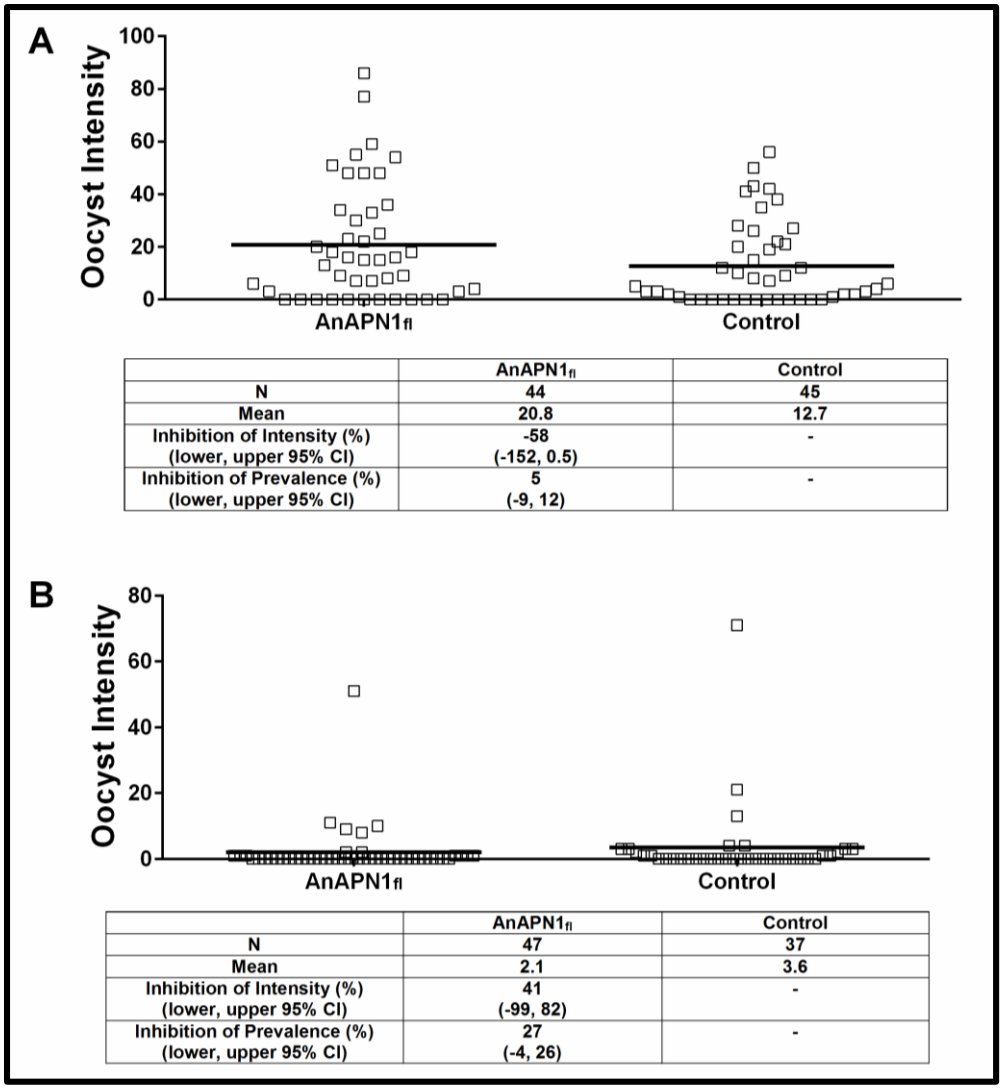
To determine whether a low anti-AnAPN1$_{fl}$ antibody concentration had an effect on the infectivity of *P. berghei*, two independent membrane feeding assays were performed using sera at two different concentrations. Thus the parasitised RBCs fed to each mosquito species were the same for each concentration tested in each assay.

At an antibody concentration of 763AU, anti-AnAPN1$_{fl}$ had no effect at inhibiting parasite intensity or prevalence in both *A. stephensi* and *A. gambiae* (**Figure 7.3**). In *A. stephensi*, there was a possible enhancement of parasite intensity at 58% (p=0.06) and a non-statistically significant reduction in prevalence of 5% (p=0.7) (**Figure 7.3A**). On the other hand, infectivity in *A. gambiae* revealed a non-statistically significant impact of 41% on intensity (p=0.4) and 27% on prevalence (p=0.3) (**Figure 7.3B**).

At an antibody concentration of 1,526AU, anti-AnAPN1$_{fl}$ had non-statistically significant inhibition of *P. berghei* infectivity with a 40% reduction in intensity (p=0.3) and 32% in prevalence (p=0.2) in *A. stephensi* (**Figure 7.4A**). In *A. gambiae*, there was a possible enhancement of parasite intensity of 80%, (p=0.6) whilst a non-statistically significant effect of 26% (p=0.7) was observed on prevalence (**Figure 7.4B**). In comparison, anti-rAnAPN1

IgG had a statistically significant effect on parasite development with a 76% reduction in intensity (p=0.03) and a 62% reduction in prevalence (p=0.03) in *A. stephensi*. However, in *A. gambiae* there was a non-statistically significant effect on both parasite intensity and prevalence.

Considering that the same parasitised RBCs were used to infect mosquitoes in these assays, verification of whether there was a mosquito species influence on parasite intensity and prevalence efficacy estimates was undertaken. There was statistically significant evidence that inhibition of both infection intensity and prevalence were more likely to be lower in *A. gambiae* than in *A. stephensi* ($p<0.0001$, log-likelihood ratio test). This observation was independent of the whether anti-AnAPN1$_{fl}$ or anti-rAnAPN1 antibodies were used as there was no evidence that these interventions behaved differently in the different mosquitoes regardless of whether intensity or prevalence was used as the efficacy estimate.

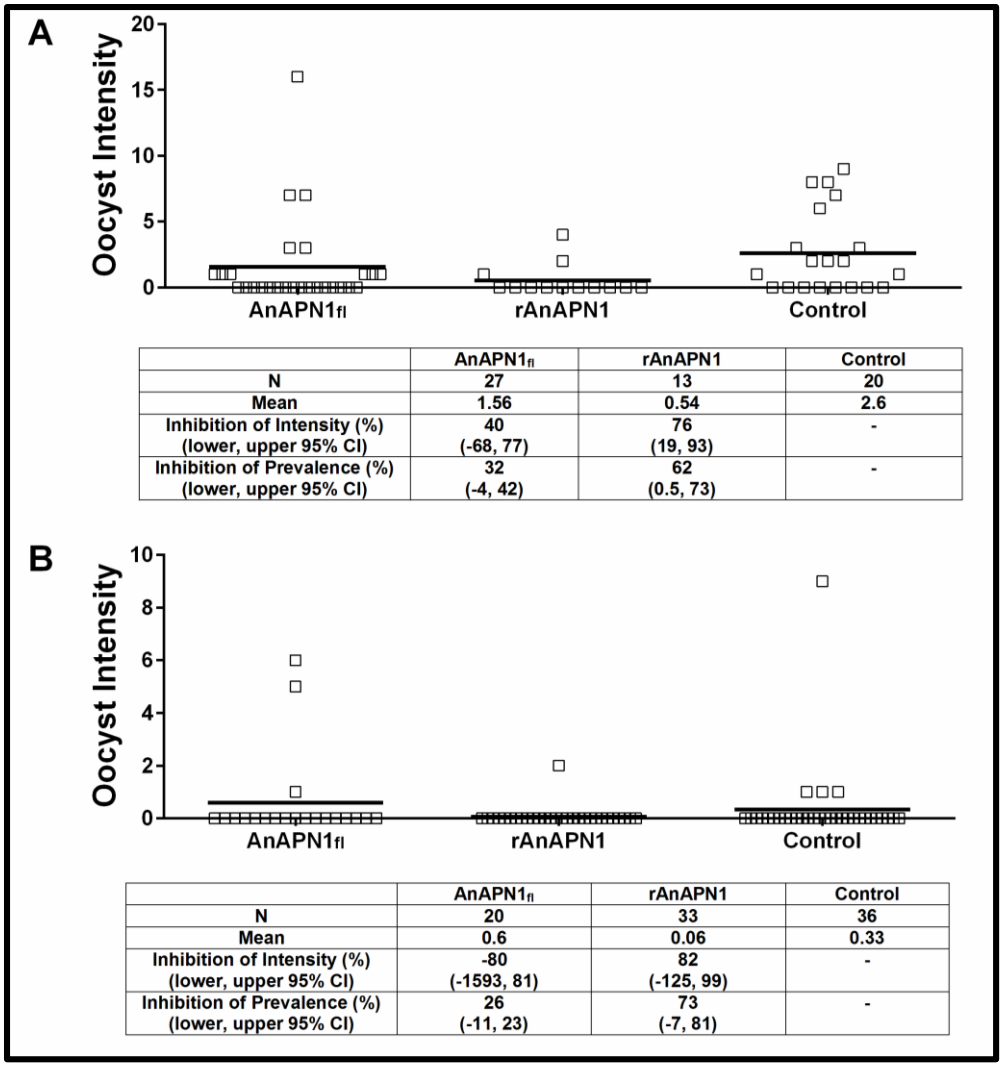


	AnAPN1$_{fl}$	Control
N	44	45
Mean	20.8	12.7
Inhibition of Intensity (%) (lower, upper 95% CI)	-58 (-152, 0.5)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	5 (-9, 12)	-

	AnAPN1$_{fl}$	Control
N	47	37
Mean	2.1	3.6
Inhibition of Intensity (%) (lower, upper 95% CI)	41 (-99, 82)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	27 (-4, 26)	-

Figure 7.3. Effect of anti-AnAPN1$_{fl}$ sera against *P. berghei* infectivity.

Sera from mice vaccinated with ChAd63 and MVA – AnAPN1$_{fl}$ or – GFP (control) (**4.2.2.1**) at a 1:10 dilution (763AU) in PBS was mixed with parasitised RBCs from mice infected with *P. berghei*. The mixtures were immediately fed to (**A**) *A. stephensi* or (**B**) *A. gambiae* in SMFA. Midguts were dissected 11-13days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.



	AnAPN1fl	rAnAPN1	Control
N	27	13	20
Mean	1.56	0.54	2.6
Inhibition of Intensity (%) (lower, upper 95% CI)	40 (-68, 77)	76 (19, 93)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	32 (-4, 42)	62 (0.5, 73)	-

	AnAPN1fl	rAnAPN1	Control
N	20	33	36
Mean	0.6	0.06	0.33
Inhibition of Intensity (%) (lower, upper 95% CI)	-80 (-1593, 81)	82 (-125, 99)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	26 (-11, 23)	73 (-7, 81)	-

Figure 7.4. Effect of anti-AnAPN1fl sera against *P. berghei* infectivity.

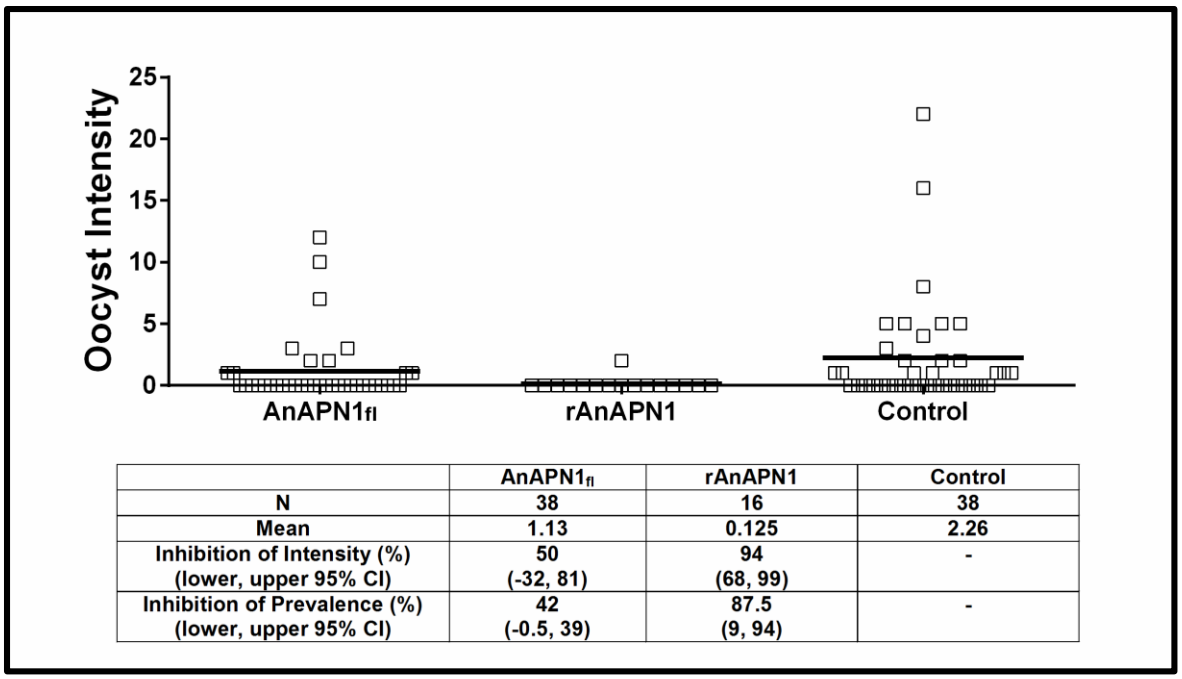
Sera from mice vaccinated with ChAd63 and MVA – AnAPN1fl or – GFP (control) (**4.2.2.1**) at a 1:5 dilution (1526AU) in PBS was mixed with parasitised RBCs from mice infected with *P. berghei*. In addition, anti-rAnAPN1 IgG at 100µg/ml in PBS was also mixed with parasitised RBCs. The mixtures were immediately fed to (**A**) *A. stephensi* or (**B**) *A. gambiae* in SMFA. Midguts were dissected 11-13days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.

7.2.1.2.2. Effect due to a high antibody concentration

Independent experiments against both *A. stephensi* and *A. gambiae* were conducted to further determine whether a high antibody concentration would have a significant impact on parasite infectivity. Therefore, parasitised RBCs used in either mosquito species were different.

At an antibody concentration of 2,115AU, there was a non-statistically significant effect on both intensity (50%, p=0.2) or prevalence (42%, p=0.06) of *P. berghei* infectivity to *A. stephensi* (**Figure 7.5**). Even though this result wasn't non-statistically significant, there was an improvement in the outcome observed compared with a lower antibody concentration (**Figure 7.3A and Figure 7.4A**). However, the wide confidence intervals imply that there is no evidence that the antibodies induced have any impact on either intensity and/or prevalence. This is more evident considering that anti-rAnAPN1 IgG was able to significantly reduce intensity by 94% (p=0.001) and prevalence by 88% (p=0.001) (**Figure 7.5**). Regardless of the antibody concentration of anti-AnAPN1$_{fl}$ antibodies, there was no effect at inhibiting *P. berghei* infections in *A. stephensi*. However, the inhibitory activity reported with anti-rAnAPN1 antibodies has been confirmed in these experiments.

