Open Research Online

The Open University's repository of research publications
and other research outputs

Clinical and immunological evaluation of new malaria vaccines encoding the Thrombospondin Related Adhesion Protein

Thesis

oro.open.ac.uk

Clinical and immunological evaluation of new malaria vaccines encoding the Thrombospondin Related Adhesion Protein.

A dissertation submitted for the degree of Doctor of Philosophy.

Open University

Philip Bejon, B Sc, MB BS.

June 2007

Candidates contribution to the work undertaken.

1 Sporozoite Challenge in Oxford: PCR Data.

   Statistical Analysis. (Studies designed and conducted by collaborators)

2. Phase 1 Studies in Kilifi: Safety.

   Study design, medical aspects of clinical trial, training and supervision of field workers.

3. Phase 1 Studies in Kilifi: Immunogenicity

   Study design, laboratory work, training and supervision of research assistants.

4. Efficacy in the field

   Study design, training and supervision of research assistants, training and supervision of all field staff.

5. Malaria and Immunosuppresion.

   Study design, training supervision of research assistants.

The analysis, interpretation and presentation of all work contained here is the candidate's work only.

Preface

The work is dedicated to my wife, Sarah, and to my son, Moses.

"Muganga ni kulaza mukongo na tumai"

…which translates "a (traditional) doctor

makes the sick lie down with hope."

Mijikenda proverb

Abstract

Malaria causes around 2 million deaths per year, and the mortality appears to be rising. A prophylactic vaccination is urgently needed. A viral vectored vaccination regimen has been developed in Oxford to induce T cell responses reactive to pre-erythrocytic antigens of *Plasmodium falciparum* malaria. I conducted a series of studies to evaluate the clinical outcome and immunological response to the vaccine. I analysed PCR data from experimental sporozoite challenge studies in the UK, and showed that vaccination reduced the numbers of parasites completing their pre-erythrocytic development. I conducted preliminary studies to explore the safety and immunogenicity of vaccination in adults and then children in Kilifi, Kenya.

Antigen insert, multiple versus single priming, different batches of vaccine (in one instance) and pre-vaccination immunity influenced the reactogenicity and immunogenicity of vaccination. Enhanced memory responses were seen with novel alternating vector regimens.

Having established immunogenicity, safety, and preliminary evidence of efficacy, I then conducted a large efficacy trial in the field, randomizing 406 children to either active vaccination or control. The primary outcome was time to first episode of febrile malaria. Although the regimen was immunogenic, the magnitude of T cell responses was lower than in previous studies. 346 children were vaccinated according to protocol. Episodes of febrile malaria were more frequent in the FFM ME-TRAP group (52/171 vs 40/175 among controls), but this was not statistically significant (95% confidence interval 0.83 to 2.08, P=0.55 by logrank). Among children vaccinated with FFM ME-TRAP, there was no correlation between immunogenicity and malaria incidence.

I examined factors in the cohort of children that may have influenced immunogenicity. Malaria was more immunosuppressive than other parasitic infections, and is therefore the factor most likely to impact the development of investigational T cell inducing vaccines in malaria endemic areas.

Contents

Index of Tables

Index of Figures

.

1 Introduction

The worldwide mortality and morbidity from *Plasmodium falciparum* malaria is high, and has risen over the last 20 years (Hay et al. 2004; Snow et al. 1999; Snow et al. 2005). There is evidence that Insecticide Treated Nets (Lengeler 2004) and newer anti-malarials (White & Olliaro 1998) might reduce this mortality, but these are not yet adequately implemented (White et al. 2004) and it seems unlikely that these measures alone will halt the rising mortality of the disease. It has been argued that a malaria vaccine is required in order to bring about a lasting improvement in public health (Malkin et al. 2006).

1.1 Vaccine Development.

The traditional approach to vaccines development was to attenuate or inactivate the organism or toxin against which immunity is desired. Although irradiated sporozoites generate protective immunity in humans, this is laborious and does not currently appear to be feasible on a large scale (Herrington et al. 1991; Hoffman et al. 2002).

Molecular technology allows cloned falciparum antigens and epitopes to be manufactured on a large scale, and delivered by a variety of vectors; as particulate protein with adjuvant, recombinant bacteria, recombinant viruses or as naked DNA (Moore & Hill 2004). This approach has resulted in licensed vaccines to Hepatitis B and A (Peetermans 1992; Peetermans 1987) and *Streptococcus pneumoniae* (Eskola et al. 2001).

Vaccines designed to protect against *Plasmodium falciparum* malaria can be divided into 3 main groups, according to which life cycle stage the chosen antigen is derived. This thesis concerns an approach to generate immunity against the pre-erythrocytic stage. Other approaches attempt to generate blood stage immunity, or transmission blocking immunity designed to prevent gametocytes from infecting mosquitoes.

The sequenced malaria genome offers a large choice of antigens for inclusion in a candidate malaria vaccine (Gardner et al. 2002). The choice of antigen is influenced by expression, the degree of antigenic variation in field isolates, and data from field studies suggesting that responses directed against it are protective in the field, and recent advances in proteomics might facilitate rational choices of antigens (Hall et al. 2005). Exposure necessarily correlates with the immunological responses measured and with protection against malaria, perhaps as a consequence of a number of protective responses (Marsh & Kinyanjui 2006). This means that observational studies are difficult to interpret, since exposure is a confounding factor.

Having selected an antigen, animal models are generally used to measure immunogenicity and protection against infective challenge. This allows the vector delivery and vaccination regimen to be optimised. Animal studies are often poorly predictive of human responses, and given that the correlates of natural immunity in the field are not clearly understood (Marsh & Kinyanjui 2006), a safe challenge model for human subjects is highly desirable. Exposure of human volunteers to mosquito bites infective for *Plasmodium falciparum* consistently results in infection in non-immune volunteers, and is safe when carried out under strict clinical supervision (Herrington et al. 1988). This allows valuable preliminary evidence of efficacy in studies of small numbers of volunteers in countries that are not endemic for malaria.

1.2 Blood Stage Vaccines

Blood stage parasitisation is associated with clinical disease and chronic parasitisation of the host. Early studies in monkeys with *Plasmodium knowlesi* demonstrated antibody dependent protection against challenge with malaria (Mitchell et al. 1974). Most approaches to replicate immunity in humans use merozoite antigens. Antibody responses to MSP -1 (merozoite surface protein 1), MSP-2, MSP-3 and AMA-1 (apical membrane antigen 1) have been associated with protection in field studies (Cavanagh et al. 2004; Polley et al. 2006; Polley et al. 2004; Polley et al. 2007). Vaccines based on all these antigens are in clinical development.

The "Combination B" vaccine combined MSP-1, MSP-2 and a third antigen, RESA (ring-stage erythrocyte surface antigen). Partial efficacy was observed in a fall in parasite density. However, there was no reduction in the frequency of episodes of malaria after vaccination. (Genton et al. 2002)

A fragment of the MSP-1 antigen, MSP-1 42, has been formulated with a novel lipid adjuvant (AS02). This formulation appears to be safe and immunogenic, but a recent field trial has not demonstrated efficacy (Stoute et al. 2007). The AMA-1 antigen has also been the subject of Phase 1 trials, but efficacy trials in the field have not been conducted.

MSP-3 has been developed as a long synthetic peptide (Druilhe P 2002). Vaccination raises antibodies which interact with monocytes in an *in vitro* assay, and sera from vaccinated volunteers inhibits the growth of parasites *in vitro* (Druilhe et al. 2005). However, *in vivo* efficacy data is still lacking.

Schizont derived antigens that are expressed on the surface of red blood cells, and so accessible to host immunity, are typically very polymorphic, and so difficult targets for vaccination. However, a vaccination approach targeting the Glutamate Rich Protein (GLURP) using synthetic peptides is in phase 1 trials (Sauerwein R 2002), and sera from vaccinated volunteers also inhibits parasite growth *in vitro*.

None of these approaches have been associated with efficacy against experimental or natrurally acquired malaria in human volunteers. It has been argued that sporozoite challenge is not appropriate in the clinical development of blood stage vaccines, since efficacy in the field might be seen at higher parasitaemias despite absence of efficacy in experimental infection. However, given the large number of vaccines now in development, it seems highly desirable to select the most promising candidate vaccines prior to large field trials.

1.3 Sexual stage vaccines.

Transmission blocking vaccines aim to elicit an antibody response capable of neutralizing the oocyst stage in the mosquito. Unusually, this vaccination would not benefit the individual, but would prevent transmission to others. Proof of principal has been achieved in vitro and in animal models (Stowers et al. 2000), and Phase 1 trials in human volunteers are underway.

1.4 Pre-erythrocytic vaccines.

1.4.1 SPf66

This synthetic vaccine, derived from the sequences of the circumsporozoite protein and 2 other blood stage proteins, has been the subject of 4 randomized trials in Africa, and 5 outside. In metanalysis, efficacy against clinical infection was not demonstrable in Africa (OR 0.96, CI 0.81 to 1.14) (Graves & Gelband 2003). Limited protection was demonstrable outside Africa (OR 0.77, CI 0.67 to 0.88). The vaccine is unlikely to be the subject of further large field trials.

1.4.2 Circumsporozoite protein

The circumsporozite protein is a logical choice of antigen, since it is present on the surface of sporozoites (Nussenzweig & Nussenzweig 1985), and therefore a target for antibody mediated immunity, as well as highly expressed during intracellular hepatocytic development (and so a target for T cell mediated immunity), and has conserved antibody and T cell epitopes (Aidoo et al. 1995). The circumsporozoite protein contains a conserved N terminal section, a central region with NANP repeats and a C terminal section with a relatively conserved region, and a highly polymorphic region, divided into the TH2R and TH3R sections.

Individuals in malaria endemic areas acquire antibody responses to the circumsporozoite protein, but these do not appear to be protective (Nussenzweig & Nussenzweig 1985). However, studies in mice (Egan et al. 1987) confirmed that some degree of protection could be induced by antibody responses to the circumsporozoite protein. Studies in humans showed that a synthetic peptide of the NANP repeat was

immunogenic, and when the highest responders were exposed to infective mosquito bites, some limited protection was seen (Herrington et al. 1987).

1.4.3 Thrombospondin Related Adhesion Peptide

TRAP is the second most abundant pre-erythrocytic protein (Robson et al. 1988). Although it has an important role in hepatocyte invasion, the molecule is not accessible to antibody mediated immunity at the sporozoite stage (John et al. 2005). However, it is highly expressed during intra-hepatocytic development, and so is accessible to T cell mediated immunity (Robson et al. 1995). The molecule consists of a small extra-cellular domain, with adhesive modules. Naturally acquired T cell responses are distributed along the length of the molecule, and single nucleotide polymorphisms are distributed along the length of the molecule (Flanagan et al. 1999).

1.4.4 RTS,S

Greater efficacy has been obtained with a formulation of the circumsporozoite antigen called RTS,S. RTS,S contains the carboxyl terminal (a.a. 207-395) of the 3D7 circumsporozoite protein fused to the hepatitis B surface antigen, co-expressed in yeast with the non-fused hepatitis B surface antigen. The co-expression with hepatitis B surface antigen results in particle formation, and this strategy is designed to enhance the immunogenicity of the vaccine. Adjuvants were used to enhance immunogenicity further, and novel adjuvants were found to be more immunogenic than alum (Gordon et al. 1995). Most studies in humans have used the proprietary adjuvant AS02. This is an oil-in-water emulsion containing the two immunostimulants QS21 and MPL, following relatively small studies to compare alternative formulations of the adjuvant (Stoute et al. 1997). A subsequent field trial was conducted in The Gambia, using asymptomatic parasitaemia in

6

adults as a marker of efficacy. (Bojang et al. 2001) 71% protection against infection was observed in the first 2 months (although this did not persist beyond 2 months), and this has encouraged further studies in children. 30% efficacy against disease has been demonstrated in 1 to 4 year old children in Mozambique. This was originally documented during the first 6 months of follow up (Alonso et al. 2004), but now efficacy has been seen up until 18 months (Alonso et al. 2005).

1.4.5 *Other pre-erythrocytic vaccinations.*

A protein particulate vaccine was prepared comprising CS repeats expressed with the hepatitis B core protein, given with the adjuvant Montanide (Birkett et al. 2002), but this vaccine did not protect volunteers in infective challenge studies (Walther et al. 2005a).