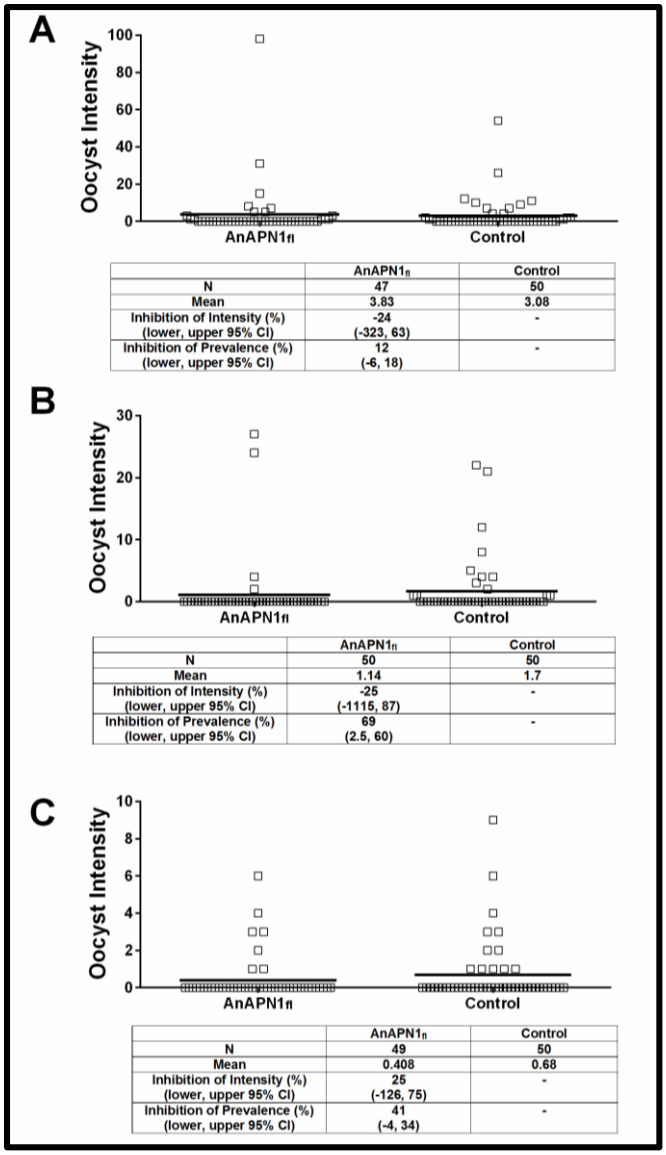
Assessment of infectivity in *A. gambiae* was tested at three different concentrations (**Figure 7.6**). Regardless of the antibody concentration, there was no statistically significant impact on parasite intensity either at 528AU, 1,057AU, or 2,115AU (**Figure 7.6A-C**). Interestingly, there was a statistically significant inhibition of prevalence of 69% (p=0.01) with an antibody concentration of 1,057AU (**Figure 7.6B**). There appears to be limited evidence of impact of anti-AnAPN1$_{fl}$ antibodies on infectivity of *P. berghei* in *A. gambiae*, a conclusion applicable to anti-rAnAPN1 IgG in these experiments.



	AnAPN1$_{fl}$	rAnAPN1	Control
N	38	16	38
Mean	1.13	0.125	2.26
Inhibition of Intensity (%) (lower, upper 95% CI)	50 (-32, 81)	94 (68, 99)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	42 (-0.5, 39)	87.5 (9, 94)	-

Figure 7.5. Effect of anti-AnAPN1 sera against *P. berghei* infectivity to *A. stephensi*.

Sera from mice vaccinated with ChAd63 and MVA – AnAPN1$_{fl}$ or – GFP (control) (**4.2.2.1**) at a 1:5 (2115AU) dilution in PBS were mixed with parasitised RBCs from mice infected with *P. berghei*. In addition, anti-rAnAPN1 IgG at 100µg/ml in PBS was also mixed with parasitised RBCs. *A. stephensi* mosquitoes were immediately fed in SMFA with the mixtures. Midguts were dissected 11-13days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.



	AnAPN1fl	Control
N	47	50
Mean	3.83	3.08
Inhibition of Intensity (%) (lower, upper 95% CI)	-24 (-323, 63)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	12 (-6, 18)	-

	AnAPN1fl	Control
N	50	50
Mean	1.14	1.7
Inhibition of Intensity (%) (lower, upper 95% CI)	-25 (-1115, 87)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	69 (2.5, 60)	-

	AnAPN1fl	Control
N	49	50
Mean	0.408	0.68
Inhibition of Intensity (%) (lower, upper 95% CI)	25 (-126, 75)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	41 (-4, 34)	-

Figure 7.6. Effect of anti-AnAPN1fl antibodies against infectivity to *A. gambiae*.

Sera from mice vaccinated with ChAd63 and MVA – AnAPN1fl or – GFP (control) (**4.2.2.1)** was mixed in PBS with parasitised RBCs from *P. berghei* infected mice and immediately fed to *A. gambiae* at (**A**) 1:5 (**B**) 1:10 or (**C**) 1:20 in SMFA. Midguts were dissected 11-13days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. Squares represent oocyst numbers from each individual mosquito dissected. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI.

7.2.2. Design and generation of AnAPN1 fragments

All the experiments examining the impact of anti-AnAPN1$_{fl}$ antibodies raised using ChAd63-MVA vaccination, show that there is limited evidence that these antibodies induced are able to inhibit either *P. falciparum* or *P. berghei* in either *A. stephensi* or *A. gambiae*. However, Dinglasan et al. (2007) has previously reported incomplete inhibition of parasite intensity against both parasites in either mosquito species by anti-rAnAPN1 antibodies [348]. Therefore, the antigen was further optimised to generate fragments that could possibly induce antibodies in a ChAd63-MVA prime-boost regimen with inhibitory effects on parasite infectivity.

7.2.2.1. Design of AnAPN1 fragments

Considering that rAnAPN1 has sequence homology with human APN1, the design of the fragment antigens was carefully considered to take this into account. The sequence homology between AnAPN1 and CD13 was determined. Protein sequences were obtained from NCBI protein database and alignments of the anopheline (AnAPN1, accession number XP_318000), human (HuAPN1, accession number P15144), and murine (MuAPN1, accession number NP_032512) APN1 sequences were created with a protein BLAST [664] and Clustal W [665] to determine the regions of complementarity and disparity. These alignments revealed that full-length AnAPN1 had 34% sequence identity against HuAPN1 and 33% against MuAPN1 with more than 200αα residue alignment score. HuAPN1 and MuAPN1 had greater sequence identity of 76%. Analysis of specific conserved domains or regions of the individual sequences revealed a greater overlap between HuAPN1 and MuAPN1 as evidenced by the degree of sequence identity (**Table 7.1**).

To ensure that the design of the AnAPN1 fragments would overcome the problem of sequence identity but still maintain the ability to induce antibodies with parasite inhibitory activity, a model of the structure of AnAPN1 was simulated using SWISS-MODEL [666], MODELLER [667] and the positioning of the sequences determined using PyMOL [668]. The codon optimised sequence of full-length AnAPN1 (20 – 996αα residues) was modelled against the crystal structure of the soluble domain of human endoplasmic reticulum aminopeptidase 1 (ERAP1, accession number Q9NZ08) with 33% sequence identity (**Figure 7.7**). The three-dimensional structure revealed that the protein was largely composed of β-pleated sheets and α-helices. The protein was subdivided into four possible structural domains (I – IV) (**Figure 7.7A**). Based on this model, 135αα residues corresponding to rAnAPN1 (located within Domain I) appeared to be part of one of the β-pleated structures with some of the strands missing that would possibly enable stability of the protein (**Figure 7.7B**). Based on this model, it was thus postulated that this might not fold properly or be stable when expressed.

Therefore, the entire Domain I and the residues corresponding to rAnAPN1 were considered for the generation of AnAPN1 fragments. Thus the fragments designed were Domain I (hereafter referred to as AnAPN1 DomI) based on residues 1 – 283αα and N terminus residues corresponding to the 135αα residues in rAnAPN1 (hereafter referred to as AnAPN1 Nterm). These sequences were also aligned against HuAPN1 and MuAPN1 protein sequences to determine sequence identity. AnAPN1 DomI had 34% and 32% against HuAPN1 and MuAPN1 respectively with 80 – 200αα residue alignment score. AnAPN1 Nterm was found to be 29% and 26% identical to HuAPN1 and MuAPN1 with a lesser residue alignment score of 40 – 50αα.

Table 7.1. Characteristics of APN1 protein sequences.

Feature	AnAPN1	HuAPN1	MuAPN1
Signal peptide	1 – 19 αα	1 – 23αα	1 – 35αα
TM Domain	N/A	9 – 32αα	9 – 32αα
GPI	997 – 1020αα	947 – 957αα	946 – 955αα
Regions			
- Peptidase M1	64 – 455αα	75 – 480αα	76 – 479αα
- Aminopeptidase N	72 – 526αα	84 – 550αα	84 – 550αα
- Ser/Thr rich motifs	952 – 992αα	33 – 68αα	33 – 68αα

AnAPN1: *Anopheles* amino peptidase N1, accession number XP_318000; HuAPN1: human amino peptidase N1, accession number P15144; MuAPN1: murine amino peptidase N1, accession number NP_032512; αα: amino acid; N/A: not applicable; GPI: glycosylphosphatidylinositol; TM: Transmembrane domain; Ser/Thr: Serine/Threonine.

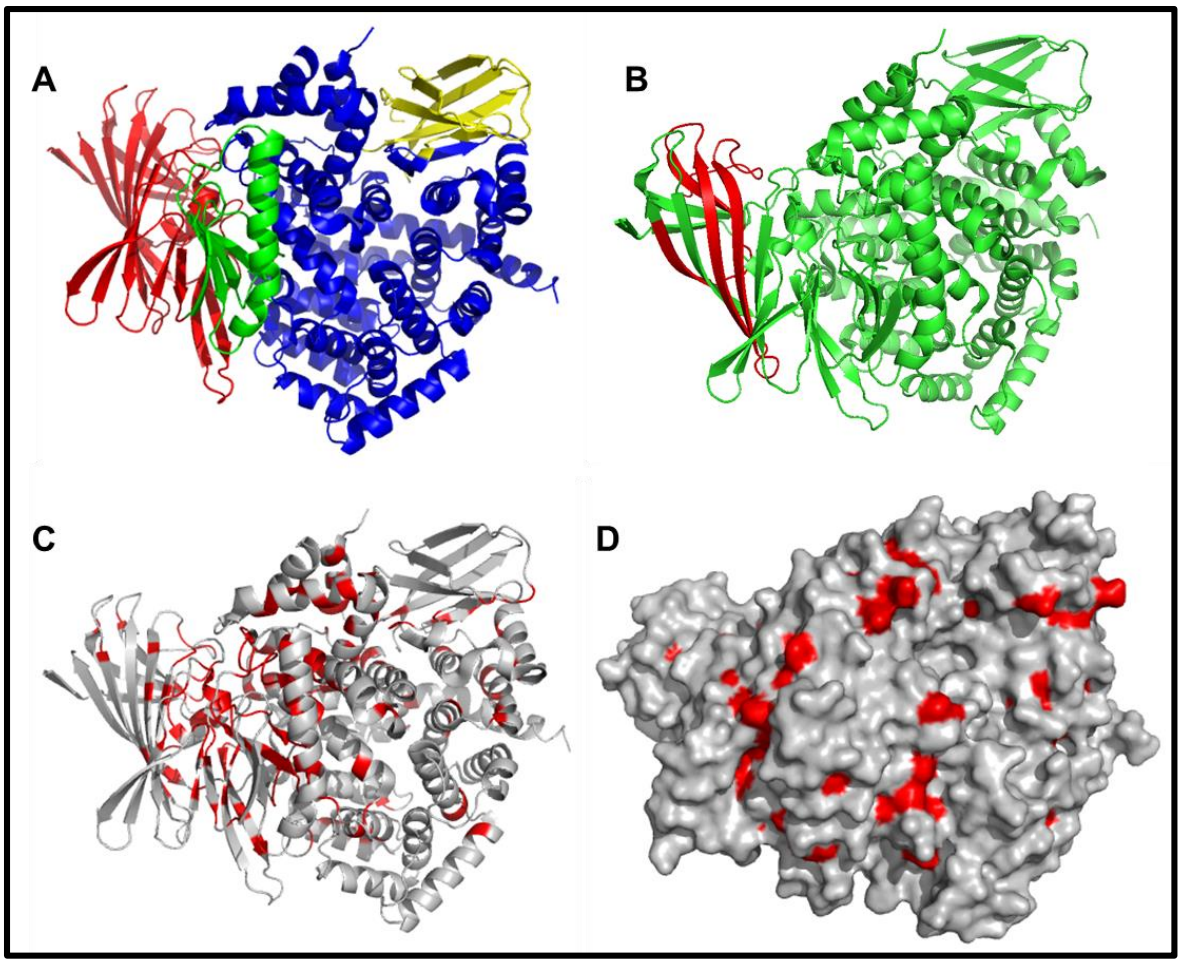


Figure 7.7. Proposed model structure of AnAPN1.

Codon optimised AnAPN1 protein sequence was modelled based on the crystal structure of the soluble domain of human endoplasmic reticulum aminopeptidase 1 (ERAP1) using SWISS-MODEL, MODELLER and PyMOL. (**A**) Shows the four possible structural domains identified: Domain I, red (1-283αα); Domain II, green (284-356αα); Domain III, yellow (538-616αα); and Domain IV, blue (357-537αα and 617-1010αα). (**B**) Shows the sequence corresponding to rAnAPN1 (1-135αα residues) in red and the rest of the sequence in green. (**C**) Shows the residues that are conserved (red) and non-conserved (grey). (**D**) Shows the surface representation of (**C**).

7.2.2.2. Generation of recombinant AnAPN1 fragment viral-vectors

AnAPN1 fragments were generated based on codon optimised full-length AnAPN1 by PCR cloning. AnAPN1 DomI (20 – 303αα) and AnAPN1 Nterm (41 – 176αα) specific primers were designed (**Table 7.2**) and used to amplify the specific sequences from the plasmid containing the codon optimised sequence of $AnAPN1_{fl}$ (**Figure 7.8**). These primers were designed to have 15bp complementary to the entry plasmid used to clone the antigens, linearised by PCR to allow fusion of the homologous fragments. PCR reactions were performed using high-fidelity Phusion DNA polymerase. The respective PCR products were in-fusion cloned into the linearised entry plasmid containing the tissue plasminogen activator signal peptide. The resulting entry plasmids were used to clone into pENTR4-LPTOS and pMVA.GFP by restriction cloning as described in sections **3.2.2.1** and **3.2.3.1** respectively. These were then used to subsequently generate recombinant ChAd63 and MVA expressing either AnAPN1 DomI or AnAPN1 Nterm as described in **Chapter 3**.

To confirm the identity and purity of the generated recombinant viral-vectors, PCR reactions were performed using primers specifically designed to detect antigen-specific sequences (ID PCR primers) and regions flanking the antigen (flank-to-flank PCR primers) (**Table 7.3**). The presence of either AnAPN1 DomI or AnAPN1 Nterm and the purity of the ChAd63 recombinant viruses was confirmed with the respective PCR products being amplified with no observed contamination (**Figure 7.9A**). The presence and purity of MVA viruses was also confirmed with only the target sequences being amplified (**Figure 7.9B**). For each recombinant virus, pre-viral plasmid amplification products, used as positive control, further confirmed that the viruses contained sequences corresponding to the respective antigens.

Table 7.2. Primers used for cloning AnAPN1 fragments.

Plasmid	Forward Primer	Reverse Primer	Product size (bp)
Entry plasmid	AGCGCCTGGTCTCATCC ACAATTTG	GGTACCAAGCTTAACCG GAGGCTGGATC	3340bp
AnAPN1 DomI	**GCCCGATTCAGAAGA**A GATCTGACAGACCCCCC TACAAG	**ATGAGACCAGGCGCT** GTCGGACACCACGAAGG	786bp
AnAPN1 Nterm	**GCCCGATTCAGAAGA**A GATCTGAGAGATACAGA CTGCCCAC	**TTTTCAAATTGTGGC** GGCCAGGTATCTCC	438bp

In bold are 15bp complementary sequences at the 5'end of forward and reverse primers.