1.5   Why T cells Inducing Vaccinations in Malaria?

The HLA-B*53 allele was associated with protection against severe malaria in a large case-control study of Gambian children, suggesting a role for CD8 positive T cells (Hill et al. 1991). It was subsequently confirmed that T cells are induced by natural infection. Adults in endemic areas develop HLA-B*53 restricted T cells recognising a conserved nonamer peptide from liver-stage antigen-1 (Hill et al. 1992), and a peptide-based approach using allele-specific motifs identified fourteen other class I epitopes in six pre-erythrocytic *P. falciparum* antigens (Hill 1999).

1.5.1 Cultured and Ex vivo ELISpots.

The Ex vivo ELISpot assay is well established as a measure of vaccine immunology (Scheibenbogen et al. 2000). This assay enumerates cells producing interferon gamma after overnight stimulation with antigen. However, other older assays used a period of culture with antigen (or cytokines) before measuring cytotoxic activity (Jerome et al. 2003) or proliferation (Harrop et al. 2004), and a culture step using antigen and Il-2 can similarly be used before conducting an ELISpot assay. This is referred to as the "cultured ELISpot". These two assays do not correlate with each other (Flanagan et al. 2001) in individuals naturally exposed to malaria. Further, although the numbers of immediate effector T cells responsive to pre-erythrocytic antigen did not correlate with protection against mild malaria in children in Kilifi (Flanagan et al. 2003), cultured ELISpots correlated with protection against natural infection in semi-immune adults (Reece et al. 2004).

Persistent responses are documented to influenza peptides in the absence of recent exposure (Scheibenbogen et al. 2000). However, after vaccination ex vivo responses are not necessarily persistent (McConkey et al. 2003). However, responses identified by the cultured ELISpot appear to be persistent, and the long term cultured response is proportional to the initial ex`vivo response (Keating et al. 2005). These durable responses were called memory responses. Memory was originally used to describe the protection afforded by vaccination or previous infection, but has since been used as a term to describe secondary antibody or T cell responses (Zinkernagel & Hengartner 2006). Further descriptions of memory T cells have been in terms of markers, homing patterns and proliferative capacity, allowing a division into central and effector memory (Sallusto et al. 1999). Limited evidence suggests that central memory cells are detected in the

cultured ELISpot assay (Godkin et al. 2002). Since these cells are persistent, and require culture in order to be detected, I have used the term "resting memory" in this thesis.

1.6 T cells in Animal Models

1.6.1 Sporozoite immunization

Multiple immunizations of both mice and human volunteers with irradiated sporozoites induce sterilizing immunity. Immunity does not correlate with antibody titres against sporozoites, and is not generated by sporozoites that are incapable of invading hepatocytes because of over-irradiation (Herrington et al. 1991). In mice, T cell clones from irradiated sporozoite immunized mice transfer protective immunity to live sporozoite challenge (Mellouk et al. 1990). These clones recognized circumsporozoite protein derived peptides on infected hepatocytes leading to cell lysis and parasite death (Weiss et al. 1990). Furthermore, gene knock out mouse experiments demonstrated that protection is dependent on interferon gamma production (Doolan & Hoffman 1997).

1.6.2 Sub-unit vaccinations: DNA

Preparing large numbers of irradiated sporozoites is laborious, and it would be impractical to deliver them in the field. Further pre-clinical development of sub-unit T cell inducing vaccines was conducted in mice. Mice can be infected with the malaria species *Plasmodium berghei* and *Plasmodium yoelii*, for pre-clinical testing of the efficacy of vaccinations. The BALB/c and C57BL/6 strains of mice have been most commonly used. These mice recognise a pre-erythrocytic malaria epitope (Pb9) from the circumsporozoite protein (CSP). Early studies of T cell inducing vaccines used DNA expressing *Plasmodium yoelii* CSP (Sedegah et al. 1994). Only limited protection was

achieved in the mouse model, and DNA alone is poorly immunogenic in humans (Wang et al. 2001). Although the route, method and schedule of DNA administration has been varied (Leitner et al. 1997), it has not been possible to obtain consistent protection with DNA vaccinations alone.

1.6.3 Sub-unit vaccinations: Poxviruses

Poxviruses can be made recombinant for malaria antigen (Satchidanandam et al. 1991). They are not effective when used alone, but are effective when used after other vectors. In the mouse model, vaccinia virus recombinant for the circumsporozoite antigen was found to be highly effective in protecting against malaria in combination with a recombinant influenza virus vector (Li et al. 1993). The order of vaccination was important. Vaccinia successfully boosted after priming with influenza virus vaccinations, but regimens that primed with vaccinia were not successfully boosted by influenza vaccination. Vaccinia is quite reactogenic in humans (Casey et al. 2005), but prime-boost vaccinations using naked DNA encoding the TRAP antigen to prime and an attenuated vaccinia virus to boost (modified vaccinia virus Ankara) achieved complete protection against malaria challenge (Schneider et al. 1998). Again, the sequence of vaccination was critical, since MVA alone or followed by DNA was not protective. A second pox virus, the attenuated fowlpox strain FP9, can also be used as a priming vector. In mouse studies, sequential immunization with FP9 and recombinant modified vaccinia virus Ankara (MVA) recombinant for malaria antigen was more immunogenic and protective than DNA/MVA prime boost immunization (Anderson et al. 2004). Better protection was not achieved by sequential (triple) immunization with a DNA vaccine, FP9, and MVA.

It is unclear why heterologous prime-boost vaccination should be so highly effective, in contrast to homologous repeated immunisations using viral vectors. It is suggested that the more strongly immunogenic virus, such as MVA, induces a strong immunological response to the vector rather than recombinant antigen when given alone. However, when a modest, but specific expansion of T cells reactive to the recombinant antigen has recently been achieved by a priming vaccination, these T cells have a selective advantage, and are preferentially expanded by the boosting vaccination (Moore & Hill 2004). Further pre-clinical studies confirmed that sequential DNA then MVA vaccinations were immunogenic (although not protective) in *Pan troglodytes* chimpanzees (Schneider et al. 2001) and sequential DNA then NYVAC vaccinations (another attenuated vaccinia virus vector) were protective in rhesus macaques (Rogers et al. 2002). Most pre-clinical development had concerned the pre-erythrocytic antigens, thrombospondin related adhesion protein (TRAP, (Robson et al. 1988)) and the circumsporozoite protein (CS). Both are well characterised antigens, and protection was seen with vaccination regimens based on either antigen.

1.7 T cell Inducing Vaccinations in Human volunteers

These recombinant vectors were then used to vaccinate human volunteers with a common pre-erythrocytic antigen construct, ME-TRAP, comprising multiple pre-erythrocytic stage epitopes (ME) and the whole pre-erythrocytic stage antigen Thrombospondin Related Adhesion Protein (TRAP). TRAP was chosen, rather than the circumsporozoite antigen, since it appears to be less polymorphic (Flanagan et al. 1999), as well as being abundantly expressed by pre-erythrocytic parasites (Robson et al. 1988). The string of multiple epitopes was defined by reverse immunogenetics (selecting epitopes based on HLA binding) (Gilbert et al. 1997). Multiple DNA priming followed

by intradermal MVA boosting (i.e. heterologous prime-boost vaccination) induced 10-fold higher T cell responses than previously achieved with homologous immunisations. Partial protection against experimental malaria was seen as a 2 day delay in time to parasitaemia after experimental malaria sporozoite challenge in a non-endemic area (McConkey et al. 2003). The regimen DNA ME-TRAP followed by boosting with MVA ME-TRAP was then subjected to Phase I and II trials in The Gambia. 300 semi-immune adults were immunised and followed weekly for asymptomatic parasitaemia. The vaccination was safe, immunogenic, but not significantly protective (Moorthy et al. 2004b).

Since the protection in mice was greater from FP9 primed regimens compared with DNA primed regimens, 17 volunteers were immunized with two sequential FP9 immunizations followed by MVA once. This was equally immunogenic, but more protective (Webster et al. 2005). Two volunteers were completely protected after sporozoite challenge in Oxford, and the overall delay to parasitaemia was 2.5 days (p=0.32 for comparison with DNA/MVA combinations, p=0.0022 with unvaccinated controls). One of these two volunteers was protected on re-challenge at 6 months and 20 months. As in the mouse model, sequential priming with DNA then FP9 before using MVA did not improve protection.

1.7.1 Modified Vaccinia Ankara (MVA)

MVA is much safer than vaccinia because it fails to replicate *in vivo*. During the smallpox eradication campaign vaccinia administration was associated with significant side effects. The most comprehensive data come from a 10 state survey of the USA in 1968: there were 2.8 cases per million of post-vaccinia encephalitis, 0.9 cases of

progressive vaccinia, 10.4 cases of eczema vaccinatum, 23.4 cases of generalised vaccinia and 25.4 cases of accidental infection per million primary vaccinations with non-attenuated vaccinia (Gurvich 1992). In an attempt to reduce these side effects several attenuated strains of vaccinia were developed. The most widely used of these was MVA, derived by multiple serial passage of vaccinia strain Ankara through chick embryo fibroblasts. MVA caused no serious side effects when given by intramuscular, subcutaneous and intradermal routes to 120,000 individuals in southern Germany and Turkey (Mayr et al. 1978). MVA was safe when administered to neonatal mice by intracerebral and intraperitoneal routes, and did not disseminate in mice immunosuppressed by irradiation (Mayr et al. 1985). It appears to be attenuated as a consequence of genetic deletions, with a unique cytokine receptor profile (Blanchard et al. 1998).

The safety data acquired during 100 immunisations with prime boost regimes in healthy volunteers in Oxford and over 200 immunisations with MVA in The Gambia has been favourable (Moorthy et al. 2004a; Moorthy et al. 2004b; Moorthy et al. 2003b; Webster et al. 2005). Local reactions occurred in 70% of subjects, limited to discolouration, blistering and itching, typically resolving over one week. 25% had a mild flu-like illness for not more than 24 hours after the first dose of MVA. Stocks of MVA encoding ME-TRAP are produced to GMP standard by Impfstoffwerk Dessau-Tornau, Roslau, Germany.

1.7.2 The Attenuated Fowlpox Strain, FP9.

Fowlpox is an avian poxvirus which causes disease in chickens but not mammals. Recombinant attenuated fowlpox has been used as a rabies vaccine in mammals. FP9 was derived from wild type fowlpox by 400 passages in tissue culture, leading to marked

attenuation and loss of pathogenicity in chickens, including one-day old chicks (Taylor & Paoletti 1988; Taylor et al. 1988). In the UK a clone of FP9 has been developed and characterised in detail at the Institute for Animal Health in Compton (Laidlaw & Skinner 2004). The stock of FP9 from which FP9 ME-TRAP was manufactured was derived from a sample obtained of this clone. Like all avipoxviruses, FP9 and the recombinant FP9 ME-TRAP cannot replicate in mammalian cells. Attenuated strains of fowlpox virus and other avipoxviruses have been used safely as recombinant viral vaccines in many human clinical trials and have been demonstrated to be non-virulent in human volunteers (Cavacini et al. 2002; Moorthy et al. 2003b; Rosenberg et al. 2003; Webster et al. 2005). 40 volunteers have been immunised on 1-2 occasions with FP9 ME-TRAP in Oxford and 16 with 2 immunisations in The Gambia. As for MVA, GMP standard production is by Impfstoffwerk Dessau-Tornau, Roslau, Germany.

1.7.3 ME-TRAP

The malaria DNA sequences cloned into FP9 ME-TRAP and MVA ME-TRAP are derived from a number of different genes.

The single largest part of the synthetic protein ME-TRAP consists of the entire amino-acid sequence of thrombospondin–related adhesive protein (TRAP) from the human parasite *P. falciparum* strain T9/96.

The remainder of the synthetic protein ME-TRAP is made up of a multiple epitope (ME) string, containing a total of 20 short peptide sequences. Of these, 17 are derived from previously-defined immunogenic regions of six human malaria antigens (Gilbert et al. 1997) while one (pb9 in cassette C) is derived from the mouse malarial parasite *P. berghei* (Romero et al. 1989).