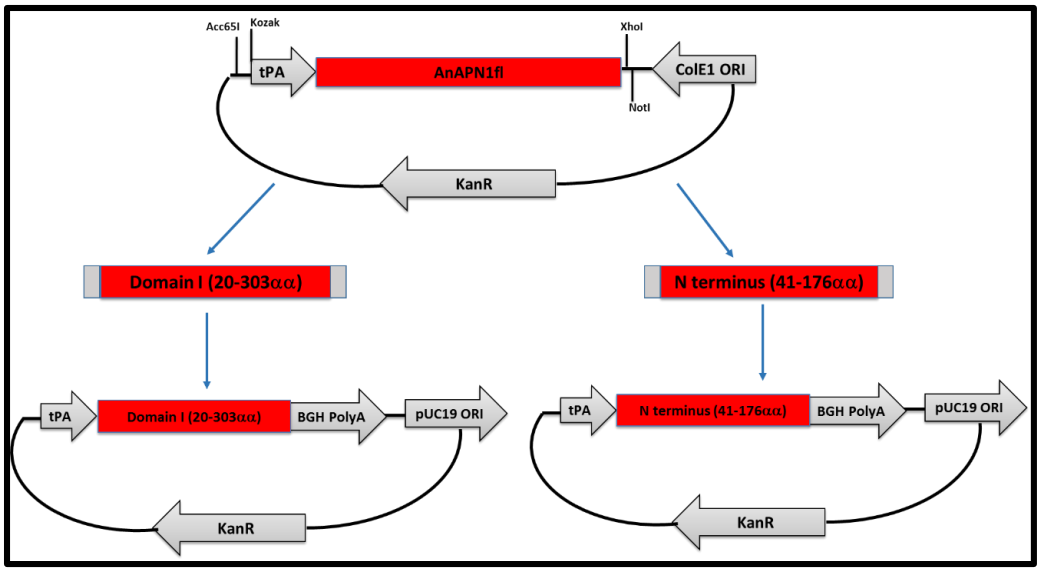


Figure 7.8. Schematic for the cloning of AnAPN1 fragments.

pAnAPN1$_{fl}$ DNA (**Figure 3.1**) was used to PCR amplify sequences specific for Domain I and N terminus. The primers used had 15bp (shown as grey bars at either end) complimentary to an entry plasmid containing tPA and BGH Poly A. The entry plasmid was Phusion PCR linearised and used to perform In-fusion reactions with Domain I and N terminus PCR products to obtain plasmid vectors containing the respective constructs for restriction cloning into ChAd63 and MVA. tPA: tissue plasminogen signal peptide; BGH Poly A: bovine growth hormone polyadenylation sequence; ORI: origin of replication; and KanR: Kanamycin resistance cassette.

Table 7.3. List of primers used to determine identity and purity of viral-vectors.

Primer pair	Sequence	PCR Purpose
J1212 J1251	GCAACAGCTTCTCCTCGTTC CTTCGAGCACATCACCTTCA	ID for ChAd63*
J1209 J1210	TCAGAGGTAACTCCCGTTGC CAGCGAGCTCTAGCATTTAGG	Flank-to-flank for ChAd63*
J1205 J1251	CCATCGAGTGCGGCTACTAT CTTCGAGCACATCACCTTCA	ID for MVA*
J1204 J1205	TGTGGGTAATGTTCTCGATGTC CCATCGAGTGCGGCTACTAT	Flank-to-flank for MVA*

*The same primer pairs and PCR conditions were used for both fragments.

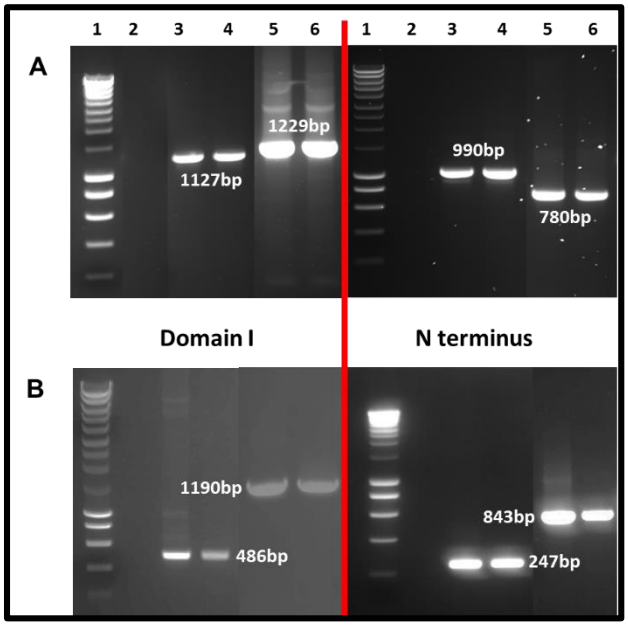


Figure 7.9. PCR confirmation of recombinant viral-vectored virus identity and purity.

PCR reactions were performed using purified viral DNA and products were run on agarose gels. Respective pre-viral plasmid DNA and water were used as positive and negative controls respectively. Depicted are purified recombinant (**A**) ChAd63 and (**B**) MVA virus for each antigen. Expected product sizes are shown alongside each respective corresponding band. Lanes: 1 – Smart DNA ladder; 2 – water; 3 – pre-viral plasmid DNA; 4 – viral DNA; 5 – pre-viral plasmid DNA; 6 – viral DNA. Lanes 3 and 4 show identity; and, 5 and 6 purity PCR products. Red line demarcates PCR products for the respective antigens.

7.2.3. Efficacy of AnAPN1 fragments – P. falciparum NF54

7.2.3.1. Immunogenicity of AnAPN1 fragments

To ascertain whether AnAPN1 fragments were immunogenic when expressed in recombinant ChAd63 and MVA viral-vectors, BALB/c mice were prime-boost vaccinated with ChAd63 and MVA individually expressing AnAPN1 DomI or AnAPN1 Nterm. Total IgG responses were determined by endpoint ELISA using full-length recombinant AnAPN1 protein and boosting assessed by Mann-Whitney test comparing day55 and day70 (**Figure 7.10**). Anti-sera from AnAPN1 Dom1 and AnAPN1 Nterm vaccinated animals reacted with full-length antigen at all time-points assessed. AnAPN1 DomI responses were boosted by a 5-fold magnitude (p=0.03) (**Figure 7.10A**). This same was true for AnAPN1 Nterm with an 11-fold increase in antibody responses post-boost (p=0.009) (**Figure 7.10B**). However, AnAPN1 Nterm wasn't as reactive as AnAPN1 DomI with variability in the number of responders post-boost. Only 67% had an observed reactivity in the AnAPN1 Nterm group whilst all the animals were reactive in the AnAPN1 DomI group (**Figure 7.10**).

Therefore, the expression of the antigens in HEK293 cells was determined. pENTR4-LPTOS DNA expressing either AnAPN1 DomI or AnAPN1 Nterm was used to transfect HEK293s. Supernatant and cell lysate were harvested after 24hours, mixed with non-reducing and reducing SDS buffer and used to detect the presence of the antigens by western blotting. Anti-sera specific for AnAPN1 DomI was able to detect proteins migrating at 30kDa and 60kDa (**Figure 7.11A**). The predicted size of AnAPN1 DomI is 27.5kDa, indicative of the lower molecular weight species observed at 30kDa. Thus the higher observed band could be as result of protein dimerisation or aggregation. On the other hand, AnAPN1 Nterm was weakly recognised closer to the expected size of 14.9kDa (**Figure 7.11B**). Expression of AnAPN1 DomI was predominantly observed in the supernatant whilst AnAPN1 Nterm was

only observed in the lysate leading to the assumption that the protein might be poorly secreted.

7.2.3.2. Kinetics of the ChAd63 antibody priming response

Investigation of the kinetics of the ChAd63 priming response to AnAPN1$_{fl}$ previously showed that antibodies increased to time of boosting (**4.2.2.1**). Assessment of whether the kinetics of the ChAd63 priming response to AnAPN1 DomI and AnAPN1 Nterm would also follow a similar trend was performed. BALB/c mice were prime-boosted with ChAd63 and MVA and sera was assayed for antibody induction on days14, 35 and 55 by endpoint ELISA using full-length recombinant protein. Statistical significance was ascertained by Mann-Whitney test.

There was no difference between responses at day14 and day35 or day14 and day55 for both antigens (**Figure 7.12**). However, as observed (**7.2.3.1**), MVA vaccination significantly boosted the reactivity to AnAPN1$_{fl}$; AnAPN1 DomI 2-fold increase (p=0.04) and AnAPN1 Nterm 9-fold increase (p=0.005). Ditto, AnAPN1 Nterm reactivity was inferior compared to that of AnAPN1 DomI. Likewise, there was variability in the number of responders in the AnAPN1 Nterm group with only 60% showing reactivity.

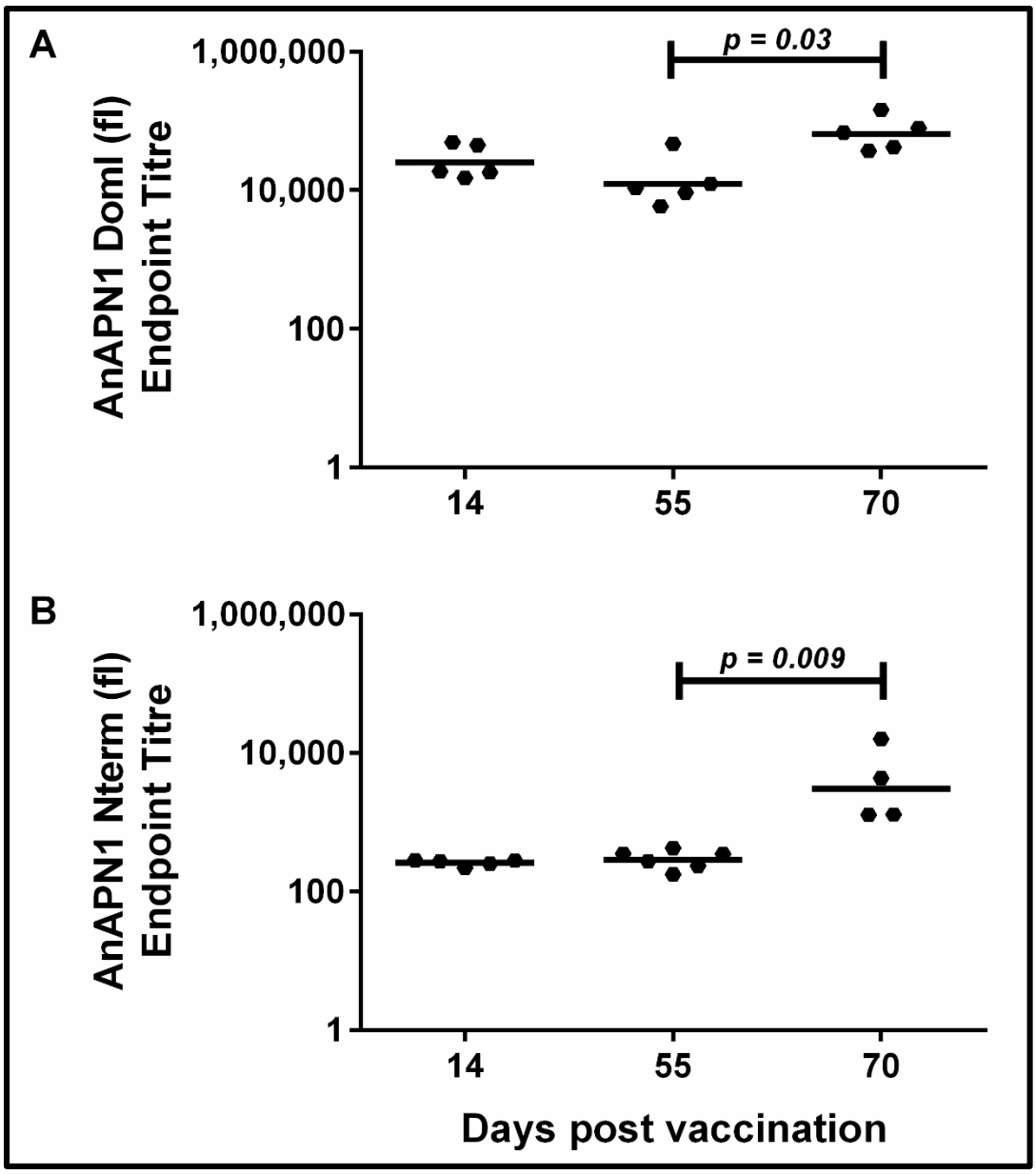


Figure 7.10. Vaccine-induced IgG responses after ChAd63-MVA prime-boost.

BALB/c mice (n=5-6/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with the respective MVA ($1x10^7$pfu, day56) expressing (**A**) AnAPN1 Dom I; and (**B**) AnAPN1 Nterm. Whole IgG responses were assayed on days 14, 55 and 70 by endpoint ELISA using AnAPN1 full-length (fl) recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points. Dots represent responses from individual mice. Significance shown and considered at $p<0.05$.

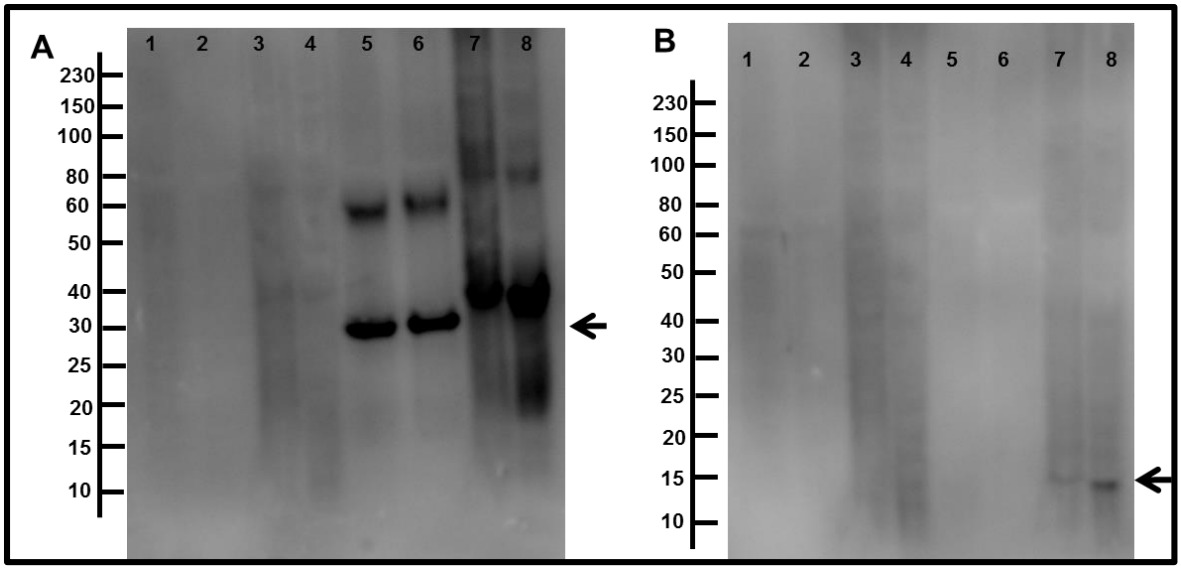


Figure 7.11. Expression of AnAPN1 fragments in HEK239s.

pENTR4-LPTOS DNA expressing (**A**) AnAPN1 DomI; and (**B**) AnAPN1 Nterm was used to transfect HEK293s for 24hours. Supernatant and cell lysate were harvested and mixed with either non-reducing or reducing SDS buffer. Samples were run on 4-20% polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using day70 pooled sera. Untransfected supernatant and cell lysate were used as negative controls. Lanes: 1 – control supernatant in non-reducing buffer; 2 – control supernatant in reducing buffer; 3 – control lysate in non-reducing buffer; 4 – control lysate in reducing buffer; 5 – 8 antigen-specific supernatant or lysate respectively. Arrowheads indicate the relative migration mobility expected for each respective antigen.