Pb9 was included to allow evaluation of immunogenicity in mice. The remaining two

epitopes, BCG and TT, are non-malarial T cell helper (universal) epitopes for CD4 T

cells, from tetanus toxin and BCG vaccine respectively. and were included to help

promote a strong cellular immune response. The TT epitope has a single amino acid

change (tyrosine to phenylalanine at position two of the peptide sequence to attempt to

broaden further the range of HLA class II molecules for which it will act as an epitope.

A complete list of the gene cassettes containing the epitopes included in the ME.TRAP

construct is given in appendix 9.1.3.

1.7.4 Semi-immune adults: Time to asymptomatic parasitaemia.

A field trial in Gambian semi-immune adults had already assessed two DNA and one

MVA:ME-TRAP immunisations and observed an efficacy of only 10% (P=0.49) in

reducing rates of asymptomatic parasitaemia. However, efficacy of FP9-MVA is likely

to be higher than DNA-MVA vaccination (see 1.6.3). Furthermore, the efficacy against

disease in children should also be higher than efficacy against parasitaemia in adults.

The reasons for this are described below.

- Early acquisition rates of parasitisation are much lower than expected from

  calculations of entomological inoculation rates (Appawu et al. 2004). If there was

  no significant contribution of host immunity to infection rates, it would be

  expected that adults become parasitaemic at the rate they are bitten by infective

mosquitoes. Since the acquisition rate of parasitaemia is much lower than expected, this suggests that studies of asymptomatic parasitaemia in adults are strongly influenced by pre-existing host immunity.

- Parasite incidence rates should be corrected for frequency of mosquito biting; evidence suggests this is proportional to surface area, and so lower for children than adults(Smith et al. 2004). Reanalysis correcting for surface area would then suggest lower infection rates in adults.

- In both the field study of DNA:ME-TRAP prime, MVA ME-TRAP boost and the study of RTS,S (a particulate protein vaccine that encodes the circumsporozoite antigen) conducted in The Gambia, gradually increasing protection against asymptomatic parasitaemia was seen when comparing older with younger age groups (Bojang et al. 2001).

- Recent work suggests that blood stage immunity to a merozoite antigen protects against parasitisation in a longitudinal study of adults after curative chemotherapy (John et al. 2004). This is further evidence that incidence studies in semi-immune adults are complicated by naturally acquired immunity. This immunity would act after parasites emerge from the liver, but before they reach sufficient numbers to be seen by microscopy.

Furthermore, in endemic areas infection rates of adults are so high that it is very hard to observe efficacy against infection, in effect the experimental model is "saturated". When studies following parasitaemia clearing chemotherapy have been conducted in areas at approximately ten fold different transmission rates (Northern Ghana (Owusu-Agyei et al. 2001) and Mali (Sagara et al. 2002)), the rate of acquisition of parasitaemia during follow-up was very similar.

16

Finally, there is evidence that other measures reducing pre-erythrocytic parasites do reduce clinical disease despite a limited action on parasitaemia rates.

- Extensive clinical trials on insecticide treated nets (ITNs) have been performed, and the data subjected to meta-analysis recently. Limited impact on parasitisation rates per se is demonstrated (10%), albeit studied in cross-sectional surveys rather than longitudinal cohort studies after radical cure. Efficacy is harder to demonstrate in areas at higher transmission rates. Nevertheless, 40-60% efficacy against mild malaria in young children is described, as well as efficacy against anaemia and severe disease in larger studies (Lengeler 2004).

- During both the RTS,S study and the DNA/MVA:ME-TRAP study in The Gambia, although use of bed nets was not randomised, bed net use was not associated with any lower incidence of malaria parasitisation (Bojang et al. 2001).

- In observational studies, although the prevalence of parasitisation changes little according to season (relative risk of 1.5 for the rainy season) the risk of clinical disease changes dramatically (relative risk of 4.4) (Baird et al. 2002).

Importantly, a field trial in The Gambia demonstrated an overall 34% efficacy of RTS,S against asymptomatic parasitaemia in adults; 70% in the first 9 weeks, and none thereafter. However, phase I trials of RTS,S in non-endemic areas demonstrate long lived partial efficacy against experimental challenge with a large inoculum of sporozoites, with a delay to parasitaemia following sporozoite challenge still seen 6 months after vaccination (Stoute et al. 1998). The discrepancy between the duration of efficacy in these studies may reflect the limitations of studying time to asymptomatic parasitaemia in adults, and the protection seen against disease in children may be greater than that seen for asymptomatic semi-immune adults.

In summary the incidence rate of parasitaemia in semi-immune adults is a measure that appears to be insensitive to a ten fold reduction in malaria challenge rate, with between-individual variation determined heavily by blood stage immunity, and this incidence rate is poorly predictive of the potential protection afforded against disease in children, the main group requiring protection by a vaccine.

1.7.5 Objectives.

The objective of further studies in humans was to develop vaccinations using FP9 and MVA vectors to deliver the antigen construct ME-TRAP, with the aim of conducting an efficacy trial in children in a malaria endemic area. I conducted a preliminary analysis of efficacy in sporozoite challenge studies of volunteers in a non-endemic area, phase 1 safety and immunogenicity studies in adults and then children in a malaria endemic area, and finally a large phase 2b efficacy trial on children.

2 Sporozoite Challenge in the UK: An Analysis of PCR Data

(Calculation of Liver-to-Blood Inocula, Parasite Growth Rates and Pre-Erythrocytic Vaccine Efficacy from Serial Quantitative PCR Studies of Malaria Sporozoite Challengees)

2.1 Introduction

Sporozoite challenge of human volunteers can assess efficacy of pre-erythrocytic vaccines before large field trials (Herrington et al. 1988). Volunteers are exposed to the bites of *Plasmodium falciparum* infected mosquitoes, monitored with regular blood film examinations, and treated after the first positive blood film. Parasitaemia-free intervals after sporozoite inoculation are then analysed as a survival function. A longer parasitaemia-free interval for a vaccine group has been considered to represent a reduced inoculum of parasites seeding the blood from the liver (McConkey et al. 2003; Witney et al. 2001). PCR detects lower parasite numbers than detected by blood smears. Blood films detect between twenty and fifty thousand parasites per ml, but PCR can detect 20 parasites per mL (Andrews et al. 2005). Analysis of these data allowed us to test if vaccine induced immunity reduced parasite numbers emerging from the liver or subsequent growth rates.

Several immunization regimens were examined. Five used the pre-erythrocytic antigen ME-TRAP (malaria epitope-thrombospondin-related adhesion protein), encoded by three vectors; FP9, an attenuated strain of fowlpox, modified vaccinia virus Ankara (MVA) or plasmid DNA. A second pre-erythrocytic antigen, the circumsporozoite protein, was delivered by recombinant MVA and RTS,S/AS02, a particulate vaccine fusing most of the circumsporozoite protein to hepatitis B surface antigen with a proprietary adjuvant (Stoute et al. 1997). The particulate Apovia vaccine, ICC-1132, comprises the hepatitis B core antigen containing T and B cell epitopes from the circumsporozoite protein, with Montanide ISA720 adjuvant (Birkett et al. 2002; Walther et al. 2005a).

Heterologous prime-boost regimes using DNA and MVA encoding ME-TRAP were associated with significant delays in time to parasitaemia by blood film, but pre-date quantitative PCR (McConkey et al. 2003). A similar regimen (two sequential DNA immunizations followed by MVA and then FP9, designated "DDMF"), among others, has been assessed here. Two FP9 immunisations followed by MVA ("FFM"), and two prime-boost regimes of RTS,S/AS02A and MVA encoding the circumsporozoite protein protected some volunteers and significantly delayed parasitaemia in others (Dunachie et al. 2006; Webster et al. 2005). Other ME-TRAP-based regimes, two DNA immunizations followed by FP9 then MVA ("DDFM"), a single FP9 before MVA ("FM") and MVA before FP9 ("MF") did not delay onset of parasitaemia by blood film (Webster et al. 2005). The protection data available from blood film studies are summarized in table 2.1.

Table 2.1 Summary of vaccination regimes, numbers of volunteers and efficacy by blood film data.

Vaccine	N	Delay to parasitaemia (days)	Fully protected
DDDMM	0	2.2*	0
DDMF	4	1.7*	0
FFM	17	2.5*	2
DDFM	3	0	0
FM	4	0.6	0
MF	4	0.4	0
ICC-1132	11	0	0
RRM/MRR	12	3*	4

Vaccine abbreviations are; D – DNA expressing ME-TRAP, M- MVA expressing ME-TRAP, F- attenuated fowlpox strain FP9 expressing ME-TRAP, R- the particulate vaccine RTS,S/AS02 encoding most of the circumsporozoite protein. ICC-1132 is a hepatitis B core-based circumsporozoite vaccine. M appearing in regimes with RTS,S/AS02 denotes MVA expressing the circumsporozoite protein. * indicates significance (p<0.05) by log rank survival analysis.

Parasites first emerge from the liver 6.5 days after inoculation of sporozoites by mosquitoes (Daubersies et al. 2000; Hermsen et al. 2001). Parasite numbers in peripheral blood then depend on multiplication rates and sequestration of parasites in blood vessels. Parasites sequester in the second half of their 48 hour life cycle; the progeny of sequestered parasites then appear in peripheral blood until they too sequester. If perfectly

synchronous, parasites would be absent from peripheral blood on alternate days. However, partial synchrony is usual, with marked periodic fluctuations in parasitaemia. Fluctuations in blood film parasitaemia, measured in *P. falciparum* treated neurosyphilis patients, have been modelled using a sine wave function for sequestration and a logarithmic function for growth (Simpson et al. 2002). Another approach splits parasites into broods depending on the time of sampling, and was applied to volunteers receiving identical blood stage inocula to calculate growth rates (Cheng et al. 1997). I adapted both these models to determine growth rates and liver-to-blood inocula, and thereby protective efficacy, resulting from various immunisation strategies.

2.2 Methods

2.2.1 Challenge Studies

Vaccinated and unvaccinated volunteers were exposed to the bites of 5 *P. falciparum* (strain 3D7) infected *Anopheles stephensi* mosquitoes. Twice daily blood smears and PCR measurements were made from the 6th until the 14th day and then once daily until the 21st day. These vaccination and challenge trials are reported in full elsewhere (Dunachie et al. 2006; Walther et al. 2005a; Webster et al. 2005). Visualizing a single parasite by blood film examination prompted immediate treatment with standard doses of chloroquine over 3 days, and defined the study endpoint. Volunteers were considered fully protected if not parasitaemic by 21 days. Neither managing clinicians nor microscopists were aware of PCR data during the trial. The PCR method is described elsewhere (Andrews et al. 2005). Briefly, EDTA anticoagulated blood samples were filtered to remove leukocytes, DNA purified from 0.5ml filtered blood, and eluted into 50μl. A portion of the multicopy 18S (small subunit) ribosomal RNA genes of *P. falciparum* was amplified by PCR and the increase in PCR product detected by binding

the fluorescent dye SYBR green using the Rotor-Gene Real-Time PCR machine (Corbett Research), using 5μl extracted DNA in duplicate. All volunteers gave informed consent for immunisations, challenge studies, and blood sampling. Procedures were reviewed by OXREC, the local ethics committee.

2.2.2 Modelling

2.2.2.1 Individual modelling using the sine wave function.

Volunteers with less than six PCR readings and those completely protected were excluded. Of 80 volunteers, six volunteers were completely protected and six volunteers had less than six points. Data for the logarithm of each PCR result, described by time as a continuous variable and volunteer were analysed using Stata 8^{TM} (Timberlake). Non linear regression to determine best fit by least squares was used, using the model; log (P) = tm+a+c sin(πt+k); P is the number of parasites per ml, t is time minus 6.5 days after sporozoite inoculation, m (gradient) is the logarithm of the daily multiplication rate, a (intercept) the logarithm of the starting parasite population per ml of peripheral blood. c (the sine wave amplitude) is the logarithm of the sequestered parasites and k (phase shift) the time between day 7 and the first peak. Hence, $10^{[c+a]}$ approximated the first peak of parasites per ml as the number emerging from the liver after day 6.5. A 5 litre blood volume was assumed to calculate total parasite numbers. 95% confidence intervals were calculated using the root mean square of the standard error of each parameter, derived by asymptotic approximation. Day 6.5 readings were excluded, since the change between days 6.5 and day 7 is generated by parasites emerging from the liver, rather than growth or sequestration. Samples taken before the first detection of parasites were excluded from analysis. Where parasites were undetected but previous timepoints had detected parasites, the lower limit of parasite detection was used (20 parasites per ml).

The line was fitted by non-linear least squares regression and checked by visual inspection. Alternative models, including linear regression, other sine functions (e.g. $\sin^2$ or $\sin^3$ based functions and Fourier transformations) and using the sine function without log transforming PCR results were considered. These strategies were no better, judged by least squares and r squared values (without increasing the number of parameters). Volunteers with more observations generated parameter estimates with lower standard errors ($p<0.0005$). An example of the line fit is shown in figure 1. Most individuals' data were well represented by the modelling strategy: the distribution of r squared values is shown in figure 2.

I assessed methods for estimating the liver burden from these parameters by using a step function simulation of parasites emerging from the liver with a 10 fold multiplication rate in each 48 hour life cycle and 24 hour patent, non sequestered period. I simulated 12 hourly sampling over 4 days, and found $10^{[c+a]}$ to be an accurate approximation for total liver burden, with a 10% average error.

Figure 2.1 Example of line fit by individual sine wave modelling approach.

Volunteer 184 received FP9 followed by MVA expressing the antigen ME-TRAP, and became blood film positive on day 11. The line drawn here is given by log(PCR)=0.56 time+1.54+0.57sin(π time − 1.02)
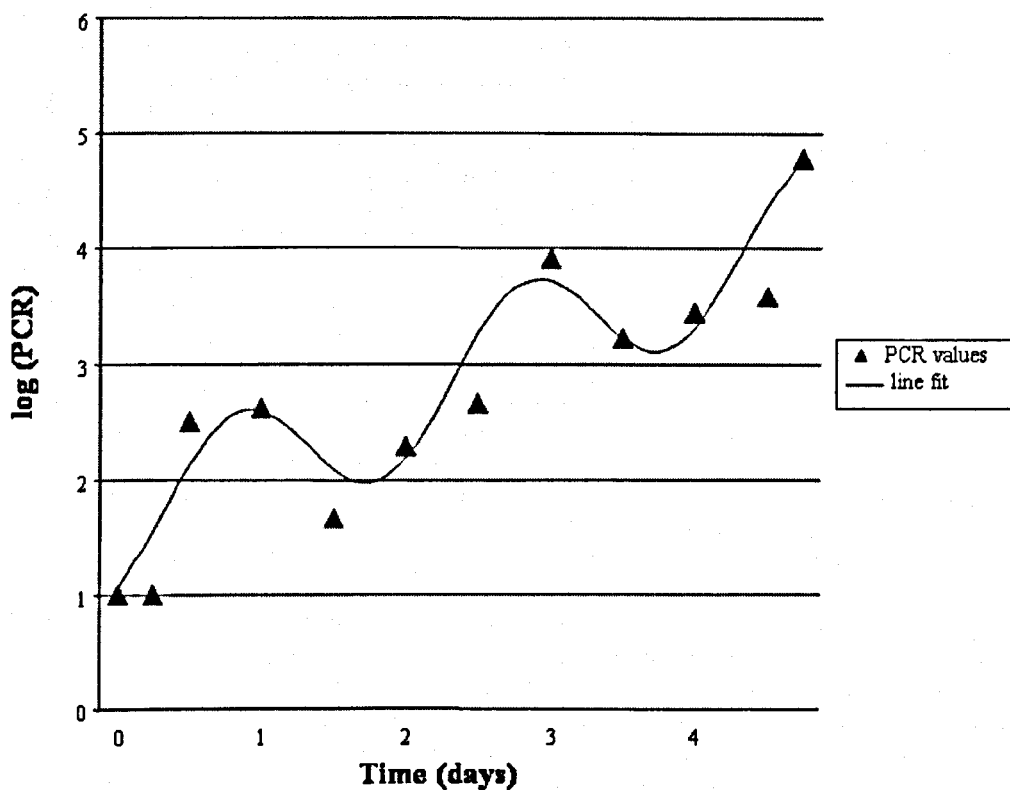
Figure 2.2 R squared values for line fit by individual sine wave modelling.
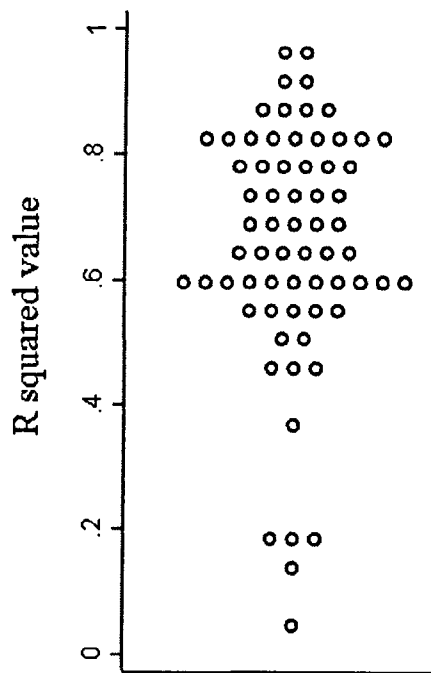
2.2.2.2 Group modelling using the sine function.

A mixed effects model, applied separately to each group, allows individuals with fewer than four PCR readings to be included in analysis. Mixed effects models were used for groups with eight or more individuals. A group mean and each volunteer's deviation from the mean were calculated, assuming normal distributions for the group. Parameters m, c, a and k are analogous to those in individual fits, where m0 represents the mean value for the group, and mi the separate factor for each individual., i.e. $\log(P)=t(m0+mi)+a0+ai+(c0+ci)\sin[\pi t+(k0+ki)]$. Mixed effects modelling methodology has previously been described in detail (Burton et al. 1998). Extensions of this model allowed us to examine periodicity for the overall group, i.e.

$\log(P)=t(m0+mi)+a0+ai+(c0+ci)\sin[\pi t(p+p0)+(k0+ki)]$, and change in growth over time, $\log(P)=t(m0+mi)\exp(v0+v1)+a0+ai+(c0+ci)\sin[\pi t(p+p0)+(k0+ki)]$.

A Bayesian Markov Chain Monte Carlo method (WinBUGS ™ Version 1.4, August 2002, MRC Biostatistics Unit, and Imperial College, London) was used. Fully protected individuals were excluded, and day 6.5 values and undetectable values treated as above.

2.2.2.3   Group modelling using divided parasite populations.

An alternative model links the peaks, troughs and midpoints of the oscillations (assuming 48 hours periodicity) by a logarithmic growth function. The volunteers in these trials have four blood tests in each 48 hour period, so are considered as infected by four partially overlapping populations of parasites. For example, parasites observed in peripheral blood on day 9 are the progeny of those observed on day 7. Few individuals would have sufficient time points to allow modelling of four different parasite broods, but mixed effects modelling allows analysis of a group of volunteers, each with four broods. Normal distributions are assumed for parameters and WinBUGS ™ Version 1.4 used for analysis.

2.2.3 Statistical comparisons

Liver-to-blood inocula and growth rates for individuals were compared with those from unvaccinated individuals by non-parametric testing (Stata 8™). Kruskal-Wallis, non parametric testing compared vaccination regimes within the individual modelling.

2.3 Results

2.3.1 Individual modelling using sine wave function

The numbers of parasites emerging from the liver are displayed by individual grouped into different vaccination groups. (Figure 2.3, below). There were no significant differences between the control group and ICC-1132, DDFM, FM or MF. DDMF was associated with an 86% decrease in liver parasite burden (p=0.074). The FFM and RRM/MRR groups showed 92% and 97% reductions in liver-to-blood inocula compared with the control group (Kruskal Wallis p=0.004 and p=0.001,). Growth rates were similar between groups (fig. 2.4, below), with an average multiplication of 14.4 fold (95% confidence interval 11 fold to 19 fold) in 48 hours. There were no differences between groups for sequestration of parasites or time to parasites emerging from the liver. Estimates of the reduction in liver stage parasite numbers corresponded with blood film estimates of vaccine efficacy. Correlating liver burden estimate with time to blood film patency gave r=-0.552, p<0.0005. No correlation was seen with growth rate.

Figure 2.3: Estimated liver parasite load on day 6.5 plotted by volunteer with 95% confidence intervals. Grouped according to vaccine, and ordered according to liver burden. Groups are ordered according to efficacy (significant delay to parasitaemia by blood film data). A dashed horizontal line marks the average liver burden for controls. Vaccine abbreviations are; D – DNA expressing ME-TRAP, M- MVA expressing ME-TRAP, F- attenuated fowlpox strain FP9 expressing ME-TRAP, R- the particulate vaccine RTS,S/AS02 encoding most of the circumsporozoite protein. ICC-1132 is a hepatitis B core-based circumsporozoite vaccine. M appearing in regimes with RTS,S/AS02 denotes MVA expressing the circumsporozoite protein.
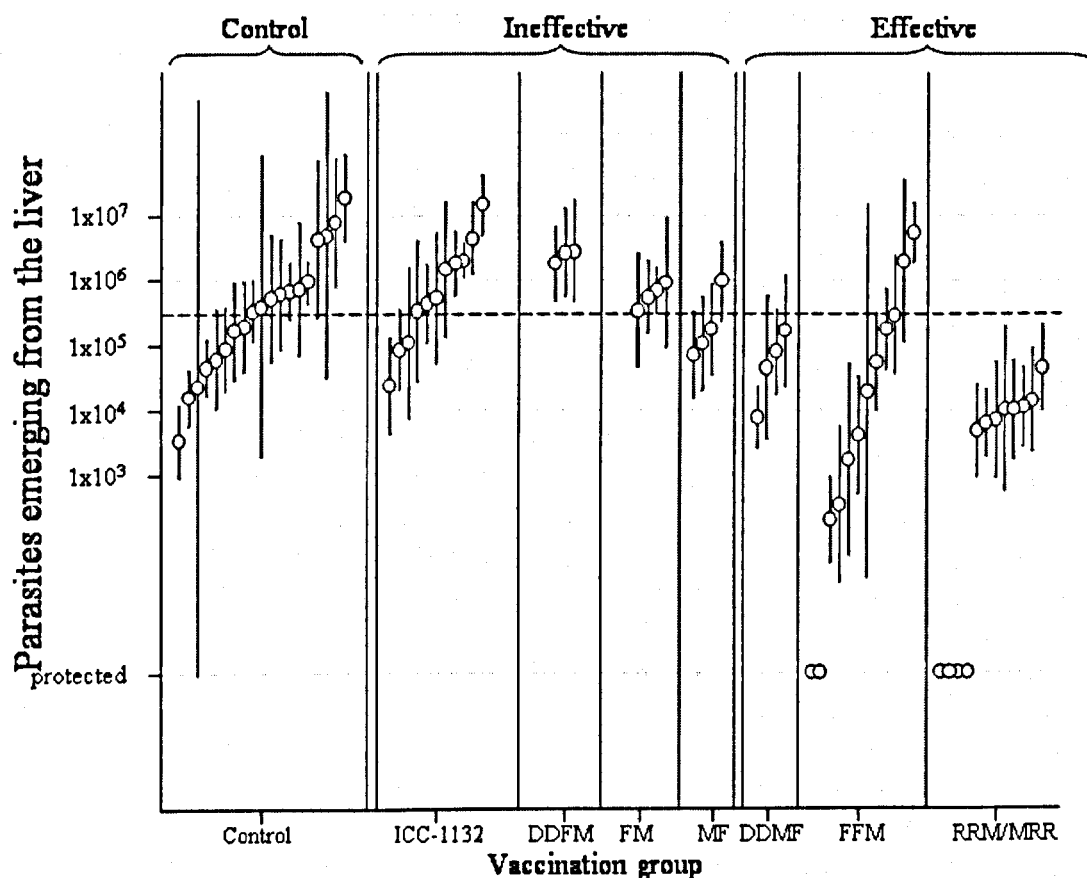
Figure 2.4: Growth rates with 95% error bars. Volunteers are grouped according to immunization and ordered according to growth rate. A dashed horizontal line marks the average growth rate. Vaccine abbreviations as above (Fig 2.3).
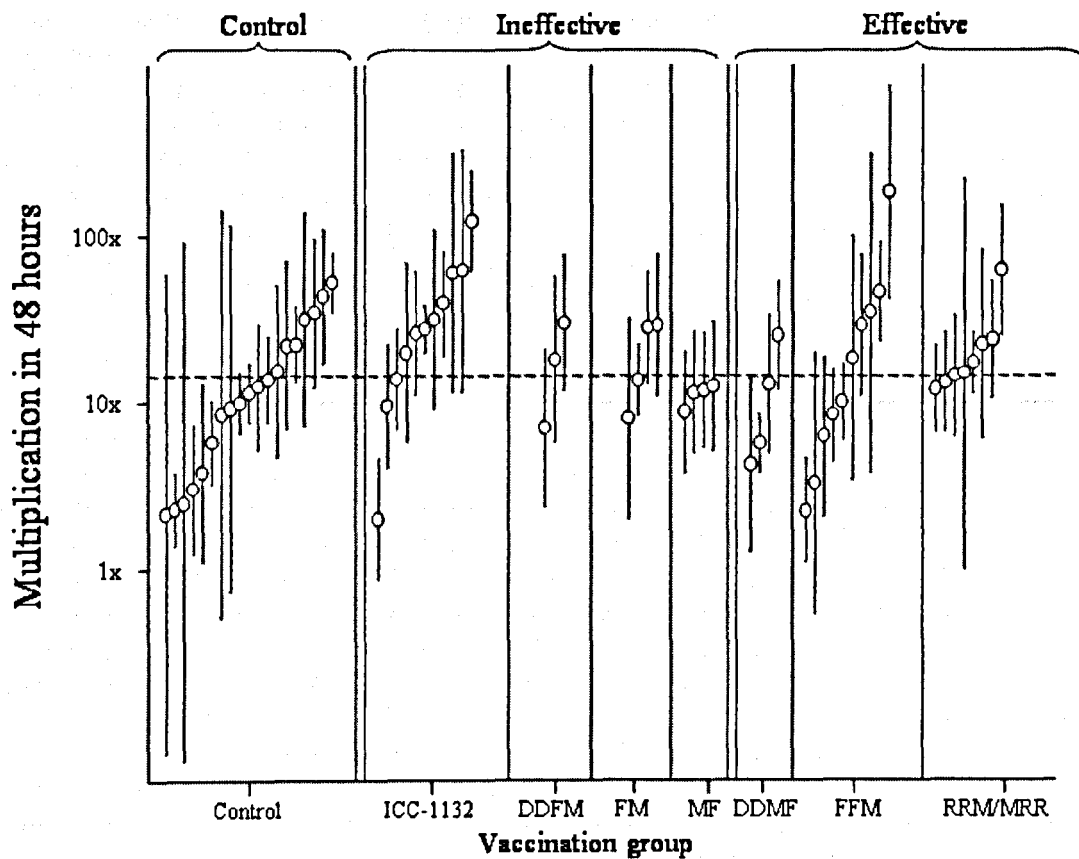
2.3.2 Group modelling using the sine function