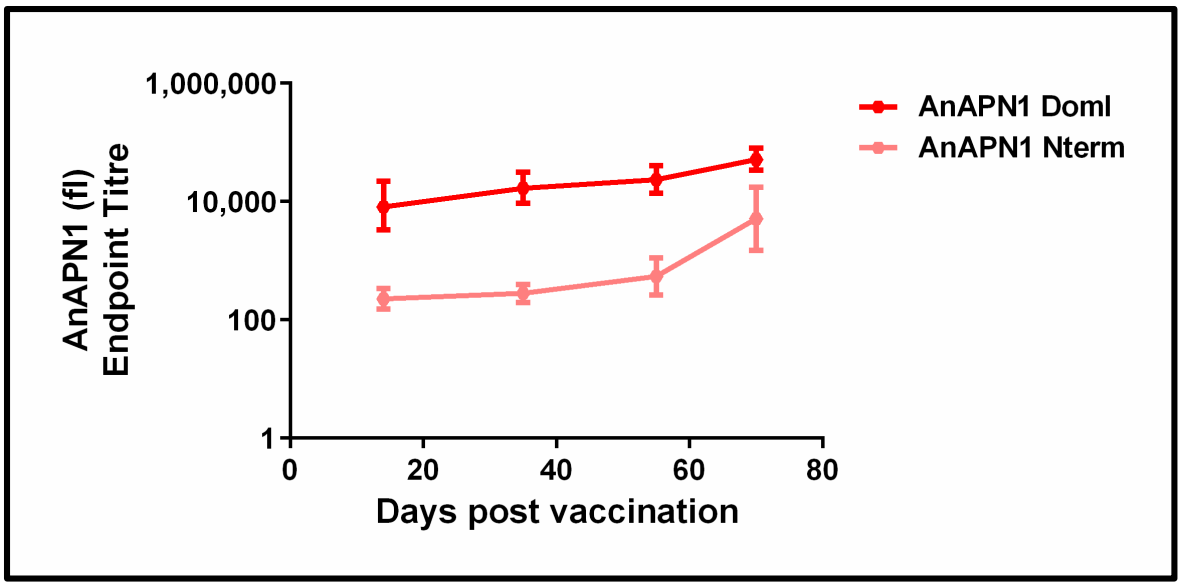


Figure 7.12. Kinetics of the antibody priming response following ChAd63 vaccination.

BALB/c mice (n=10/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with the respective MVA ($1x10^7$pfu, day56) expressing either AnAPN1 DomI or AnAPN1 Nterm. Whole IgG responses were assayed on days 14, 35, 55 post-prime and day70 post-boost by endpoint ELISA using AnAPN1 full-length (fl) recombinant protein. The data was log-transformed and presented as geometric mean ± 95%CI for the different time points.

7.2.3.3. Reactivity of AnAPN1 fragments to human CD13

Antibodies raised against rAnAPN1 have been previously shown not to cross-react with CD13 expression in frozen human tissue sections from the kidney, liver and small intestine [349]. To completely rule out the possibility of antibodies induced by vaccination using viral-vectors against $AnAPN1_{fl}$, AnAPN1 DomI and AnAPN1 Nterm cross-reacting with CD13, immunohistochemistry staining of frozen human tissue sections was also carried out. The monoclonal antibody to CD13, WM-47, was used as a marker for positive CD13 staining [656, 669]. The scoring of the tissue sections was independently assessed by Prof. Kevin Gatter and Helen Turley (Nuffield Division of Clinical Lab Sciences, University of Oxford).

The staining pattern representative of CD13 observed in tonsil, liver, endometrium and ovary tissue sections was as previously reported [656, 670-672] (**Table 7.4**). Anti-sera against $AnAPN1_{fl}$ weakly exhibited cross-reactivity to CD13 on Kupfer cells in comparison to that of anti-AnAPN1 Dom I and anti-AnAPN1 Nterm. Even though anti-sera to all the AnAPN1 fragments reacted with lipofuscin in the liver and granulocytes in the tonsil, this didn't appear to be as a result of CD13 cross-reactivity. This could be due to non-specific interactions with lipofuscin granular contents or other surface granulocyte proteins. Overall there appears to be limited evidence to suggest that anti-sera to the AnAPN1 fragments cross-reacted with CD13 on frozen tissue sections. In addition, any possible reactivity to MuAPN1 is unlikely as a number of mice have been vaccinated with these constructs with no evidence of any vaccine-associated illness. More so, there was no evidence of this against $AnAPN1_{fl}$ (longevity experiment, **4.2.2.4**) which has the highest degree of sequence identity.

Table 7.4. Reactivity of AnAPN1 fragments against tissue sections.

Tissue	**AnAPN1$_{fl}$**	**AnAPN1 DomI**	**AnAPN1 Nterm**	**CD13**
Tonsil	Scattered staining of granulocytes	Weak staining of granulocytes	Weak staining of granulocytes	Staining of macrophages
Liver	Lipofuscin staining; light staining of Kupfer cells	Lipofuscin staining; no staining	Lipofuscin staining; no staining	Staining of Kupfer cells
Endometrium	No staining	No staining	No staining	Staining of endometrial stroma cells; scattered staining of macrophages
Ovary	No staining	No staining	No staining	Scattered staining of macrophages

7.2.3.4. Inhibition of *P. falciparum* NF54 infectivity to *A. stephensi*

Anti-rAnAPN1 IgG though effective at inhibiting *P. falciparum* infection intensity in *A. stephensi*, doesn't have complete TBA [348]. Thus, it was imperative to determine whether antibodies induced to AnAPN1 fragments would be able to inhibit parasite development. Antibodies raised against $AnAPN1_{fl}$ haven't shown any efficacy but were included in these experiments to comparatively assess the ability of vaccine-induced responses against AnAPN1 DomI and AnAPN1 Nterm. Mice were prime-boost vaccinated with ChAd63-MVA expressing the individual antigens. In addition, a control group was vaccinated with ChAd63-MVA-GFP. Vaccine-induced responses were measured by ELISA against full-length AnAPN1 and any statistically significant boosting determined by Mann-Whitney test. There was consistent boosting of the ChAd63 priming response to; $AnAPN1_{fl}$ (10-fold, $p<0.0001$) (**Figure 7.13A**); AnAPN1 DomI (3-fold, $p=0.001$) (**Figure 7.13B**); and AnAPN1 Nterm (10-fold, $p=0.0002$) (**Figure 7.13C**). As observed previously in the AnAPN1 Nterm group, not all the animals responded (64% responders).

Purified total IgG from pooled sera was tested at two concentrations in SMFA (**Figure 7.14**). Anti-$AnAPN1_{fl}$ IgG was ineffective at inhibiting both parasite intensity and prevalence. In addition, anti-AnAPN1 DomI IgG was also unable to statistically significantly inhibit both parasite intensity and prevalence regardless of the antibody concentration. In comparison, anti-AnAPN1 Nterm IgG was only able to have a statistically significant impact of 48% on parasite intensity ($p=0.02$) at the least concentration tested of 375μg/ml. In this experiment, there was possibility that a dose effect or an optimal concentration of anti-AnAPN1 Nterm antibodies might be required to potently inhibit parasite infectivity. However, this needs to be further validated but it can be concluded that antibodies raised against AnAPN1 fragments have limited or no evidence of efficacy against parasite development.

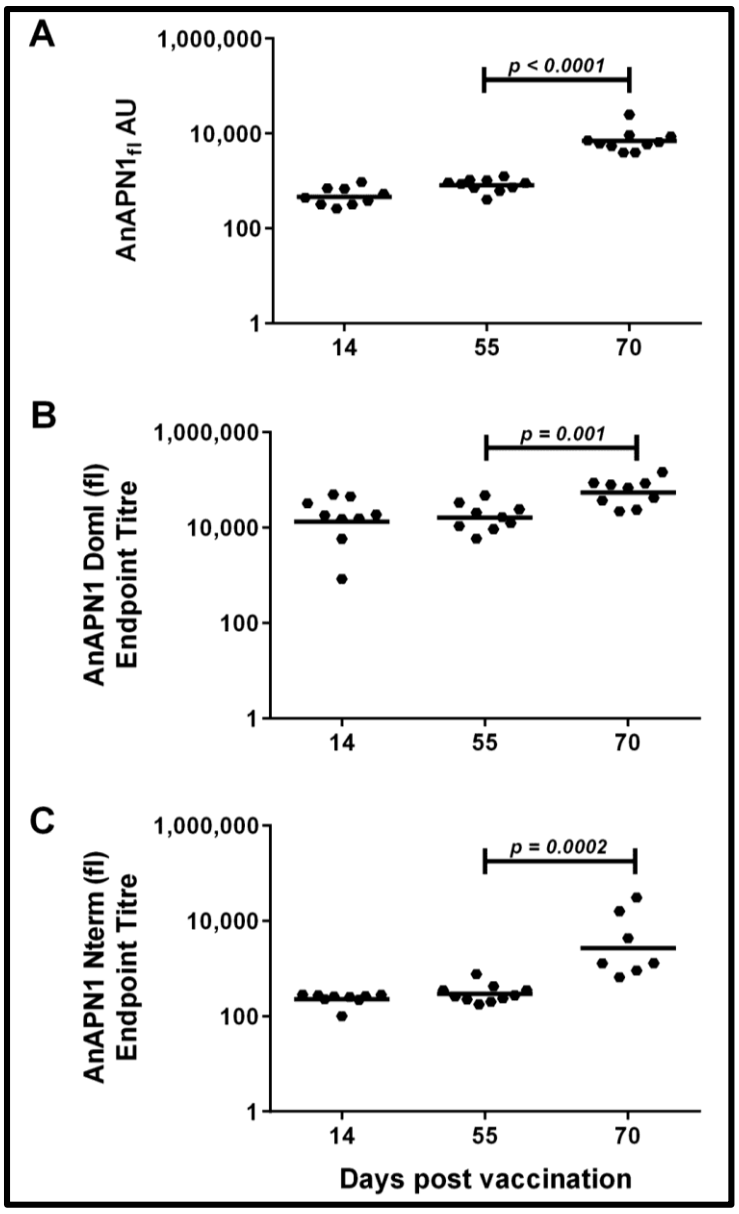
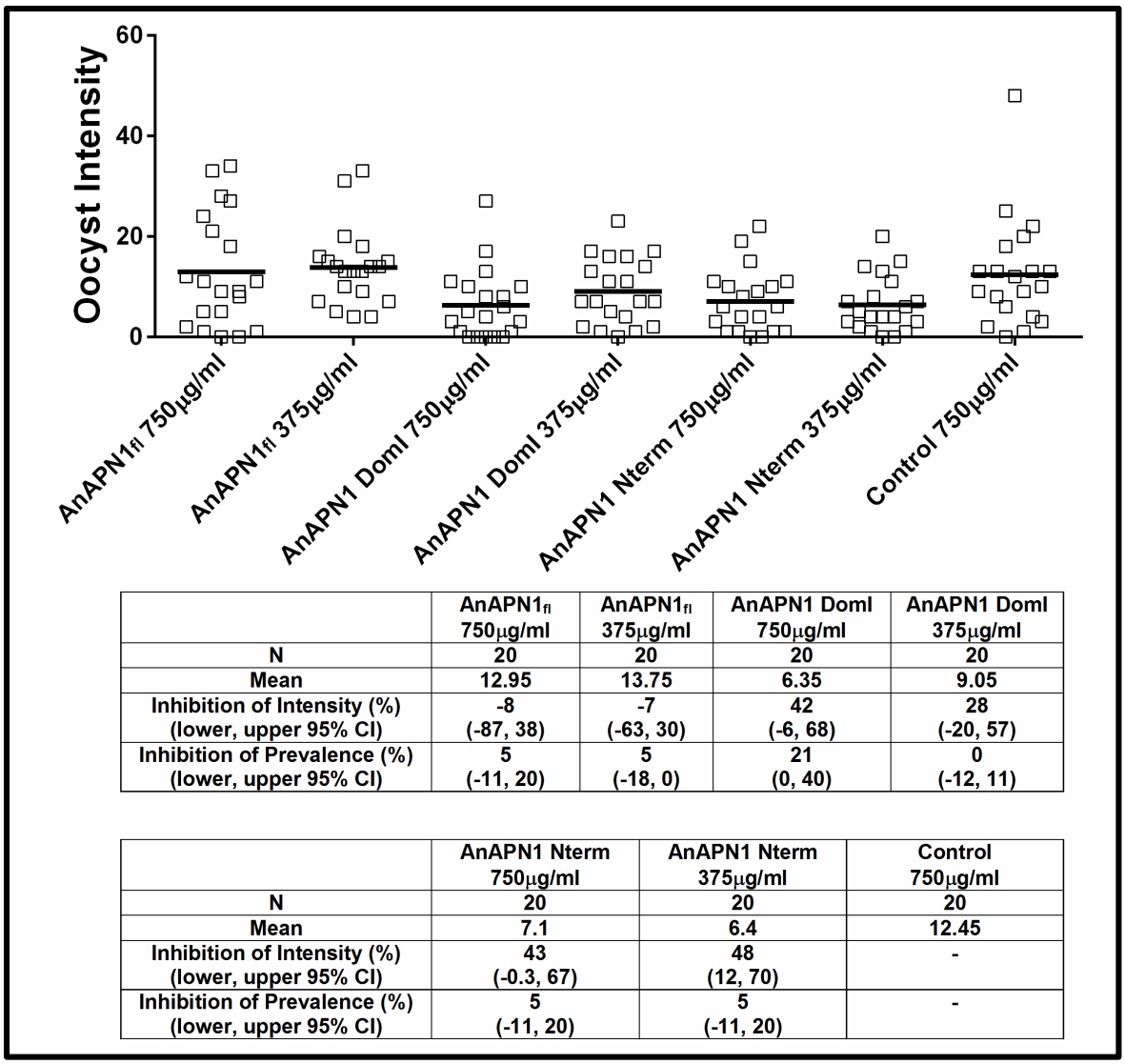


Figure 7.13. Vaccine-induced IgG responses to full-length recombinant protein.

BALB/c mice (n=9-11/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with respective MVA ($1x10^7$pfu, day56) expressing (**A**) $AnAPN1_{fl}$; (**B**) AnAPN1 Dom I; and (**C**) AnAPN1 Nterm. Whole IgG responses were assayed on days 14, 55 and 70 by either standardised (**A**) or endpoint (**B** and **C**) ELISA using AnAPN1 full-length (fl) recombinant protein. The data was log-transformed and presented as geometric mean for each of the different time points. Dots represent responses from individual mice. Significance shown and considered at $p<0.05$.



	AnAPN1$_{fl}$ 750µg/ml	AnAPN1$_{fl}$ 375µg/ml	AnAPN1 DomI 750µg/ml	AnAPN1 DomI 375µg/ml
N	20	20	20	20
Mean	12.95	13.75	6.35	9.05
Inhibition of Intensity (%) (lower, upper 95% CI)	-8 (-87, 38)	-7 (-63, 30)	42 (-6, 68)	28 (-20, 57)
Inhibition of Prevalence (%) (lower, upper 95% CI)	5 (-11, 20)	5 (-18, 0)	21 (0, 40)	0 (-12, 11)

	AnAPN1 Nterm 750µg/ml	AnAPN1 Nterm 375µg/ml	Control 750µg/ml
N	20	20	20
Mean	7.1	6.4	12.45
Inhibition of Intensity (%) (lower, upper 95% CI)	43 (-0.3, 67)	48 (12, 70)	-
Inhibition of Prevalence (%) (lower, upper 95% CI)	5 (-11, 20)	5 (-11, 20)	-

Figure 7.14. Comparative inhibition against *P. falciparum* infectivity in *A. stephensi*.

Total IgG was purified from sera pooled (day70) from mice vaccinated with each respective construct or control. The total IgG was diluted at the indicated concentrations in PBS, mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* in SMFA. Midguts were dissected 7-8days post-feeding and the number of oocysts in individual mosquitoes and the prevalence of infection were determined. The results are represented as arithmetic mean and N the number of mosquitoes dissected. Inhibition of oocyst intensity and prevalence were calculated relative to the control as percentages ± 95% CI. Squares represent oocyst numbers from each individual mosquito dissected.