A group model allowed inclusion of individuals whose data had too few points for individual modelling. The reduction in parasite load was 89% for RRM/MRR and 82% for FFM. (fig 2.5). By weighted means including completely protected individuals the reductions are 93% and 85% for RRM/MRR and FFM respectively. The mean 48 hour multiplication rate was 11.5 fold (95% CI 6.6-20.9). Growth rates were not significantly different by vaccination; control 12.3 fold (95% CI 8.3-16.6), ICC-1132 23 (95% CI 11-60), FFM 9.6 fold (CI 8-23), RRM/MRR 15.8 fold (CI 8.7-30). In models examining periodicity, a cycle of 2.2 days was found (CI 1.5-2.9 days). There was no change in growth rate over time (change in rate of 0.97 fold per day, CI 0.7-1.3).

2.3.3 Group modelling using divided parasite populations.

Assuming a 48 hour life cycle, it was possible to fit a logarithmic growth pattern for observations taken 48 hours apart for each volunteer. A mixed effects model for 4 different broods per volunteer showed a reduction in parasite load of 95% for RRM/MRR and 86% for FFM (Fig 2.5). The overall growth rate was 11.5 fold (95% CI 5.8-22.9), by group growth rates were; control 10.5 fold (95% CI 4.7-23), ICC-1132 23 fold (95% CI 8-60 fold) FFM 10.5 fold (5.2-20), RRM/MRR 15.9 fold (7.9-31 fold).

Figure 2.5: Comparison of estimates for liver burden by different modelling strategies. Vaccine abbreviations are as for figure 2.3 above.
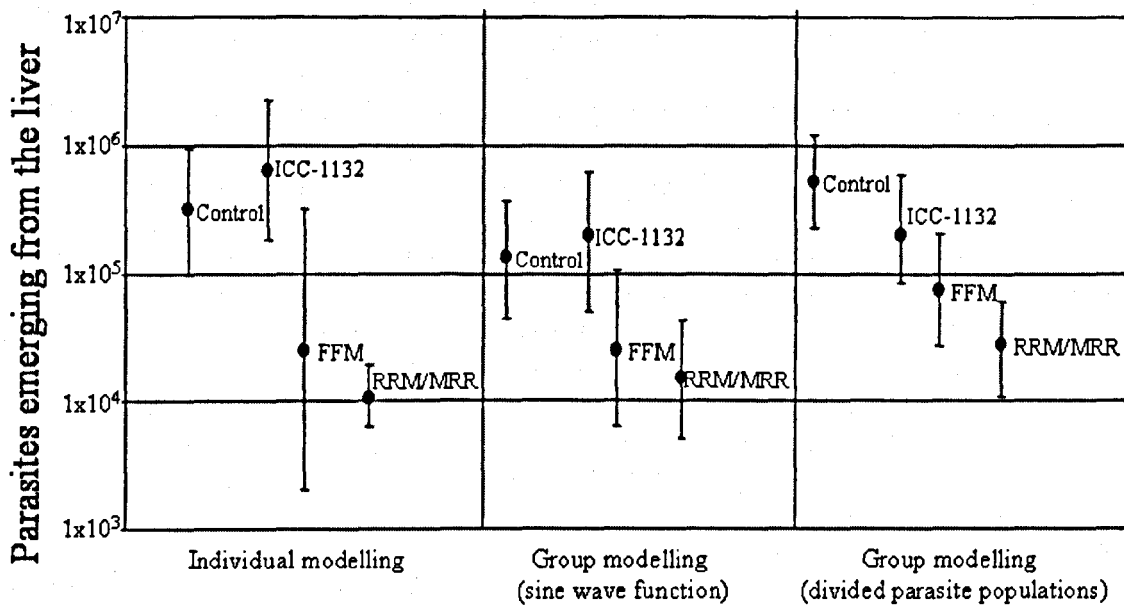
2.4 Discussion

In all 3 models, reduced numbers of parasites emerging from the liver were found for vaccinations associated with protection by conventional blood film assessments, (RRM/MRR, FFM and DDMF, fig 2.3). In contrast, no differences in growth rates were seen (fig 2.4). Estimated liver inoculums from the individual sine wave model (model 1) correlated closely with the time until blood film patent infection ($r=-0.552$, $p<0.0001$), but no correlation was seen with growth rate.

Estimating 4 parameters from 6-16 (mean ten) points per individual gave large standard errors. (figs 2.3,2.4). Parameters are fitted as log values, amplifying standard errors when described in linear terms. This contributes to the large range of values returned. Nevertheless, the efficacy of immunization with FFM and RRM/MRR is clear

and attributable to a reduction in parasites emerging from the liver rather than reduced blood stage growth rates. The mixed effects models (models 2 and 3) gave equally large standard errors. I used group models to avoid bias from excluding those with few points from individual modelling, and have not selected volunteers whose data best fits the model to reduce standard errors, as done previously (Simpson et al. 2002). Results were similar to the individual model using a sine wave function (fig 2.3); I have therefore emphasized the more parsimonious individual model in presentation and discussion.

Morphological studies suggest twenty thousand merozoites are released by a single schizont, (Meis et al. 1984; Meis 1986) an estimated eleven sporozoites (range 3-17) are injected by a single mosquito bite (Beier et al. 1992), and the challenge model uses five infective bites. Fifty five sporozoites would therefore theoretically produce over one million merozoites, three times the PCR-based estimate, if every sporozoite reaches a hepatocyte. However, the average unvaccinated subject was blood film positive on day 11. Assuming parasites emerged from the liver on day 6.5, grew in a blood volume of five litres at a rate of ten fold in 48 hours and were seen by blood film microscopy at ten parasites per microlitre, then the initial inoculum from the liver-to-blood would be 280 thousand, very similar to the PCR derived estimate. (Fig 2.6) This suggests that only a fraction of sporozoites successfully invade liver cells.

.

Figure 2.6: Schema relating sporozoite inoculum, initial merozoite release, multiplication rate and blood film diagnosis.

The text in the figure reads:

14 sporozoites successfully invade hepatocytes

20,000 merozoites released per sporozoite: 280,000 total

10 fold multiplication in 48 hours: parasite numbers given by $280,000 \times 10^{(\text{life cycles})}$

Blood film diagnosis at 50,000,000 parasites: 4-5 days later (ie 2-2.5 life cycles).
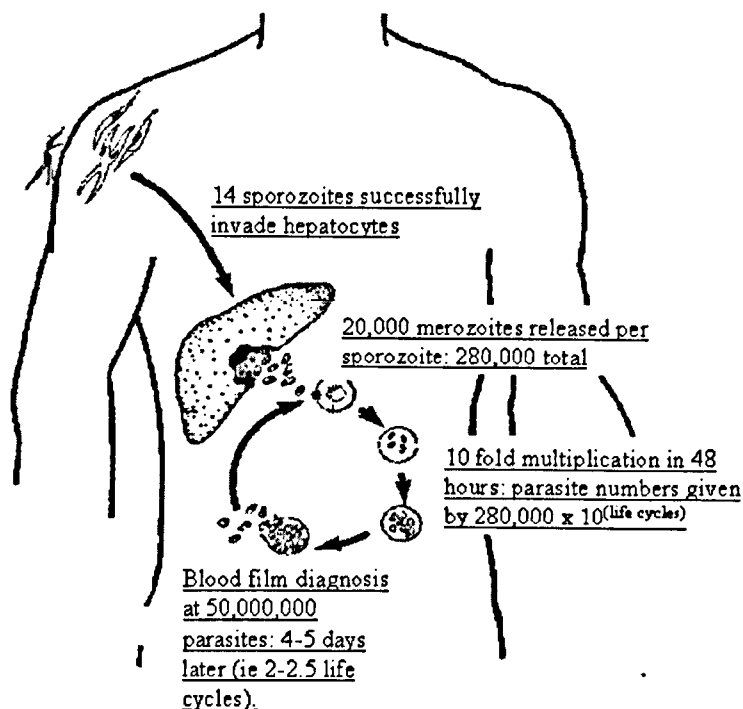
The average liver-to-blood inoculum in the RRM/MRR group was in the ten to thirty thousand range, suggesting that these volunteers have only one infected hepatocyte. Individuals clearing 13 out of 14 hepatocytes might have had similar immunity to those clearing all 14, perhaps explaining difficulties in correlating individual immunity with protection (Stoute et al. 1998). There are several caveats in this calculation, however; estimates of nascent merozoites within a single schizont are not precise and do not allow for inter-schizont variation *in vivo*, only a fraction of the 20 thousand merozoites will invade red cells, and it is possible that schizonts may be damaged but not killed by vaccine effects, so releasing reduced parasite numbers. Delay in schizonts releasing merozoites rather than reduction in numbers of released merozoites would also reduce the intercept. However, there was no difference in the timing of the first peak (as determined by modelling) between FFM vaccinees and controls (p=0.61) or between RTS,S

vaccinees and controls (p=0.75), and similarly no difference in timing of the first PCR positive result (p=0.45 and 0.52 respectively). It is therefore unlikely these results reflect prolonged hepatic schizogony in vaccinees rather than killing of parasites.