7.2.3.5. AnAPN1 fragment reactivity to rAnAPN1

Hitherto, there has been no consistent and convincing evidence to show that any form of AnAPN1 expressed in ChAd63-MVA is able to induce potent antibodies with TBA against *P. falciparum* infectivity in *A. stephensi*. However, responses to rAnAPN1 expressed in *E. coli* have been shown to exhibit TBA [348]. Therefore, I sought to examine any possible differences in the responses and reactivity of the ChAd63-MVA expressed AnAPN1 fragments compared to rAnAPN1.

7.2.3.5.1. Reactivity to HEK293 expressed AnAPN1 fragments

Firstly, reactivity of anti-rAnAPN1 to mammalian expressed AnAPN1 fragments was assessed to determine the protein expression profile. pENTR4-LPTOS DNA expressing the individual AnAPN1 fragments was used to transfect HEK293s and after 24hours, supernatants and cell lysates were harvested, mixed in non-reducing and reducing SDS buffer and used to generate immunoblots using anti-rabbit rAnAPN1 IgG. As previously observed with antigen-specific sera, the AnAPN1 fragment proteins migrated at the expected sizes (**Figure 7.15**). The intensity of reactivity against AnAPN1 Nterm was greater compared to that observed with antigen-specific sera (**Figure 7.15C**). For $AnAPN1_{fl}$ and AnAPN1 DomI, expression was predominantly observed in the supernatant, confirming secretion of the antigens. However, the dominant species against AnAPN1 Nterm were observed in the cell lysate, further confirming the speculation that protein was poorly secreted. Therefore, antibodies directed at rAnAPN1 were able to recognise AnAPN1 expressed in mammalian cells.

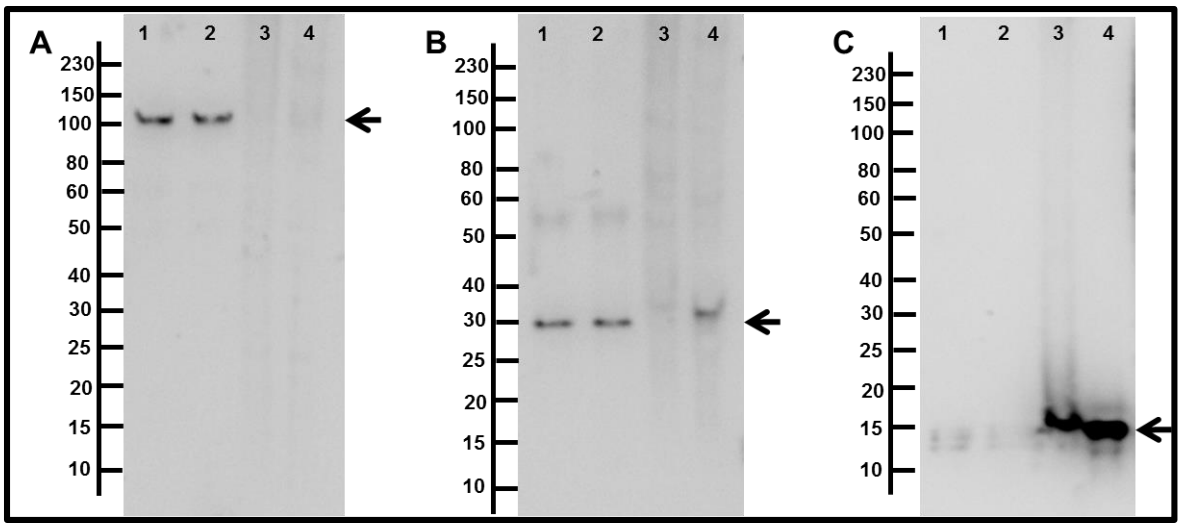


Figure 7.15. Reactivity of AnAPN1 fragments against anti-rAnAPN1.

pENTR4-LPTOS DNA expressing (**A**) AnAPN1$_{fl}$; (**B**) AnAPN1 DomI; and (**C**) AnAPN1 Nterm was used to transfect HEK293s for 24hours. Supernatant and cell lysate were harvested and mixed with either non-reducing or reducing SDS buffer. Samples were run on 4-20% polyacrylamide gels. These were then transferred onto nitrocellulose membranes and immunoblots obtained using purified anti-rAnAPN1 IgG. Lanes: 1 – antigen-specific supernatant in non-reducing buffer; 2 – antigen-supernatant in reducing buffer; 3 – antigen-specific lysate in non-reducing buffer; and 4 – antigen-specific lysate in reducing buffer. Arrowheads denote the relative migration mobility expected for each respective antigen.

7.2.3.5.2. Reactivity of vaccine-induced AnAPN1 fragment antibodies to rAnAPN1

The positive reactivity of anti-rAnAPN1 IgG to mammalian expressed protein further led to the determination of reactivity of antisera against rAnAPN1. Sera from previously vaccinated mice for each antigen including the pooled sera used in SMFA (**7.2.3.4**) were used in ELISA. These experiments were performed with blinded samples against the vaccine construct, rAnAPN1, at Johns Hopkins University. The identity of the samples is shown in **Table 7.5.**

Antigen-specific responses were detected with the control samples not showing any reactivity (sera from mice vaccinated with ChAd63-MVA expressing GFP) (**Figure 7.16**). All the samples against AnAPN1$_{fl}$ reacted positively to rAnAPN1 and the same was true for AnAPN1 DomI (**Figure 7.16**). However, there were non-responders in the AnAPN1 Nterm group with only 81% of the samples showing positive responses to rAnAPN1 (**Figure 7.16**). Overall, this was consistent with responses observed against full-length recombinant AnAPN1 though there was an improvement of responsiveness to rAnAPN1 considering that the same samples were assayed (64% vs. 81%, **Figure 7.13C** and **Figure 7.16** respectively). Thus the variability in the AnAPN1 Nterm immune responses is suggestive of the fact that this AnAPN1 Nterm construct might be poorly immunogenic or is poorly expressed as observed in the mammalian expression profile. This possibly supports the assumption that the protein wouldn't fold properly or be stably expressed thus affecting the induction of antibodies (**Figure 7.7B**).

B-cell epitope analyses of responses against rAnAPN1 have shown that antibodies are raised against two dominant peptides at the N and C terminus of the antigen (Rhoel Dinglasan, personal communication). Responses directed against the N terminal peptide (referred to as peptide 1) don't inhibit development of lab and field *P. falciparum* parasites in *A. gambiae* whilst responses directed against the C terminal peptide (referred to as peptide 9) have TBA.

Therefore, sera against the AnAPN1 fragments were assessed to ascertain whether the antibody responses were directed to either the non-transmission-blocking peptide 1 or the transmission-blocking peptide 9. Synthetic peptide-based ELISA was used with either peptide 1 or peptide 9 (**Table 7.6**) on the same blinded samples used to detect rAnAPN1 responses at Johns Hopkins University.

Interestingly, individual anti-AnAPN1$_{fl}$ sera didn't contain antibodies to either peptide 1 or peptide 9 (**Figure 7.17**). Majority of the peptide 1 responses (78%) were attributed to anti-AnAPN1 DomI sera with only 22% to anti-AnAPN1 Nterm (**Figure 7.17A**). On the other hand, both anti-AnAPN1 DomI and anti-AnAPN1 Nterm sera had equal numbers of responders even though only four samples showed a positive response to peptide 9 (**Figure 7.17B**). Furthermore, half of these (either anti-AnAPN1 DomI or anti-AnAPN1) only responded to peptide 9 and not peptide 1. None of the pooled sera against either AnAPN1$_{fl}$, AnAPN1 DomI or AnAPN1 Nterm neither contained responses to the transmission-blocking peptide 9 nor peptide 1. However, inhibition of *P. falciparum* intensity was observed with total IgG purified from the AnAPN1 Nterm pooled sera. Overall, it would be interesting to determine whether individual samples with only peptide 9 responses would be able to inhibit parasite infectivity in comparison to those that respond to both peptides. Considering that the AnAPN1 Nterm specific sample with the highest peptide 9 response is also responsive to peptide 1.

Table 7.5. Identity of samples in ELISAs performed at Johns Hopkins University.

Sample number	Sample identity
27 – 36	$AnAPN1_{fl}$
37 – 45	AnAPN1 DomI
46 – 56	AnAPN1 Nterm
57	$AnAPN1_{fl}$ pool
58	AnAPN1 DomI pool
59	AnAPN1 Nterm pool
60	Control pool
61 – 65	Control

Table 7.6. rAnAPN1 peptide sequences

Peptide name	Sequence	Amino acid position
Peptide 1	DERYRLPTTSIPIHY	41 – 56αα
Peptide 9	TNDDGFYVSSYVADNGERRYLA	114 – 135αα

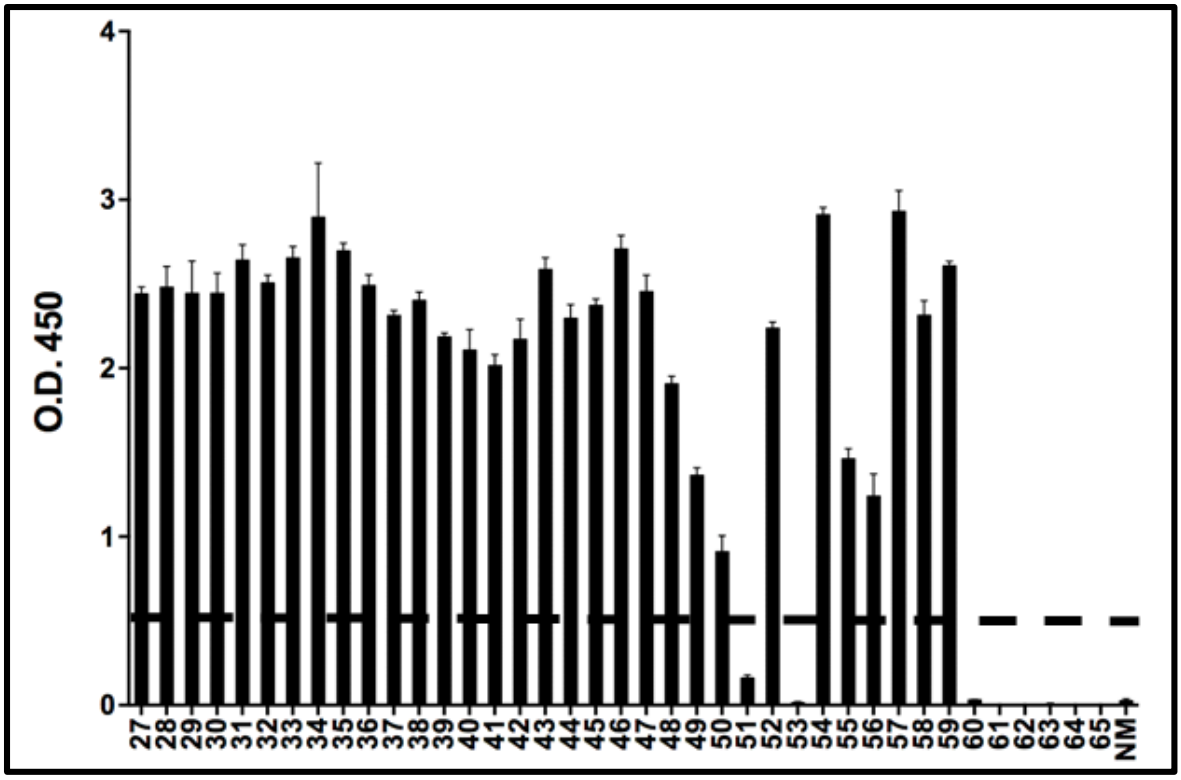


Figure 7.16. Vaccine-induced IgG responses to rAnAPN1 recombinant protein.

BALB/c mice (n=9-11/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with respective MVA ($1x10^7$pfu, day56) expressing $AnAPN1_{fl}$ (samples 27-36); AnAPN1 Dom I (samples 37-45); AnAPN1 Nterm (samples 46-56); or GFP (samples 61-65). Whole IgG responses were assayed on day70 by synthetic peptide-based ELISA using rAnAPN1 recombinant protein. The data is presented as single bars for each individual sample shown as mean ± 1SD of OD_{450} absorbance readings from triplicate wells. Dashed line represents the cut-off for positive signal with values above the line being considered positive. Samples 57, 58, 59 and 60 are day70 pooled sera from mice from each group. NM: normal mouse serum.

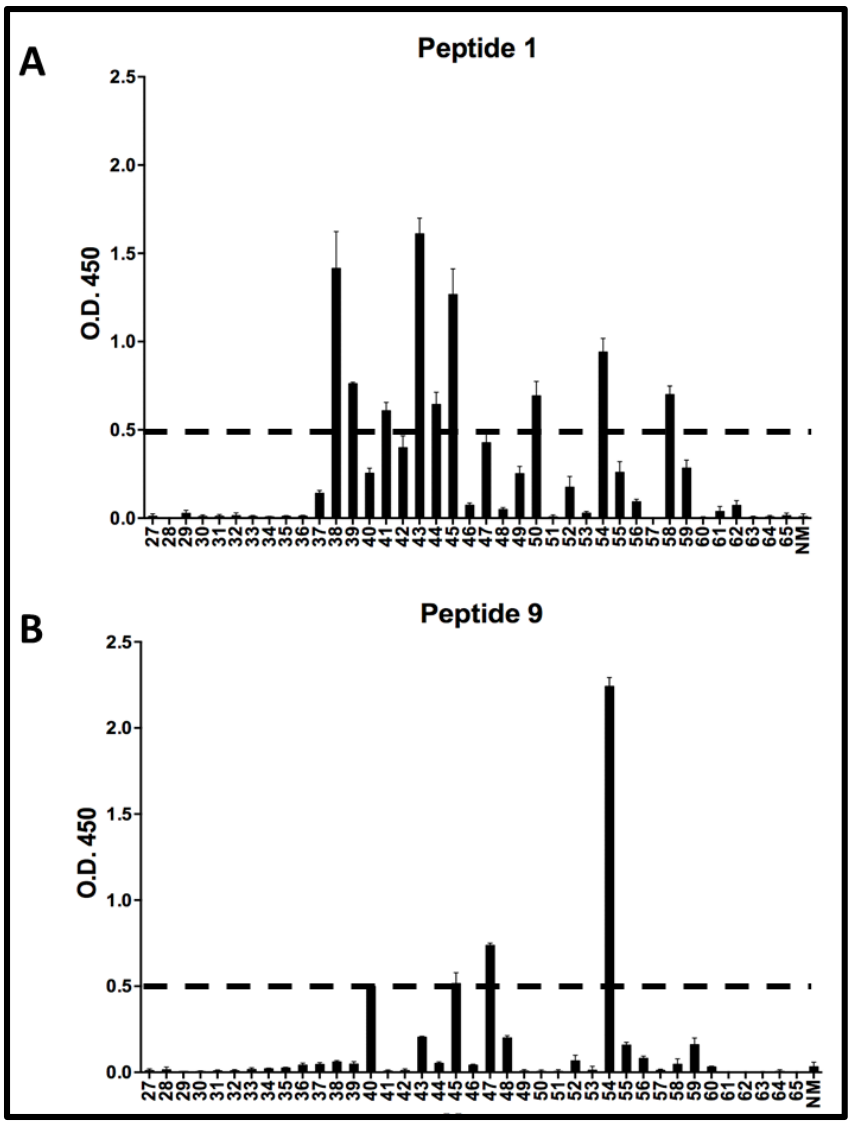


Figure 7.17. Vaccine-induced IgG responses to rAnAPN1 peptides 1 and 9.

BALB/c mice (n=9-11/group) were vaccinated i.m. with ChAd63 ($1x10^8$iu, day0) and boosted with respective MVA ($1x10^7$pfu, day56) expressing $AnAPN1_{fl}$ (27-36); AnAPN1 Dom I (37-45); AnAPN1 Nterm (46-56); or GFP (samples 61-65). Whole IgG responses were assayed on day70 by synthetic peptide-based ELISA using peptides 1 and 9. The data is presented as single bars for each individual sample shown as mean ± 1SD of OD_{450} absorbance readings from triplicate wells. Dashed line represents the cut-off for positive signal with values above the line being considered positive. Samples 57, 58, 59 and 60 are day70 pooled sera from mice used in SMFA (**Figure 7.11**). NM: normal mouse serum.

7.3. Discussion

Development of a malaria vaccine that targets more than one *Plasmodium* species and affords heterologous protection would be vital for the fight against the disease. More so, development of a TBV candidate antigen that would have a pan-malaria impact is desirable as this could be incorporated in existing elimination and control programmes in more than one geographical endemic region. AnAPN1 can be categorised as a potential pan-malaria antigen. Therefore, the optimisation of this candidate antigen, expressed in ChAd63-MVA viral-vectors, was undertaken to improve the efficacy of the vaccine-induced antibody response. Two fragments of the antigen were designed, used to induce antibody responses and to assess the infectivity of *P. falciparum* infectivity to *A. stephensi*. Only the AnAPN1 Nterm fragment, based on the rAnAPN1 construct, was able to have a potent TB effect although the efficacy observed wasn't comparable to that reported for the recombinant protein vaccine construct [348]. There has been no reported evidence of the need for optimal antibody concentration (or dose effect) in efficacy against rAnAPN1 [348], however, the evidence observed here supports this possibility with anti-AnAPN1 Nterm IgG. Nevertheless, this has only been observed in one experiment and will require further experimental validation to support this conclusion.

Expression of all the AnAPN1 fragments in the heterologous prime-boost ChAd63-MVA regime that has been shown to induce potent antibodies to a number of transmission-blocking antigens (**Chapter 4, 5 and 6**), was able to induce antibodies to these fragments. Even though these antibodies were reactive against full-length and rAnAPN1 recombinant proteins, the quality of this response didn't mediate any TBA especially for $AnAPN1_{fl}$ and AnAPN1 DomI. There was evidence that the vaccine-induced antibody response generated was directed to induce non-transmission-blocking responses. This in part could be a possible

explanation as to why there has been no efficacy observed with antibodies directed against both AnAPN1$_{fl}$ and AnAPN1 DomI. It is left to further fully determine whether within these responses generated there could be epitopes targeted that instead of blocking parasite development, promote or enhance infectivity or have no activity at all. There are an additional seven predicted B-cell epitopes within rAnAPN1 that could be tested to ascertain the full specificity of the antibody response (Rhoel Dinglasan, personal communication). Antibody responses to MSP-1 have been reported to be composed of different classes being; those essential for parasite inhibition; irrelevant antibodies; and those that block inhibitory antibodies [121, 673-676]. Mutation of sites within the blocking epitope regions has led to the conclusion that vaccine design could be achieved by directing the antibody response to that which is relevant for parasite inhibition [675, 677].

Thus the quality and specificity of the AnAPN1 response is vital for the induction of potent TBA. Epitope mapping could thus be a consideration for the designing of this mosquito-based TBV candidate antigen; a strategy that has been employed against the mosquito-borne zoonotic, West Nile virus [678, 679]. A comprehensive characterisation of AnAPN1 transmission-blocking epitopes could result in the incorporation of these peptides with other TBV candidate antigens such as Pfs230-C and Pfs25 that induce antibodies that mediate complete parasite inhibition against *P. falciparum*. This TBV combination would circumvent the occurrence of any polymorphisms in parasite antigens and enable a pan-TBV that would be effective against more than one *Plasmodium* species. Another approach would be to incorporate the transmission-blocking epitopes into transgenic mosquitoes, an approach that has been reported to be effective at inhibiting *P. falciparum* development in *A. stephensi* [680, 681]. Isaacs et al. (2012) were able to generate transgenic *A. stephensi* mosquitoes co-

expressing the Pfs25 monoclonal antibody 4B7 and two other monoclonal antibodies targeting parasite-specific antigens [681].

Investigation of the impact of anti-AnAPN1$_{fl}$ antibodies against *P. berghei* infectivity yielded limited evidence of this being a possibility as observed against *P. falciparum*. There was no evidence of efficacy in *A. stephensi* whilst in *A. gambiae* overall efficacy was only seen in reduction of infection prevalence. There was also evidence that efficacy estimates in *A. gambiae* are likely to be lower compared to *A. stephensi*. However, infectivity of *P. berghei* in *A. gambiae* has been shown to be less efficient compared to that in *A. stephensi* especially at the oocyst formation stage [682]. Thus the observed efficacy could be as a result of a lack of infection establishment (based on the overall mean in the control group) and could explain the mosquito species-specific influence of efficacy estimation. This could also be in part explained by the fact that *P. berghei* might be better at infecting *A. stephensi*. It would be interesting to determine whether the same is pertaining in other anopheline species. *A. funestus* has recently been shown to be permissive to *P. berg*hei infectivity [683] and thus consideration of use the *P. berghei – A. funestus* model would be able to validate these results. Thus when measuring the efficacy of an intervention, it would be worth testing more than one mosquito species as each mosquito species or strain will respond differently to infection [684, 685]. However, this conclusion is based on one data set and therefore it would need to be further investigated taking into consideration a number of parasite-mosquito combinations. It would also be interesting to determine whether inhibition of *P. berghei* infectivity in both *A. stephensi* and *A. gambiae* by anti-AnAPN1 DomI or anti-AnAPN1 Nterm would be potent and also result in a mosquito species-specific effect.

Hitherto, there has been no evidence of comparable inhibition of parasite development between the anti-AnAPN1 fragment antibodies and anti-rAnAPN1 antibodies although not

completely inhibitory. Thus there is need to consider other potential mosquito-specific moieties with known parasite interactions as possible pan-TBV strategies. Other midgut-specific proteins that appear to be secreted and up-regulated after mosquito blood-feeding could serve as potential vaccine targets. For instance, *Anopheles gambiae* peritrophic protein (Aper14) and Mucin-1 have been shown to be expressed abundantly on the midgut epithelium and could be explored as potential mosquito-based vaccine antigens [686, 687]. On the other hand, carboxypeptidases have been explored as potential TBV candidate antigens and could be expressed in the ChAd63-MVA heterologous system to identify any transmission-blocking potential [688, 689]. Alternatively, mosquito-based targets such as genes mediating ookinete interaction and/or invasion as possible TBVs could be considered [654, 690]. There is a plethora of genes to choose from (including and not exclusive to immunity genes) with the possibility of additive and/or synergistic effects as observed with salivary gland and midgut peptide 1 (SM1) and rAnAPN1 antibodies [348]. SM1 has been shown to inhibit the invasion of *P. berghei* ookinetes and sporozoites in *A. stephensi* midguts and salivary glands respectively [691]. Thus epitopes or peptides with TBA could be used together or in conjunction with other antigens as highlighted above. Exploration and elucidation of strategies that would enable development of a pan-TBV candidate vaccine is imperative as the current advanced pan-TBV candidate antigen, rAnAPN1, doesn't induce antibody responses that have complete efficacy to-date.

Summary of findings:

- ChAd63-MVA prime-boost regimen induces antibody responses to AnAPN1 fragments that don't mediate complete inhibition of parasite development.
- Quality, quantity and specificity of antibody responses to AnAPN1 are vital for inhibition of parasite infectivity with only responses specific for AnAPN1 Nterm inhibiting parasite intensity.
- Antibodies raised against AnAPN1 fragments, despite sequence homology, don't cross-react with human CD13.
- Efficacy estimates against *P. berghei* infectivity in *A. gambiae* are likely to be lower compared to *A. stephensi* regardless of the method used to assess inhibition.

8. Conclusions and Future Plans

8.1. Conclusions and significance

This body of work describes the design, development, and assessment of leading malaria TBV candidate antigens in a viral-vectored delivery platform. ChAd63-MVA heterologous prime-boost regimen has been utilised as an antigen delivery platform leading to the induction antigen-specific antibody and T-cell responses [130, 133, 247, 426]. This platform has been translated into the clinic [expressing other antigens] and provides a robust and useful tool for advancing subunit vaccines into clinical development [138, 265, 510]. From this study, the viral-vector antigen delivery platform has been shown to be able to induce antibodies with TBA against a number of candidate antigens. Using this platform, antigens of varying sizes were generated and it was demonstrated that antigen design and expression was critical for the efficient induction of antibodies with TB potential.

The demonstration that antigen design is very crucial for the induction of relevant transmission-blocking antibody responses has been a significant finding. The case for this comes from evidence using two variants of Pfs48/45. It can be concluded that mutation of N-glycosylation sites had an impact on immunogenicity and in turn TBA. However, there is need to further validate this for other antigens such as Pfs230-C and Pfs25, where N-glycosylation sites were also substituted. Another case in point is that for AnAPN1, where only the N-terminal fragment, AnAPN1-Nterm, utilised as an antigen had antibody responses exhibiting transmission-blocking activity. The ability to manipulate antigen sequences and use different expression systems to enhance immunogenicity and in turn efficacy has been demonstrated for other malaria candidate antigens including CSP in RTS,S [90, 203], MSP1 [247, 252, 676], and AMA1 [248]. The same can be said of Pfs230-C and Pfs28 where only domains inducing antibodies with the most potent TBA have been utilised as a candidate

antigens and thus impacting efficacy [321, 488, 529]. On the other hand, most of the antigens utilised in this study are membrane-bound antigens with GPI anchors and/or TMDs (**Table 2.1**). These protein features have been omitted in this study, thus expression of correctly folded protein could be hampered. GPI anchor sequences allow for retaining of the protein on the membrane surface, as a post-translational modification, resulting in among other modifications, correct disulphide bond formation [496, 692]. This is evidently crucial for such cysteine-rich proteins and therefore, correct protein conformation is essential for induction of antibodies with TBA. Fanning et al. (2003) reported that Pfs230-C expressed with a GPI sequence induced better antibody responses compared to Pfs230-C without a GPI anchor sequence [496]. However, the addition of a GPI sequence to CSP had the opposite effect [482]. Thus elucidation of the protein structures of these antigens would shed more light on what epitopes are crucial for TBA and whether these epitopes are conformation-dependent. Gregory et al. (2012) have analysed the structure of algae-produced Pfs25 and showed that the expression system was able to produce a protein comparable to the structure of native Pfs25 [500].

Of importance is the observation that the most potent antibodies have the most effect regardless of parasite exposure. This is supported by previous work by Ponnudurai et al. (1987), where they demonstrated that anti-Pfs25 and anti-Pfs48/45 antibodies had varying effects on different parasite exposures, with the former being more potent than the latter [376]. Thus, if an intervention induces potent antibodies, these would be very effective at inhibiting both parasite intensity and prevalence. However, at low parasite exposures it was evident that the effect of antibodies was mediated against inhibiting both parasite intensity and prevalence. Whilst at higher parasite exposures, one required a higher, more potent antibody titre/concentration to enable inhibition of both outcomes. On the other hand,

observations by Churcher and colleagues, indicated that efficacy [against Pbs28 (monoclonal antibody 13.1)] was dependent on parasite exposure (assessed in repeated SMFAs) [378].

Thus for an intervention to be considered as a potential candidate warranting further development, parasite exposure and potency of the antibody response needs to be taken into consideration. Therefore it is imperative to test intervention(s) either against different lab parasite lines (and/or in independent experiments) or exposures relevant to field settings. Hence the relevance of the comparative screening approach in this study that helps to determine which candidate antigen(s) are important for further development. This study identifies that both Pfs230-C and Pfs25 are strong candidates that warrant progression to clinical assessment. This is supported by a TBV efficacy model that suggests that multi-antigen-component vaccines: a) with 100% vaccine coverage, would require both antigens generate synergistic and/or additive TBA or induce relatively similar individual efficacies; and b) with <100% coverage, 3times less antibodies would be required compared to single-component vaccine [693].

The priming antibody response, which correlates with the frequency of the memory B cell response, has been shown to be vital for maintenance and persistence of antigen-specific responses after boosting and enables a robust recall responses to be generated [694]. Evidence from this study suggests that the magnitude of the priming response is key to enable a potent, robust induction of antibodies after boosting. This has been shown to have a spill over effect on efficacy. However, if antibodies to another antigen are present, there is possibility for compensating for the lack of a robust priming response to any one antigen in the combination. More importantly, the antibody response needs to be sustained in order to have a lasting effect on efficacy. Thus longevity of antibody responses shown in Chapter 4 is a significant finding.

On the other hand, the presence of antigen-specific ASCs after vaccination could be an explanation for the persistent antibody responses observed. Plasma cells can persist in the absence of antigen for up to a year [569, 695, 696] and could explain the presence of antibody in serum even up to eleven months post-boost. Pandey et al (2013) have demonstrated in a Group A *Streptococcus pyrogenes* animal model that memory B-cells and long-lived plasma cells are crucial in mediating protection in the absence or presence of T-cells [697]. Memory B-cells have been shown to be induced and importance for vaccine-induced efficacy even half a century post-vaccination [698]. Therefore, it is pertinent to induce antibody responses that not only are robust and potent, but also a B-cell response that would enable the persistence of the antibody response. This is more so as antibody titres need to be sustained in order to at least last for one transmission season to have an overall impact on transmission-blocking.

Interestingly, Blagborough et al. (2013) have recently demonstrated that a transmission-blocking drug was able to eliminate malaria from a mouse population, in a *P. berghei* model, after three 'transmission seasons' [699]. In this significant finding, and in the context of this study, the intervention used didn't have the same potency as that observed against Pfs230-C and/or Pfs25. Therefore, based on the finding that antibody titres are sustained over time, ability to induce antibodies with additive TBA and efficacies of ≥85% (current efficacy required for a target product profile [24, 495]) regardless of the parasite exposure, one can conclude that an intervention with this combination would be able to eliminate malaria in a hypoendemic area. It is argued that individuals residing in these areas are exposed to less infectious mosquito bites comparable to those observed to clear parasites within the mouse population [699, 700]. Thus, in the current elimination and eradication climate, the introduction of a transmission-blocking intervention, such as a TBV with ≥85% efficacy,

would enable the elimination and possibly eradication of malaria in the majority of populations exposed to the parasite. Therefore, TBVs would augment existing vector control strategies aimed at reducing contact with potentially infectious mosquitoes. More importantly, the ability to employ a comparative assessment and down selection of leading candidate antigens, as utilised in this thesis, has the potential to inform on what candidate antigen combination strategies need to be advanced for clinical development.

8.2. Future plans

8.2.1. Improving immunogenicity

A useful correlate of vaccine efficacy already exists, being the antibody response and/or titre. However, the magnitude required to inhibit parasite development and whether it can be sustained over time are important considerations. Antibodies are vital but the type, quality, quantity and potency is paramount. As evidenced with AnAPN1, not just any antibody response is able to mediate TBA. Therefore there is need to either improve on existing responses, optimise antigen design or optimise antigen delivery mechanisms to ensure that the most efficacious responses are induced.

8.2.1.1. Improving Pfs25 immunogenicity

It is worth improving the immunogenicity of Pfs25 due to the potency of the antibodies raised against the antigen and the possible downstream effects they have on parasite development. Lensen et al. (1992) have observed that targeting Pfs25 has an effect on the development of sporozoites [701]. In the clinical trial investigating immunogenicity of Pfs25, as a protein-in-adjuvant formulation, there was huge variability in the antibody response across all time-points assessed [285], also observed in this study. If Pfs25 would be used as a single-component vaccine, immunogenicity would need to be improved in order for antibody responses generated to impact efficacy at either 100% or <100% vaccine coverage [693].