If schizonts are not partially injured by vaccine effects, (i.e. either they escape completely or are killed), FFM vaccinees and RTS,S regimen vaccinees should have 2.2 and 0.9 infected hepatocytes on average, respectively. A Poisson distribution would then predict that 13% FFM and 40% RTS,S regimen vaccinees would be completely protected, close to the actual results of 12% FFM and 33% RTS,S regimen vaccinees. This supports the view that these vaccines are working by affecting hepatocyte parasite yield in an all-or-none manner rather than quantitatively affecting the parasite yield of individual parasitized hepatocytes.

I considered that chance sampling of parasites present in the volume of blood drawn and gene copies in the DNA eluted might be a source of variability in our data. Estimates from the Poisson distribution, the volume of blood sampled and the fraction of DNA analysed suggest that parasites were reliably sampled (i.e. with greater than 95% chance) above 10 parasites per ml, and that quantitation was accurate to within 20% at 250 per ml.

Growth rates here were slightly higher than the 8 fold previously calculated using blood film data (Simpson et al. 2002), but similar to the higher growth rates (ranging from 12 to 25 fold) estimated using PCR after inoculation of low numbers of blood stage parasites (Cheng et al. 1997; Pombo et al. 2002). PCR examines lower parasite densities, suggesting that at lower densities growth may be more rapid. However, in mixed effects model variation in growth rates over time did not occur during the monitoring period. It is possible that growth begins to slow at higher densities.

The protective vaccine regimes assessed here are shown to act by reduction of liver parasite load rather than altered growth patterns or time of emerging from the liver.

This is an important endpoint of phase IIa malaria vaccine trials and complements data obtained by the traditional blood film microscopy endpoint. The substantial effect quantified here on parasite liver burden of each of the two partially protective vaccination regimes (FFM and RRM/MRR) suggests that relatively minor improvements of either of these pre-erythrocytic vaccination approaches could lead to a major increase in sterile protection rates.

The combination with RTS,S and MVA was not more protective than RTS,S given alone (Dunachie et al. 2006), and so RTS,S alone is being developed in GSK sponsored trials elsewhere (Alonso et al. 2005; Alonso et al. 2004). I therefore went on to conduct further field trials using FP9 and MVA vectored vaccinations to deliver the antigen construct ME-TRAP.

3 Phase 1 in Kenya: Safety

The safety profile of viral vectors FP9 and MVA recombinant for either of two pre-erythrocytic malaria antigens, ME-TRAP or CS, in children and in HIV positive and negative adults in Kenya.

3.1 Background

This section describes the first immunisation of adults in East Africa with FP9 and MVA, the first immunisations of children with FP9 ME-TRAP and MVA ME-TRAP, and the first immunisations of HIV positive adults with attenuated pox viruses in a malaria endemic area. The aims were to establish whether adverse events in this series of Phase I trials would support further testing, and to describe how reactogenicity varies by sequence of immunization, dose and antigen insert. Immunogenicity will be described later (section 4).

3.2 Methods and volunteers.

3.2.1 Location

The study was carried at the Kenya Medical Research Institute, Centre for Geographic Medicine Research (Coast), located in Kilifi, Kenya (KEMRI, CGMR-C). Kilifi is

malaria endemic, with all year round transmission and two high transmission seasons (Mwangi et al. 2005).

3.2.2 Study Design

The study was open label. If safety data for component vaccinations of a regimen were not already available, vaccinations began with half then full doses of the individual component vaccines. These results were reviewed by safety monitors before the complete regimen was used. The safety monitors reviewed all serious adverse events, and their approval was required before further subjects were immunised. Two Safety Monitors worked in Kilifi, the third in western Kenya. Ethical approval was obtained from the Kenyan Medical Research Institute National Ethics Committee, and the Oxford University Tropical Research Ethics Committee. Research was conducted in accordance with the Helsinki Declaration of 1975 (revised 1983).

3.2.3 Volunteers

The adults worked on local sisal and dairy plantations, were male (to avoid unsuspected pregnancy) and aged 18-45 years. Children were from the families of workers, aged 1-6 years and of both sexes. After public meetings and detailed individual discussions, consent was sought and a screening date suggested. Subjects were immunized no sooner than one week after signing consent.

Volunteers were screened by history, examination and blood tests (full blood count, creatinine, alanine transaminase, malaria slide and HIV serology). Subjects with clinically significant illness were excluded. Subjects with haemoglobin below 100 g/l (adults) or 80 g/l (children) were also excluded. Subjects with parasitaemia were treated

with Sulfadoxine-Pyrimethamine before immunisation. Determine[TR] HIV rapid tests were used, confirmed by the Serozyme[TR] ELISA. Pre-test HIV counselling was offered. Individuals testing positive were referred to an HIV clinic and offered vaccination at a later date, excluding those with stage 3 disease. Pre-vaccination viral loads ranged from 600 to 500,000 per ml, and CD4 counts from 1 to 644×10^3 per ml. Only HIV negative children were immunised.

3.2.4 Vaccines

The antigen inserts used were TRAP (joined to a multiple epitope (ME) string from six *P. falciparum* pre-erythrocytic antigens (Gilbert et al. 1997)) and CS. The ME string contains 14 pre-erythrocytic MHC class I epitopes, three class II epitopes, two pre-erythrocytic B cell epitopes and pb9 (a *Plasmodium berghei* T-cell epitope that allows pre-clinical potency and stability testing). CS is coupled to the *P. falciparum* class I epitope ls6 (from Liver Stage Antigen 1) and pb9.

The vectors used were an attenuated fowlpox virus, FP9, and modified vaccinia virus Ankara. Recombinant vaccine stock was supplied to contract manufacturer IDT (Rosslau, Germany), who produced clinical lots under GMP conditions.

Two different batches of FP9 ME-TRAP were used. Batch 020602 was used for 33 adult immunisations, and 040803 for 18 adult immunisations and all the children. Single batches of FP9 CS (010703), MVA CS (020703) and MVA ME-TRAP (031099) were used.

Vaccines were stored at -80°C, and transported in cool boxes for use within 6 hours. Vaccines were given intradermally over the deltoid area of the non-dominant arm, using a 27 gauge needle to raise a visible intradermal bleb. Volunteers were observed for 1 hour with advanced resuscitation facilities available. Children received vitamin A supplements, as per Government of Kenya guidelines. The immunization regimes used are shown in Table 3.1.

Table 3.1. Vaccination Regimens used in Phase 1 trials

Regimen	Insert	Number of volunteers
HIV negative adults		
FFM	METRAP	10
FM	METRAP	13
FMF	METRAP	5
MFM	METRAP	5
M	METRAP	3
F	CS	3
F	CS	3
M	CS	3
M	CS	3
FFM	CS	6
FM	CS	6
F_M	CS	6
HIV positive adults		
F	METRAP	2
M	METRAP	2
F	METRAP	1
M	METRAP	2
Children		
fm	METRAP	2
FM	METRAP	2
ffm	METRAP	6
ffM	METRAP	6

The following abbreviations are used:

f   half dose FP9 immunisation, i.e. 5×10^7 plaque forming units (pfu) for FP9 ME-TRAP and FP9 CS

m   half dose MVA immunisation, i.e. 7.5×10^7 pfu for MVA ME-TRAP or 5×10^7 pfu for MVA CS

F   full dose FP9 immunisation, i.e. 1×10^8 pfu FP9 ME-TRAP and FP9 CS

M   full dose MVA immunisation, i.e. 1.5×10^8 pfu MVA ME-TRAP or 1×10^8 pfu for MVA CS

These abbreviations are then used to describe the sequence of vaccinations in each regimen, so that ffM refers to 2 half doses of FP9 (given sequentially) followed by a full dose of MVA. Vaccination intervals were 3 weeks for ME-TRAP insert using regimes, 4 weeks for those using CS. The underscore (_) refers to an 8 week interval between immunisations.

3.2.5 *Assessment of safety.*

The same two field workers made observations throughout the study. They saw subjects daily for three days after vaccination, supervised by a medically qualified investigator for the first 50 visits. Unsupervised observations were checked by digital photographs taken in the field. The investigator also saw subjects at one week and 2-3 months after vaccination. Solicited adverse events were recorded, and the diameters of skin discolouration, and blistering measured. Loss of the epidermis or upper part of the dermis was described as a deroofed blister. Unplanned assessments could be requested, and further weekly visits were made until resolution of persistent side effects.

Symptoms were graded according to the impact on activities of daily living, i.e. "mild" if without impact, "moderate" if an activity could only be completed with difficulty, and "severe" if an activity of daily living was prevented. Events were considered serious if they were life threatening, caused serious disability, or required hospital admission.

Repeat blood tests were taken 7 days after every immunisation, and 2 months and 9 months after the final immunisation. For HIV positive subjects, viral loads and CD4 counts were measured before, 1 week after and 4 weeks after immunisation.

3.2.6 Electron Microscopy of vaccine

Samples of FP ME-TRAP batches 020602 and 040803 were examined by transmission electron microscopy after negative staining. Formvar carbon grids were glow discharged for 30 seconds before applying the vaccine diluted 1:50 in Tris buffer (0.1M, pH6.8). They were then stained with 2% phosphotungstic acid pH 7.0 and examined with an FEI F30 microscope at 300kV.

3.2.7 Statistical analysis.

Frequencies of side effects were compared using Fishers exact test. Blistering was described by frequency, and the mean diameter among those with blistering. The diameter contributed by each subject was the maximum recorded during the 7 days follow up. A non parametric test (Kruskal-Wallis) was used to compare diameters. Log transformed ELISpot data were analysed by regression models.

3.3 Results

Eighty nine adults and 23 children were screened. Five adults (with active pulmonary tuberculosis, chronic renal failure, orchitis and two with anaemia) and one child (relapsing nephrotic syndrome) were excluded. Sixty six HIV negative adults were randomly selected and offered immunisation. Six withdrew consent, and were replaced. Of the ten HIV positive adults, 7 opted for immunisation. All 22 children continued with vaccination, and all vaccinees completed the immunisation regimes scheduled.

3.3.1 Serious adverse events (SAE)

An HIV positive volunteer was immunized with FP9 ME-TRAP, and admitted to hospital 22 days later with a swollen, painful left leg. Doppler Ultrasound scanning confirmed ileo-femoral deep venous thrombosis. He recovered after treatment with intravenous heparin and warfarin.

A 4 year old girl developed fever 10 days after immunization with MVA ME-TRAP, and was admitted with acidosis and a 24% *Plasmodium falciparum* parasitaemia. There were no unusual features of the illness, and she was discharged after 5 days. There were no other severe adverse events.

3.3.2 Moderate adverse events

One adult experienced a headache lasting 3 days after being immunised with MVA ME-TRAP, and had difficulty working. One child developed persistent vomiting 2 days after receiving fowlpox ME-TRAP. A heavy trichuris infection was concurrently identified and treated, and the vomiting resolved after 4 days.

3.3.3 Laboratory safety analysis

There were no clinically significant abnormalities in full blood count or biochemistry during the trial. In HIV positive subjects, the median viral load was 1.1×10^5 RNA copies per ml before vaccination (95% CI 1.0-3.8×10^5), 1.4×10^5 RNA copies one week after (95% CI 0.7-5.8×10^5) and 1.8×10^5 (CI 0.6-6.3×10^5) at one month. Mean CD4 counts were 231×10^3 per ml (95% CI 60-400) before, 194×10^3 per ml (CI 56-330) at one week and 127×10^3 per ml (CI 50-320) at one month. Viral load and CD4 count did not change significantly over time ($p=0.2$ to 0.5 by signed rank test for the various possible comparisons).

3.3.4 Mild adverse events: Local reactions

All vaccinations caused local skin discolouration. Erythema and slight cutaneous swelling began within 24 hours, and faded to leave a slightly hyperpigmented area after 4-7 days. In some volunteers, blistering began on day 2 or 3 and deroofed blisters were seen on day 3 or 4 (median duration of 2 days for both). Deroofed blisters were shallow, with sloping edges, and deroofed and intact blisters were strongly associated ($p<0.0005$, Fisher's exact). The presence of either intact or deroofed blisters was not significantly associated with pain or itching ($p=0.2$, 0.3, 0.1 and 0.26). Hypertrophic scars or keloid reactions were not observed.