There are a number of efforts that have been demonstrated to better the immunogenicity of Pfs25 including the conjugation with itself or highly immunogenic molecules such as OMPC, and *Pseudomonas aeruginosa* ExoProtein A (EPA) [453, 702-704]. Studies by Qian and colleagues were able to demonstrate less variability in Pfs25 responses when conjugated with EPA leading to an increase in responders [703, 705]. This approach has also employed to improve the immunogenicity of Pfs28 [706]. The Pfs25-EPA conjugate formulated with Alhydrogel is currently undergoing clinical testing in Phase I trials in non-exposed and exposed individuals (ClinicalTrial.gov, Identifiers: NCT01434381 and NCT01867463 respectively). Other efforts include expressing Pfs25 as viral-like particles (VLPs) in adjuvant inducing antibodies with TBA. This formulation has also been advanced to clinical testing (ClinicalTrial.gov, Identifier: NCT02013687). Currently, efforts are ongoing by Biswas and colleagues which include expressing Pfs25 with hepatitis B surface antigen, as achieved for RTS,S. Also at preclinical stage are efforts to develop Pfs25 fused to a multimerisation domain of largely chicken complement 4b binding protein (C4bp) resulting in multiple copies of the protein being expressed in a nanoparticle and hence more immunogenicity. This C4bp, branded as IMX313 has been used to enhance the immunogenicity of tuberculosis antigen 85 [707] and this has advanced to clinical development in an MVA viral-vector (ClinicalTrial.gov, Identifier: NCT01879163).

8.2.1.2. Antigen delivery platforms

As much as improving the antigen design is important for immunogenicity, the antigen delivery platform is also essential for the effective induction of potent responses with TBA. A number of delivery platforms are currently in various phases of pre- and clinical testing and are not limited to the use of VLPs and IMX313 [707, 708]. Nano-/micro-particle antigen delivery has been employed to induce antibodies with TBA against both Pfs25 and rAnAPN1 [495, 704]. Depending on the antigen immunogenic potential, these delivery platforms have

to be administered in highly safe, but immunogenic adjuvants [709, 710]. However, this platform promises to be an effective way of generating robust antibody responses [710]. Additionally, an alternative platform has recently been demonstrated as a novel antigen delivery mechanism especially for malaria antigens [711]. The tricomponent immunopotentiating system described by Miyata and others involves the antigen of interest, antigen-presenting cell targeting ligand and carrier protein that enables antigen oligomerisation [711]. Antibody responses were induced to both Pvs25 and MSP1 however, robust responses were dependent on all three components.

Furthermore, responses generated by ChAd63-MVA viral-vectors can be improved upon to enable efficient antigen delivery. This could include co-administration with safe immunogenic adjuvants (Hill et al., unpublished findings). A clinical trial is currently underway looking at the possibility of adjuvanting ChAd63-MVA-ME-TRAP with Matrix M (ClinicalTrials.gov Identifier: NCT01669512), an adjuvant that was observed in this study to induce antigen-specific responses to both Pfs230-C and Pfs25 recombinant protein. Thus more pre-clinical evaluation of this approach (especially with Pfs25-Pfs230-C fusion vaccines) would enable determination of the usefulness of this approach for antibody-inducing vaccines. On the other hand, Draper and colleagues have demonstrated that Ad-Protein regimens induce induce potent antibody responses compared to Ad-MVA and/or PPP regimen [130, 131, 583]. Biswas et al. (unpublished findings) in an Ad-MVA, A-P and PPP adjuvant comparative analysis demonstrated that the magnitude of Pfs25-specific antibody responses were dependent on the adjuvant utilised. In this context, this approach should be further investigated including comparisons of A-P regimes involving Pfs25-Pfs230-C fusion vaccines with co-administration of the protein-in-adjuvant component.

8.2.2. Further screening of potential candidate antigens

There has been no comprehensive screen of malaria transmission-blocking candidate antigens as has been achieved for blood-stage [277, 712] with the targets that were initially discovered being historically used as vaccine candidates. From this comparative assessment, undoubtedly Pfs230-C and Pfs25 are the main targets for further vaccine development. However, these antigens have historically been developed without comparison to other potential candidate antigens as achieved in this study. Thus a comprehensive, comparative screen would enable identification of other antigens able to induce either equally or more potent responses, with TBA, that could potentially be further developed alongside these two leading antigens and in combination. This screen would enable the rational, development of effective vaccines. This strategy is currently being pursued by Biswas et al. with the hope of identifying both parasite and mosquito-based target antigens.

There are a number of candidates that have been identified being involved in sexual-stage development and those that have been shown to be essential for parasite traversal in the mosquito midgut. The potential list of these targets is currently growing with renewed interest in the understanding of the biology of sexual-stage development [713-719]. Studies by Young et al. (2005), Lemieux et al. (2009) and Silvestrini et al. (2010) have provided a wealth of information on the transcriptome and protein profiles during the developmental process [717-719]. Of importance is the identification of a number of genes that have been characterised to play a role in the maturation and differentiation of sexual-stages and could thus be exploited and investigated for their potential as TBV candidates. These are not limited to early and late markers of gametocyte maturation. For instance, Lal et al. (2009) have identified male development gene-1 which has been shown to have an impact on female gametocyte activation and ookinete development resulting in reduced ookinete transition to

oocyst stage [715]. In addition, paralogous antigens such as Pfs230p and Pfs47 are potential TBVs due to the possibility of having redundant roles in fertilisation with their respective Pfs230 and Pfs48/45 pairs [294, 720, 721]. Pfs47 has recently been shown to be essential for parasite survival in the mosquito [722] and thus has potential as a TBV candidate.

On the other hand, it would also be important to identify targets that have the potential to have a multistage effect. The characterisation of CELTOS with possible anti-sporozoite and TB effects provides precedence for the development of such candidate antigens [230, 231]. An additional approach as illustrated by Bergmann-Leitner et al. (2013) would be to characterise specific epitopes with TB potential and design vaccines resulting in a plethora of responses generated allowing for effective coverage of a number of antigens and/or parasite-vector interaction [723]. This together with epitope 9 in AnAPN1-Nterm would enable the ability to have a multi-species effect. A further approach would be to identify additional mosquito-based antigens that are utilised by parasites as ligands. Thus antibodies could be raised to these target molecules that could block the binding of parasites. Most of the targets that have been described to-date have included carbohydrate molecules on the mosquito midgut surface [342, 724, 725]. Using phage-display libraries other possible targets have been identified [726]. Perhaps a bold approach would be to test unconventional molecules involved in the mosquito immune system. Thus there is need for additional antigen testing as that demonstrated by Williams et al. (2013) [652] enabling discovery of antigens that induce antibodies with pan-TBA. As demonstrated by Lal et al. (2001), anti-mosquito midgut antibodies block more than one *Plasmodium* species and also have an effect on the mosquito's survival potential [149].

Moreover, for effective screening and progression of candidate antigens for further development, there is a need to develop surrogate assays for down-selection. Assays that

might complement or negate the use of MFAs especially for screening of potential vaccine candidate antigens are thus required. For instance, high-throughput screening assays could be generated providing reliable tools of measuring vaccine-induced efficacy. Efforts by Delves and colleagues have proved that this is possible [363, 364]. Transgenic *Plasmodium* expressing GFP has aided the evaluation of not only candidate antigens but also drug targets for transmission-blocking activity [364]. Other ways to measure mosquito and parasite infectivity and infectiousness such as in vivo imaging of mosquitoes e.g. using GFP parasites would be ideal though not feasible for field settings. A possible effective assay that could be scaled up to enable high-throughput screening would be the exflagellation assay. This, if successful has the potential to be adopted across various laboratories reducing inter-assay variability that might be an issue with MFAs.

8.2.3. Testing of multi-antigen and/or multi-stage combinations

Most malaria multi-stage studies published to-date have not tested efficacy against the sexual-stage component. For instance Shi et al. (1999) tested multi-stage and multi-antigen approach including all the stages of the life cycle [727]. The same is true for NYVAC-Pf7 and ALVAC-Pf7 vaccines [423, 424, 532]. However, if multi-stage combination approaches are to be advanced into clinical testing there is need for evidence to show that not only are antigen-specific responses induced but these result in efficacy to all vaccine components. Currently, at the Jenner Institute, testing these combinations is possible as all stages of the life cycle are under various phases of development. For instance, combination studies of ChAd63-MVA-ME-TRAP with either Pfs25-Pfs230-C fusion vaccines or Pfs25 construct with improved immunogenicity can be evaluated. Additionally, other multi-antigen TBV combinations being tested (by Biswas et al.) and any other candidates (**8.2.2)** could be

evaluated. Hence there is need to determine what multi-stage antigen approaches would be most efficacious to enable advancement of second generation malaria vaccines.

8.3. Final remarks

This study describes a head-to-head comparison approach using a human-compatible vaccine delivery platform to help inform and direct future malaria TBV development. The primary aim of this study was to comparatively assess the ability of a heterologous prime-boost viral-vectored regimen to induce antibody responses with TBA against leading candidate antigens. This assessment led to identification of both Pfs230-C and Pfs25 as leading targets for vaccine clinical development. In hindsight, Pfs25 has been considered the frontrunner in TBV development, and thus the finding of comparable efficacy from both anti-Pfs25 and Pfs230-C antibodies [either individually or in combination] supports the further development of a multi-antigen vaccine approach for TBVs.

9. References

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10. Appendix

10.1. Reagents

Table 1. List of commercially available reagents.

Material	Supplier	Catalogue number
10,000 SYBR® Safe in DMSO	Life Technologies	S33102
1M Tris pH9.0	Sigma Aldrich	T2819
70μm cell strainer	BD Biosciences	352350
A549 cell line	ATCC	CCL-185™
Agarose	Sigma Aldrich	A9539
Agarose, low gelling temperature	Sigma Aldrich	A9414
Amicon Ultra-l5 Centrifugal filter device, 30K	Millipore	UFC903024
Ampicillin	Sigma Aldrich	A5354
AP conjugate substrate colour development kit	Bio-rad	170-6432
ASB-C8ϕ	Merck4Biosciences	182730
BCA protein assay kit	Thermo Scientific	PN23225
Benzonase	Merck	70664-100KUN
Biotinylated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 mAb	BD Biosciences	553441, 553388, 553393, 553401
Blasticidin S-HCl	Melfords Labs	B1105
Blocker Casein in PBS	Thermo Scientific	PN 37528
Bovine serum albumin, Fraction V	PAA	K41-001
Brilliant Blue R Concentrate	Sigma Aldrich	B8647
Colorplus Prestained Protein Ladder, Broad Range (10-230kDa)	NEB	P7711S
DAB chromogen substrate buffer	DAKO	K3468
Diethanolamine buffer	Thermo Scientific	PN34064
DNA Releasy	Anachem	LS02
Donkey anti-mouse IgG Alkaline phosphatase	Jackson Labs	715-055-150
Donkey anti-mouse IgG Horseradish eroxidase	Jackson Labs	715-035-150
Donkey anti-rabbit IgG Horseradish peroxidase	Jackson Labs	711-035-152
Dried semi-skimmed milk	Tesco	N/A
Dulbecco's PBS, with Ca and Mg	Sigma Aldrich	D8662
Dulbecco's PBS, without Ca and Mg	Sigma Aldrich	D8537
Effectene Transfection Reagent Kit	Qiagen	301425
Ethanol	Sigma Aldrich	E7023
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E7889
ExtrAvidin alkaline phosphatase	Sigma Aldrich	E2636
Feotal calf serum (FCS)	Sigma Aldrich	F2442
Ficoll® 400	Sigma Aldrich	F2637
Freestyle™ 293 Expression medium	Life Technologies	12338002
Fructose	Sigma	F0127
G418, Geneticin® reagent	Life Technologies	10131-035
Gateway® LR Clonase® II enzyme mix	Life Technologies	11791-020
Giemsa	Sigma Aldrich	GS1L
Glucose	Sigma Aldrich	G0350500

Material	Supplier	Catalogue number
Glycine	Sigma Aldrich	G8898
Goat anti-human IgG alkaline phosphatase	Sigma Aldrich	A1543
Goat anti-mouse IgG (H+L) Alexa Fluor® 488	Life Technologies	A11001
Goat anti-mouse IgG horseradish peroxidase	DAKO	P0447
Goat anti-mouse IgG alkaline phosphatase	Sigma Aldrich	A3562
Goat anti-rabbit IgG alkaline phosphatase	Sigma Aldrich	A8025
Halt Protease inhibitor cocktail	Thermo Scientific	PN78430
HEK293A & HEK293 T-REx cell lines	Life Technologies	R705-07 & R710-07
Heparin	Sigma Aldrich	84020
HEPES	Sigma Aldrich	H3375-500G
Hydrochloric acid (HCl) (1M)	Sigma Aldrich	S7653
Hypoxanthine	Sigma Aldrich	H9636
Immersion oil	Sigma Aldrich	10890
Immunopure IgG Binding buffer	Thermo Scientific	PN21011
Immunopure IgG Elution buffer	Thermo Scientific	PN21009
Immunopure Plus Immobilized Protein G Gel	Thermo Scientific	PN22852
Imperial blue stain	Thermo Scientific	PN24615
In-Fusion® HD Cloning kit	Clontech Labs	639648
Isoflurane	OUVS	N/A
Kanamycin	Sigma Aldrich	K0254
KAPA 2G robust polymerase	Anachem	KK5005
KAPA Long Range HotStart ReadyMix	Anachem	KK3602
KCl	Sigma Aldrich	P9541
Ketastat (100mg/ml Ketamine)	OUVS	N/A
$KHCO_3$ [1mM]	Sigma Aldrich	P9144
KOH	Sigma Aldrich	P5958
LB agar tablets	Sigma Aldrich	L7025-500TAB
L-Glutamine	Sigma Aldrich	G7513
Lipofectamine™ 2000 transfection reagent	Life Technologies	11668-019
MAIP ELISPOT Plates	Millipore	MAIPS4510
Matrix-M (Abisco®-100)	Isconova	N/A
Max Efficiency®DH5α™-TIR competent cells	Life Technologies	18258-012
MEM α-modification	Sigma Aldrich	M4526
Methanol	Sigma Aldrich	34860
Microvette serum collection tubes	Sarstedt	16.444
M-Per mammalian protein extraction reagent	Thermo Scientific	PN78501
MultiScreen HTS filter plates	Millipore	MSHVN4B50
Na_2EDTA	Sigma Aldrich	ED2SS
NH_4Cl [0.15M]	Sigma Aldrich	A4514
Nitrocellulose Transfer Pack	Bio-rad	170-4159
Novocastra protein block	Leica	RE7102
Nunc-Immuno maxisorp plates	Fisher Scientific	DIS-971-030J
Nycodenz® powder	Axis-Shield	1002424
OptiMEM	Life Technologies	11058-21
Orange G	Sigma Aldrich	03756
Ovalbumin (Grade VII)	Sigma Aldrich	A7641

Material	Supplier	Catalogue number
P-amino benzoic acid	Sigma	A9878
Paraformaldehyde (PFA)	Alfa Aesar	43368-9M
Penicillin/Streptomycin solution	Sigma Aldrich	P0781
Phenol	Sigma Aldrich	P1037
Phenylhydrazine	Sigma Aldrich	P26252
Phosphate buffered Saline powder	Sigma Aldrich	P4417
Phosphate buffered saline, TWEEN®20	Sigma Aldrich	P3563
Phusion High Fidelity enzyme	Thermo Scientific	FZF-531L
Pierce ECL substrate solution	Thermo Scientific	PN32106
Plasmodipur	EuroProxima	8011Filter25u
p-Nitrophenyl phosphate disodium hexahydrate	Sigma Aldrich	N2765
Polyethyleneimine	Alfa Aesar	43896-01
Protein A/G UltraLink Resin	Thermo Scientific	PN53132
Qiagen Minelute gel extraction kit	QIAgen	28606
Qiagen plasmid midi kit	QIAgen	12143
Qiagen spin miniprep kit	QIAgen	27106
Renilla luciferase assay system	Promega	E2820
Restriction endonucleases and related reagents	NEB	Various
Rompun (2% xylazine)	OUVS	N/A
RPMI-1640	Sigma Aldrich	R0883
SIGMAFAST™ BCIP®/NBT	Sigma Aldrich	B5655
Slide-A-Lyzer Dialysis Cassettes, 10K MWCO	Thermo Scientific	66810
Smart Ladder DNA marker	Eurogentec	MW-1700-02
SOC medium	Life Technologies	15544-034
Sodium azide	Sigma Aldrich	S2002
Sodium Bicarbonate	Sigma Aldrich	S5761
Sodium dodecyl sulphate (SDS)	Gibco	15553-035
Streptavidin Coated Plates	Thermo Scientific	15125
T-REx™ HEK293 cell line	Life Technologies	R710-07
Tris buffered saline	Sigma Aldrich	T5912
Tris-acetate-EDTA	Fisher Scientific	BP1332-1
Tris-HEPES-SDS protein precast gels	Thermo Scientific	Various
Triton™ X-100	Sigma Aldrich	T8532
Trizma® HCL buffer solution (pH8.0)	Sigma Aldrich	T2694
TryLE™ Express	Life Technologies	12605010
Xanthurenic acid	Sigma Aldrich	D120804
β-mercaptoethanol	Sigma Aldrich	M3148
β-mercaptoethanol (50μM)	Gibco	31350-010

10.2. Buffers and solutions

Buffers and solutions used were as follows:

Phosphate buffered Saline (PBS): 0.01M powder pH7.4 dissolved in 10L deionized water (dH2O).

PBS/Tween (PBS/T): PBS with 0.05% TWEEN® 20 dry powder pH 7.4 dissolved in 1L dH2O.

Tris-acetate-EDTA: 50X DNase, RNase and protease free made up in 20L dH2O.

Tris Buffered Saline (TBS): 10X solution of 20mM Tris and 0.9% NaCl pH7.4 made up to 1X with dH2O.

Ammonium-Chloride-Potassium (ACK) lysis buffer: NH4Cl (8.3mg/l), KHCO3 (1mg/l) and Na2EDTA (3.722 mg/l) in 800ml dH2O. HCl was used to adjust to pH7.4 and then finalised to 1L with dH2O.

Blocking buffers: for ELISAs 5% semi-skimmed milk in PBS/T, Casein or 1% Bovine serum albumin (BSA) in PBS/T; for western blotting 3% BSA in PBS; and for immunofluorescence assays 10% goat serum, 1% BSA in TBS.

Buffer A: 50mM Tris, 100mM NaCl, 5mM MgCl2, 1% Triton X-100 in dH2O.

Complete medium (D10): 500ml Minimum Essential Medium (MEM) α modification with sodium bicarbonate supplemented with 50ml heat-inactivated foetal calf serum (FCS (10%)), 5ml L-glutamine (2mM), 5ml penicillin and streptomycin (Pen/Strep), and 500µl β-mercaptoethanol (50µM). For cell culture β-mercaptoethanol was omitted. D10B medium had 250µl Blasticidin (5µg/ml) without β-mercaptoethanol. D2 medium had 2% FCS.

Diethanolamine buffer: 5x solution diluted in dH2O to make 1x solution.

DNA loading dye: 0.4% Orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 8.0), and 50mM EDTA (pH 8.0) made up with 100ml autoclaved H2O and filtered through a 0.22µM filter.

Exflagellation medium: 500ml RPMI-1640 medium, 25mM HEPES supplemented with 20% FCS, 10mM sodium bicarbonate and 50µM xanthurenic acid pH7.6 adjusted with HCl/NaOH.

Fructose solution: 80g fructose and 0.5g of P-amino benzoic acid (PABA) dissolved in 1L of dH2O and autoclaved.

Giemsa stain: 5% or 10% Geimsa in dH20.

Laemmli buffer: For reducing buffer, 125mM Tris-HCl (pH6.3) 20%v/v glycerol, 4%w/v SDS, 20mg/ml Brilliant Blue, 10% v/v β–mercaptoethanol made up to 20ml with dH2O. The solution was filtered with a 0.45μm filter. For non-reducing buffer, β-mercaptoethanol was omitted.

Lysis buffer: 50mM Tris, 100mM NaCl, 1% Triton X-100, 50% glycerol, 100x EDTA and 100x Halt protease inhibitor made up in 100ml dH2O.

Nycodenz buffered medium: 0.3g Tris, 0.061g CaNa-EDTA, 0.11g KCl in 500ml dH2O pH7.5.

Nycodenz stock solution: 55.2g Nycodenz powder (27.6w/v) in 200ml Nycodenz buffered medium with subsequent dilutions made in PBS.

Ookinete culture medium: 500ml RPMI-1640 medium containing 25mM HEPES supplemented with 5ml L-glutamine, 5ml Pen/Strep, 0.02% sodium bicarbonate, 50mg Hypoxanthine and 100μM xanthurenic acid adjusted to pH7.4 with KOH. Solution was made up to 1L with dH2O and sterilised using a 0.45□m filter. Solution was stored at -20°C and upon use added 20% FCS.

Polyethyleneimine (PEI) solution: 1mg/ml Polyethyleneimine powder solubilised in dH2O by heating to 90°C filtered using 0.22μM filter at pH8.0.

Tris-HEPES-SDS Running Buffer: 10x working solution were prepared with 121g Trizma base, 238g HEPES, 10g SDS up to 1L dH2O.

10.3. Report from Nijmegen

Rabbit serum samples vaccinated by prime-boost with ChAd63 and MVA - Pfs48/45 (designated Pfs48/45$_{+NGln}$)

Aim: Test and compare the immune response from Rabbits immunized with Pfs48/45+NGln.

Assays:

Sera:	Rabbit serum samples vaccinated by prime-boost with ChAd63 and MVA - Pfs48/45 (designated Pf48$_{+NGln}$)
Antibody ELISA:	Ag using for coating: Pf10C (is the 10C part of Pfs48/45), detection with anti-Rabbit IgG (H+L)-HRP and TMB
ImmunoFluorescence:	Sera from the final bleed on gametocyte/gametes
SMFA	Sera from the final bleed and compared with the pre-bleed

Serum samples from individual rabbits or the pooled sample of the Pf48$_{+NGln}$ group was tested in the 1-site ELISA (Figure 1), IFA (Figure 2) or standard membrane feeding assay (Table 1).

Antibody testing

ELISA. The Pf48/45-specific serum IgG titers were measured by ELISA using 96-well MaxiSorp plates (Sterilin) coated with 0.25 µg/ml of recombinant Pf10C in PBS and incubated overnight at 4°C. After blocking with 5% Milk in PBS, serum samples were tested at a starting dilution of 1:100, titrated in 2-fold dilutions and incubated for 3 hours at RT. Target-specific IgG was detected using HRP-conjugated goat anti-rabbit IgG at 1:2,000 (DAKO) and visualized using TMB (K-Blue substrate, Neogen, Sigma, cat 010131) as a substrate with an acid stop. The EC50 value was calculated and used for comparison of the individual rabbit sera (see Figure 1).

IFA. An indirect IFA was performed with a mix of cultured sexual and asexual stage *P. falciparum* (isolate NF54) parasites air-dried onto a multispot slide. Briefly, parasites were incubated with the test serum starting at a dilution of 1:100 and titrated in 2-fold in PBS, rinsed with PBS, and incubated with Alexa Fluor® 488-labelled chicken anti-rabbit IgG (H+L) (Invitrogen). The slides were rinsed, washed, mounted under a coverslip, and examined under ultraviolet illumination with a Leitz Ortholux fluorescence microscope (x500 magnification). Reciprocal serum dilutions that gave fluorescence greater than the background were determined as the end point titers (See Figure 1 and 2).

The immune response was assessed by 10C-ELISAs and shows EC50-titers of 1/3,515, 7,638, 1,663 and 2,754 for D70 sera from rabbit 1, 2, 3 and pooled R1-R3 respectively. Serum from rabbit 2 showed an EC50 titer of approximately two times higher compared with R1 and five times higher compared with rabbit 3.

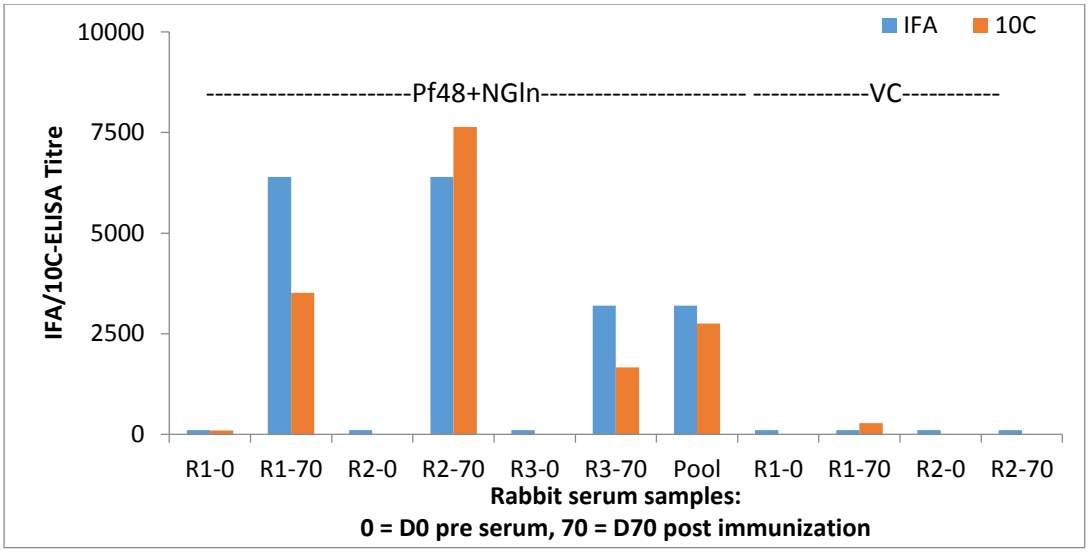

Figure 1: EC50 ELISA results and final IFA titer of bleed sera from rabbits vaccinated by prime-boost with ChAd63 and MVA - Pfs48/45 (designated Pf48$_{+NGln}$).

The ability of the antiserum to recognize native gametocyte or macrogamete protein was further assessed by IFA against whole dried sexual stage parasites. The Pf48$_{+NGln}$ vaccinated rabbits showed good reactivity to the gametocytes and macrogametes/zygotes antigen. The final titers for R1 and R2 are 1/6,400 whereas the titer for R3 and the pooled sample is 1/3,200 but this is only 1 titer (dilution step) difference. The reactivity of the R2 serum is shown in Figure 2a (1/400 diluted) and 2b (1/6,400 diluted), whereas the serum from rabbit 1 gives comparable results and rabbit 3 and the pooled sample less reactivity (data not shown). Pre sera from Rabbits or immunized with VC did not show a detectable signal (not shown). Surprisingly, the reactivity at 1/400 is very strong with gametocytes whereas at a dilution of 1/6,400, the reactivity is mainly with macrogametes/zygotes.

SMFA. Antisera obtained from rabbits immunized with the Pf48+NGln antigen were assayed for their Transmission-Blocking (TB) activities in standard membrane-feeding assays. Briefly, 30 µL of rabbit sera were mixed with 90 µL of naïve human serum and 150 µL of *in vitro* mature gametocyte culture of *P. falciparum* (NF54 line). This mixture was fed to *Anopheles stephensi* mosquitoes (Nijmegen strain) through a membrane-feeding apparatus. Pre-immune sera and sera from rabbits immunized with VC alone served as the controls. Fully engorged mosquitoes were separated and held at 26°C. Seven days later, 20 mosquitoes per feeder were dissected and the number of oocysts on the stomach wall was recorded. An experiment was deemed to be successful when at least 90% of the mosquitoes examined in the control groups carried oocysts. For comparison of groups and to see if there is a statistically significant difference between the groups, data were analyzed by a nonparametric test (not normally distributed) by comparing the medians of two groups using the Mann Whitney test or by comparing three or more groups using the Kruskal-Wallis test followed by post tests. If significance was indicated, the Dunn's analysis was used for comparison with the control group.

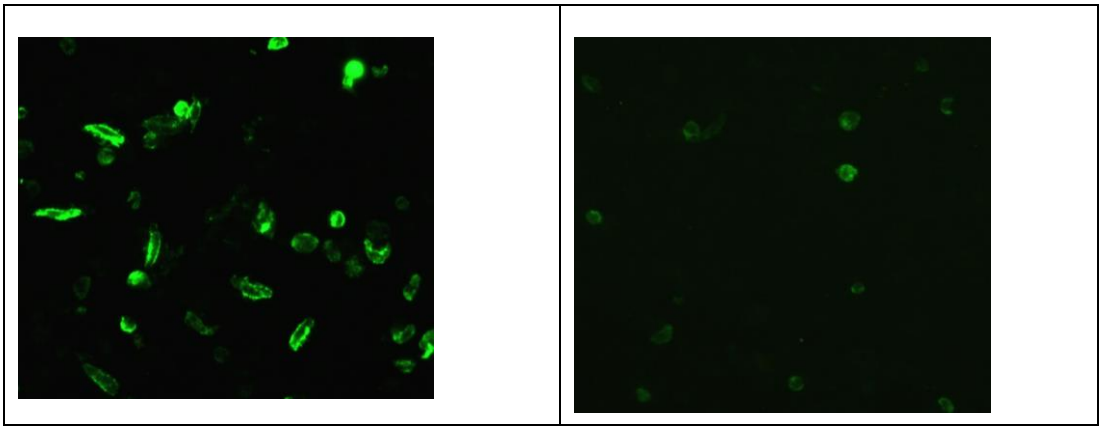

Figure 2. Immunofluorescence microscopy on *P. falciparum* gametocytes and gametes/zygotes air-dried on a multispot slide (*IFA*). (A - left) Serum from Rabbit 2 D70 at a dilution of 1/400 and (B right figure) at a dilution of 1/6,400.

Table 1: Infectivity of *P. falciparum* Gametocytes to *A. stephensi* Mosquitoes in the presence of sera from rabbits immunized with Pf48+NGln and VC in the Standard Membrane-Feeding Assay

	Group	Rabbit	Day	no. Inf/Diss	Median (IQR)	% TRA	*P*-value
R1	Pf48+N	20213	0	19	28 (10 - 38.5)		
	Pf48+N	20213	70	19	11 (3.3 - 22.8)	47	ns
R2	Pf48+N	20214	0	18	26.5 (14.3 - 42.8)		
	Pf48+N	20214	70	20	8.5 (4.3 - 13.8)	65	p<0.05
R3	Pf48+N	20215	0	19	15 (4.3 - 35.8)		
	Pf48+N	20215	70	18	11.5 (3.0 - 29)	22	ns
pool	Pf48+N	pool	70	19	13 (5.3 - 19)	30	ns
R1	VC	20216	0	19	22 (10.3 - 36)		
	VC	20216	70	19	35 (23.3 - 48.5)		ns
R2	VC	20217	0	19	43.5 (38.3 - 56.8)		
	VC	20217	70	19	30.5 (17.5 - 37)		ns

Sera (1:9 diluted) were tested in the SMFA. Seven days after membrane feed, the number of infected mosquitoes and oocyst density were determined. Sera: rabbit serum from Pf48+NGln and VC-immunized group; Control: feeder with pre-bleed serum of the group; no. Inf/Diss: total infected mosquitoes from 20 dissected mosquitoes; %TRA: percentage of Transmission reducing activity; P-value: sample of the control (pre-bleed serum) was used for calculation. NS: not significant; IQR: interquartile range

The percent inhibition was calculated using the pre-bleed serum as a control. Results from the SMFA demonstrated that only rabbit R2 with the highest EC50 titer in the 10C-ELISA shows a significant reduction (65%) in the number of oocysts (Table 1). Comparing the values of the EC50 titer with the percentage of TRA resulted in a nice correlation (R=0.95) with an equation of: %TRA = 0.007*EC50 titer + 13.86. For a TRA of ≥90% reduction in oocyst numbers an EC50 titer is needed of > 10,000.