The largest deroofed blister was 1cm diameter, and lasted 4 days (occurring after MVA CS, without a priming immunisation), but was not painful. Most of these lesions healed rapidly. Seventeen deroofed blisters lasted one day only, and 16 healed within five days, but two deroofed blisters persisted for 14 days. All local side effects were graded

mild. Thirty four subjects experienced pain for one day, 15 subjects for two days and 3 subjects for 3 days.

3.3.5 Mild adverse events: Systemic reactions

Febrile symptoms were frequent (12% of adults, 21% of children), but a measured temperature above 37.5°C was less frequent (2.5% and 10%, respectively). The highest recorded temperature was 37.8°C in adults, and 38.1°C in children. 15% of adults experienced a headache of 1 days duration, 5% had a headache of 2 or 3 days duration. 2 adults had mild headaches lasting one week (after FP9 CS and FP9 ME-TRAP). Gastrointestinal symptoms were rare in all groups (5.6%), and limitation of arm movement for one day occurred on three occasions only.

3.3.6 Variations in reactogenicity by subject group and vaccine.

Mild adverse events varied by subject group and vaccine. The different vector antigen combinations caused similar systemic symptoms, but significantly different local reactions (Table 3.2). There was more widespread discolouration after MVA ME-TRAP (p=0.0015), but MVA CS caused more deroofed (p=0.025) and intact (p=0.052) blisters.

Table 3.2. The distribution of mild adverse events by subject group and vaccination given.

	Adults				Children		HIV+ adult
Vaccine	F CS	F TRAP	M CS	M TRAP	F TRAP	M TRAP	F/M TRAP
N	30	51	24	45	40	22	7
Temp.	0 (0%)	2 (3%)	0 (0%)	2 (4%)	1 (2%)	5 (22%)	0 (0%)
Fever	3 (10%)	10 (19%)	1 (4%)	4 (8%)	9 (22%)	4 (18%)	0 (0%)
Vomiting	0 (0%)	5 (9%)	2 (8%)	2 (4%)	4 (10%)	0 (0%)	1 (14%)
Head-ache	6 (20%)	20 (39%)	2 (8%)	12 (26%)	-	-	1 (14%)
Pain	13 (43%)	17 (33%)	5 (20%)	17 (37%)	10 (25%)	5 (22%)	0 (0%)
Itch	10 (33%)	26 (50%)	10 (41%)	20 (44%)	4 (10%)	2 (9%)	3 (42%)
Disc. Diameter	0.65	0.67	0.7	0.88	0.36	0.85	0.63
95% CI for Disc.	0.57-0.73	0.62-0.73	0.60-0.82	0.78-1.00	0.29-0.44	0.38-1.31	0.44-0.90
Intact blister (freq)	8 (26%)	9 (17%)	4 (16%)	2 (4%)	8 (20%)	5 (22%)	1 (14%)
Intact blister diameter	0.25	0.21	0.3	0.2	0.23	0.24	0.2
Deroofed blister (freq)	7 (23%)	9 (17%)	6 (25%)	1 (2%)	9 (22%)	3 (13%)	0 (0%)
Deroofed blister diameter	0.25	0.36	0.3	0.2	0.27	0.26	0
Limited arm motion	1 (3%)	1 (1%)	1 (4%)	0 (0%)	0%	0%	0 (0%)

Table footnote: Each vaccination is considered separately; N refers to the numbers of vaccinations within each category. F CS refers to FP9 encoding CS, M CS to MVA encoding CS, F TRAP to FP9 encoding ME-TRAP and M TRAP to MVA encoding ME-TRAP.

Temp. refers to the number of subjects with a temperature measured above 37.5 degrees centigrade when assessed. High temperatures were only recorded the day after immunisation. Fever refers to the percentage subjectively reporting febrile symptoms, but not necessarily with a recorded temperature on measurement. Numbers of pain or itching

refer to the immunisation site only. Disc diameter refers to the mean diameter of discolouration. Blister diameter describes the mean diameter among those that had blisters. Headache is not recorded for children.

3.3.7 Subject group

Side effect profiles in adults and children were similar, except that children more frequently had a measured fever (p=0.008, only after MVA), less itching (p=0.004) and less skin discolouration (p=0.0001). There were no differences in local or systemic reactogenicity between HIV infected and uninfected subjects, excepting a trend for less frequent pain (p=0.047).

3.3.8 Sequence of immunisation

When MVA was given without prior FP9 immunisation, there was more widespread discolouration (p=0.03), intact blisters (p=0.015) and deroofed blisters (p=0.04). MVA without prior FP9 immunisation caused a 1 cm diameter deroofed blister in one subject. The largest such blister following MVA primed by FP9 was 0.3cm. These deroofed blisters healed after 1 or 2 days, but those following MVA alone persisted for 14 days in two subjects. Similarly, when FP9 immunisation was given without prior FP9 or MVA discolouration was greater (p=0.018), although other differences were marginal (p=0.04 for more frequent pain after F alone, p=0.087 for deroofed blisters) or of little significance (p=0.24 for itch).

Systemic effects were independent of sequence of immunisation. FP9 after MVA caused more frequent headache than F after F or F alone (p=0.024), but this was not corroborated by other systemic symptoms. In children, fever was less commonly reported

after the second fowlpox immunisation than after the first (p=0.02). Full details of reactogenicity by sequence of vaccination are given in table 3.3.

Table 3.3. The distribution of mild adverse events by sequence of vaccination.

	Adults					Children	
Sequence of vectors	F	FF	FM	M	MF	F	FF
N	53	16	10	18	38	22	18
Temp.	1 (1%)	1 (6%)	0 (0%)	0 (0%)	1 (2%)	1 (4%)	0 (0%)
Fever	8 (15%)	2 (12%)	3 (30%)	2 (11%)	2 (5%)	8 (36%)	1 (5%)
Vomiting	4 (7%)	0 (0%)	1 (10%)	1 (5%)	2 (5%)	3 (13%)	1 (5%)
Headache	15 (28%)	4 (25%)	7 (70%)	5 (27%)	6 (15%)	0 (0%)	0 (0%)
Pain	25 (47%)	4 (25%)	1 (10%)	9 (50%)	5 (13%)	8 (36%)	2 (11%)
Itch	25 (47%)	4 (25%)	3 (30%)	11 (61%)	13 (34%)	4 (18%)	0 (0%)
Disc. Diameter	0.7	0.58	0.63	0.99	0.75	0.37	0.31
95% CI for Disc.	0.64 -0.77	0.50 -0.65	0.51 -0.79	0.79 -1.26	0.66 -0.86	0.27 -0.45	0.24 -0.40
Intact Blister (frequency)	12 (22%)	5 (31%)	0 (0%)	4 (22%)	1 (2%)	3 (13%)	5 (27%)
Intact Blister Diameter	0.24	0.24	-	0.3	0.2	0.18	0.26
Deroofed Blister (frequency)	14 (26%)	1 (6%)	1 (10%)	4 (22%)	2 (5%)	2 (9%)	7 (38%)
Deroofed Blister Diameter	0.4	0.2	0.3	0.4	0.2	0.3	0.26
Limited arm motion	0%	0%	0%	0%	0%	0%	0%

Table 3.3 Footnote: Sequence is described left to right; F indicates the first FP9 vaccination received by a volunteer, and FF refers to the second FP9 vaccination. FM indicates the safety profile of an MVA vaccination, given after Fowlpox. Abbreviations for adverse event descriptions are as for table 3.2

3.3.9 Adverse events varied by dose of vaccine.

Since vaccination sequence modified reactions, comparisons of adverse events by dose were restricted to the first vaccination. Systemic symptoms were reduced at half doses in adults (p=0.04 for headache, p=0.18 for fever), but local reactions were similar.

In children, high dose MVA METRAP was followed by a temperature >37.5 degrees in 5 of 14 children the next day, but none of 8 children after half dose (p=0.076). Skin discolouration was greater after high dose compared to low dose (p=0.004 for FP9, p=0.06 for MVA), but the frequency and extent of blisters was similar. Full details of adverse events according to vaccine dose are given in Table 3.4.

Table 3.4. The distribution of mild adverse events by subject group and dose.

	Children				Adults			
	f	F	m	M	F	F	M	M
N	14	8	8	14	5	50	5	8
Temp.	1 (7%)	0 (0%)	0 (0%)	5 (35%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)
Fever	4 (28%)	4 (50%)	2 (25%)	2 (14%)	0 (0%)	8 (16%)	0 (0%)	1 (12%)
Vomiting	2 (14%)	1 (12%)	0 (0%)	0 (0%)	0 (0%)	4 (8%)	1 (20%)	0 (0%)
Headache	-	-	-	-	0 (0%)	15 (30%)	0 (0%)	3 (37%)
Pain	7 (50%)	1 (12%)	2 (25%)	3 (21%)	0 (0%)	25 (50%)	2 (40%)	4 (50%)
Itch	3 (21%)	1 (12%)	2 (25%)	0 (0%)	3 (60%)	25 (50%)	3 (60%)	4 (50%)
Disc Diameter	0.24	0.53	0.39	0.7	0.68	0.74	1	1.23
95% CI for Disc.	0.17 -0.32	0.32 -0.85	0.23 -0.66	0.43 -1.14	0.3 -1.0	0.67 -0.81	0.5 -1.4	0.5 -2.1
Intact Blister (frequency)	3 (21%)	0 (0%)	1 (12%)	4 (28%)	0 (0%)	12 (24%)	1 (20%)	3 (37%)
Intact Blister diameter	0.2	-	0.3	0.225	-	0.24	0.4	0.27
Deroofed Blister (frequency)	2 (14%)	0 (0%)	2 (25%)	1 (7%)	0 (0%)	14 (28%)	2 (40%)	2 (25%)
Deroofed Blister diameter	0.2	-	0.3	0.2	-	0.2	0.2	0.15

Table 3.4 Footnote: f refers to half dose FP9 vaccinations, F to full dose. Similarly, m refers to half dose MVA vaccination, M to full dose. Abbreviations for adverse event descriptions are as for table 3.2.

3.3.10 Batches of FP9 ME-TRAP had different reactogenicity

Batch 020602 of FP9 ME-TRAP caused less discolouration (p=0.0078), intact blisters (p=0.036) and deroofed blisters (p=0.0019) than batch 040803. By transmission electron microscopy, both batches contained discrete viral particles and smaller fragments

of membrane, but there were no clear differences between batches. Full details of reactogenicity according to batch are shown in Table 3.5.

Table 3.5. The distribution of mild adverse events by batch of FP9 ME-TRAP (data from adults only; 020602 not used in children).

Batch	040803	020602
N	18	33
Temp	0 (0%)	2 (6%)
Fever	3 (16%)	7 (21%)
Vomiting	1 (5%)	4 (12%)
Headache	8 (44%)	12 (36%)
Pain	4 (22%)	13 (39%)
Itch	6 (33%)	20 (60%)
Disc Diameter	0.8	0.61
95% CI for Disc.	0.66-0.95	0.56-0.66
Blister (frequency)	6 (33%)	3 (9%)
Blister diameter	0.22	0.23
Deroofed Blister (frequency)	7 (38%)	2 (6%)
Deroofed Blister diameter	0.58	0.1

Table 3.5 footnote. Abbreviations for adverse event descriptions are as for table 3.2.

3.3.11 Pre-vaccination immunity to the antigen insert reduced local reactogenicity.

There was no association between subsequent immune response to the vector or insert and local cutaneous reactions (immunogenicity will be described in full elsewhere). However, the pre-vaccination response to the insert antigen was associated with less blistering (Figure 3.1). The geometric mean response was 30 (95%CI 23-39) interferon gamma producing cells per million PBMC among subjects who went on to develop blisters, compared with 17 (95%CI 11-25) among those who did not develop blisters (p=0.022 by t-test). There was no difference among responses to the vector prior to immunisation.

Figure 3.1. Individual pre-vaccination responses to the TRAP antigen, by sequence of immunisation and presence/absence of blistering. On the category axis, subjects are divided first into immunisation category, then into "-ve" or "+ve", i.e. negative or positive for blistering. The y axis plots the summed T cell response to the antigen insert, TRAP.
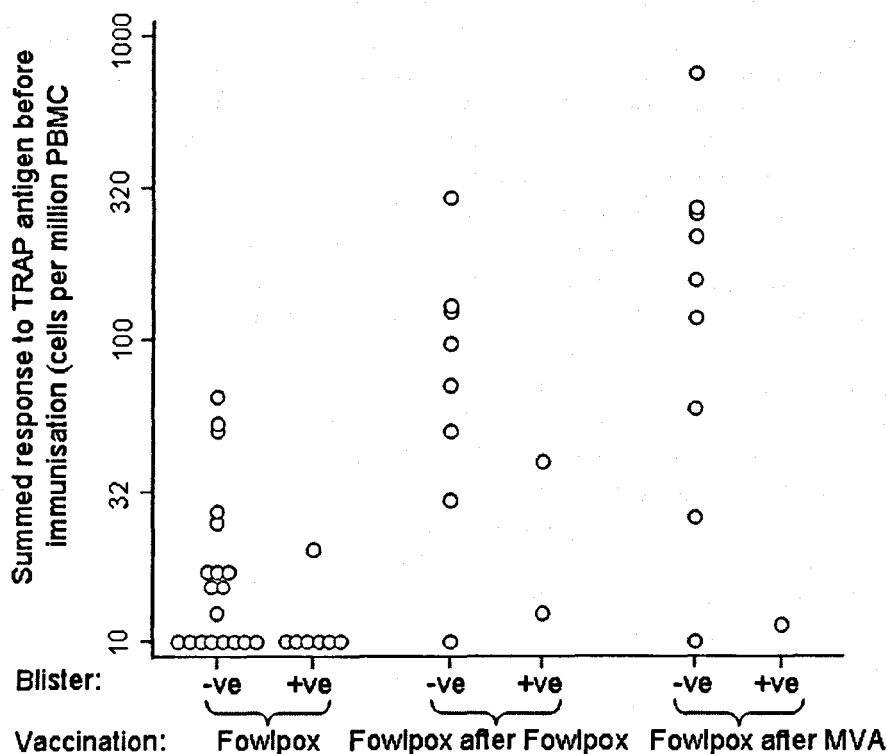
The difference in insert response was seen only in ME-TRAP vaccinees (p=0.0052) rather than CS vaccinees (p=0.4). When using a regression model to adjust for the sequence of immunisation (a possible confounder) the response was found to be 2.3 times greater among those without blistering (p=0.018). Detailed results on immunogenicity are presented in section 4.3.

Local adverse events tended to recur in individual subjects.

73 subjects were immunised more than once. Table 3.6 describes the frequency with which adverse events recurred. Systemic symptoms were randomly distributed, but if blistering occurred after the first immunisation, it was more likely to recur after the second. (p=0.01 for intact blisters and 0.005 for deroofed blisters)

Table 3.6: The frequency of adverse events by occurrence following a single immunisation, or with subsequent immunisations.

	Comparing first and second immunisations (n=73)			Comparing second and third immunisations (n=44)		
	Freq on first vaccine only	Freq on second vaccine only	Freq on both vaccines	Freq on second vaccine only	Freq on second vaccine only	Freq on both vaccines
Temp.	2	2	1	2	1	0
Headache	13	7	6	7	6	3
Vomiting	4	3	1	4	2	0
Fever	11	7	3	7	5	0
Deroofed Blister (Freq)	14	2	5*	5	4	0
Intact Blister (Freq)	11	2	5*	2	8	1
Itching	15	12	11	7	5	6
Pain	21	10	9	9	9	1

Table 3.6 Footnote: Temp. refers to the number of subjects with a temperature measured above 37.5 degrees centigrade when assessed. Fever refers to the number subjectively reporting febrile symptoms, but not necessarily with a recorded temperature on measurement. Pain or itching refers to the immunisation site only. The frequencies of subjects with blistering are given. *indicates p<0.05 for a greater than expected frequency of subjects experiencing a reaction to both vaccinations.

3.4 Discussion

There was mild subjective discomfort for the majority of vaccinees, but no serious reactogenicity. There was no plausible link between the two serious adverse events and immunisation, and the safety monitors considered these events unrelated to vaccination. Both were isolated events, and not unusual in the respective subject groups. Deep venous thrombosis is associated with HIV infection (Saber et al. 2001).

At this preliminary stage, FP9 ME-TRAP and MVA ME-TRAP appear safe in adults with HIV and there was no evidence that viral load or CD4 count was adversely affected by vaccination. This is critical, since in many populations large scale HIV testing prior to immunisations will be problematic, and a transient rise in viral load and fall in CD4 count has been seen after pneumococcal and influenza vaccines (Brichacek et al. 1996; Staprans et al. 1995). There was no evidence that adverse events were more frequent or more severe in individuals with lower CD4 counts and higher viral load, although there were few adverse events recorded in this group. Compared with adults, children showed only minor differences in local skin discolouration. There was some evidence of more frequent objective fever but less frequent symptomatic fever.

MVA CS had a different side effect profile to MVA ME-TRAP, causing less extensive skin discolouration, but more frequent blistering. MVA CS was higher titre (1×10^9 pfu/ml compared to 3×10^8 pfu/ml for MVA ME-TRAP). Blistering had a smaller average diameter (0.34 cm) than the discolouration (0.72 cm). Virus given in a larger volume might disperse more following immunisation, causing more extensive skin discolouration, but resulting in less concentrated virus, so causing less frequent blistering.

3.4.1 Dose and sequence

Systemic and local reactogenicity was less at lower doses, although few adult subjects were immunized at lower doses. However, the local reactogenicity of MVA was markedly lower if FP9 was given first. Therefore, where the intention is to use MVA as a boosting agent after FP9 in the final regimen, single dose immunisations should not be assessed first. The dose titration should start with the complete regimen at half dose, then move to full dose if reactogenicity is acceptable. Such an approach was taken for paediatric vaccinations here.

There was evidence of variable subject susceptibility to cutaneous reactions. Those with blistering after the first immunisation were likely to experience a similar reaction for the second. These host factors might differ by population. If so, this would support conducting phase I studies in new populations before larger scale trials.

3.4.2 Batch

Unexpectedly, two batches of the same vaccine (FP9 ME-TRAP) had different local reactogenicity. Batches are prepared from the same master seed lot under GMP conditions, and GMP sterility tests, repeated potency tests and titrations were performed. The FP9 supplied for the original recombination to generate FP9 ME-TRAP was clonal, having been plaque purified. Differential gene loss during the short growth required to "bulk up" virus during manufacture seems unlikely. The batches were of different titre (4×10^8 pfu per ml for 040803, compared with 1×10^8 pfu per ml for 020602). As for the comparison between MVA CS and MVA ME-TRAP, the more concentrated vaccine caused more frequent blistering. However, the less reactogenic FP9 ME-TRAP batch (020602) was also more immunogenic (results presented elsewhere), and this suggests a

more fundamental difference. Since the two batches looked similar by electron microscopy, the different reactogenicity was not due to differences in chick fibroblast debris or viral particle aggregation.

3.4.3 Immune response

After BCG local cutaneous reactions have been associated with immunogenicity (Roth et al. 2005), but this was not the case here. However, prior immunity to the malaria antigen insert protected against blistering (p=0.0052). This was only seen among the sub-group receiving FP9 ME-TRAP, probably because CS containing vaccines were less immunogenic (see 4.3.1), and blistering was rare after MVA ME-TRAP. The effect was robust to adjustment for sequence (p=0.018, 2.3 fold difference, Figure 3.1). Following natural exposure, TRAP peptides induce interleukin 10 (Flanagan et al. 2006), and MVA induces T regulatory responses (Fletcher et al, submitted). It is thus possible that TRAP specific responses down regulate local inflammation following vaccination.

In conclusion, the vaccine regimes employed here were safe, the data presented details the determinants of reactogenicity and informs the conduct of future Phase I studies.

4 Phase 1 in Kenya: Immunogenicity.

4.1 Background

The direct, or *ex vivo* ELISpot identifies T cells that produce IFN-gamma after overnight incubation with antigen. The cultured ELISpot assay uses a 10 day pre culture with first antigen then interleukin 2, in order to identify resting memory cells (Godkin et al. 2002). The cell populations identified by these two assays are different. A study of naturally acquired immunity to CS protein demonstrated no correlation between cultured ELISpot and *ex vivo* ELISpot results (Flanagan et al. 2001). The cells identified by cultured ELISpot persist for at least six months after vaccination of naïve subjects, despite waning of cells detected by *ex vivo* ELISpot (Keating et al. 2005). The cells detected by cultured ELISpot are here referred to as resting memory cells.

Mean effector T cell responses among vaccination groups predicted protection from sporozoite challenge (Webster et al. 2005), but did not predict the inter-individual variation in protection seen within vaccination groups. However, cultured ELISpot results predicted inter-individual variability in sporozoite challenge (Keating et al. 2005), and cultured ELISpot assays using a particular peptide predicted protection against both parasitaemia and febrile malaria in a field study following vaccination with RTS,S (Reece et al. 2004). In contrast to the RTS,S field study, the cultured ELISpot data from sporozoite challenge studies were analysed quantitatively (Keating et al. 2005), as are the data presented here.

I conducted ELISpots using pools of up to 17 peptides, so as to cover the full length of the antigen used for vaccinations, even where limited cells were available. Other studies have used individual epitopes (Godkin et al. 2002). However, my aim was to determine vaccine immunogenicity rather than track the kinetics of individual epitope specific responses. Protection is most likely to be related to overall immunogenicity (McConkey et al. 2003; Webster et al. 2005). Since the critical interaction is between reactive T cells and infected hepatocytes rather than peptides in solution, protection is likely to be a function of the total number of potentially protective T cells in circulation, rather than of individual epitope responses. I have therefore presented results by summing responses to the whole antigen tested, except where strain cross-reactivity for specific pools of variant peptides is examined for.

Vaccines with preliminary evidence of efficacy require immunogenicity data from adults in endemic areas before moving to immunise children. This offers a further opportunity to optimise the regimen for immunogenicity and study the acquisition of immunity. I compare prime boost regimens, single dose vaccinations and novel alternating vector regimes in a malaria endemic area, and compare CS and ME-TRAP inserts.

4.2   Methods.

The study design, subject groups and vaccination regimens have been described in section 3.2. Table 3.1 gives the numbers of subjects immunised with each vaccination regimen. Comparisons are made between the time course of responses following alternating vector vaccinations and prime boost vaccinations. Of the 23 subjects given

prime boost vaccinations, 19 were immunised 12 months before the alternating vector vaccinations, and 4 concurrently.

4.2.1 ELISpots

PBMC incubations were in RPMI (Sigma-Aldrich) with 10% Human AB serum. ELISpots used Millipore MAIP S45 plates and MabTech antibodies according to manufacturer's instructions. 4×10^5 per well of freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated in 100μl with 10 μg/ml peptides for 18-20 hours before developing. Individual 8 to 17 residue epitopes were pooled for the ME string. 20 residue peptides overlapping by 10 residues were used for TRAP. TRAP peptides were pooled first according to whether they were T996 variant specific (the vaccine strain), 3D7 specific or synonymous, and then according to region. CS was represented by 15 residue peptides, overlapping by 10 residues. The TH2R and TH3R polymorphic regions were pooled separately for 3D7 (vaccine strain) and 7G8 derived peptides. 20 μg/ml Phytohaemaglutinin (Sigma-Aldrich) was used as positive control, and PBMC cultured in media alone as negative control. Spot forming cell numbers were counted by ELISPOT plate reader (Autoimmun Diagnostika, version 3.0).

For cultured ELISPOTs, 1×10^6 PBMC were incubated in 0.5mls of 10μg/ml/peptide of pooled TRAP or CS peptides in a 24-well plate. On days 3 and 7, 250μl of culture supernatant was replaced with 250μl culture medium containing 20 IU/ml recombinant IL 2. On day 9 the cells were washed three times and left overnight before the standard ELISpot assay.

4.2.2 Analysis.

ELISpot wells were assayed in duplicate. The mean was taken, and the negative control well result subtracted from each peptide well. Assays were considered failed if the positive control well was less than 50 spots, or the negative control greater than 20 spots for *ex vivo* ELISpots, or greater than 40 spots for cultured ELISpots. Pools were summed to calculate total responses. Results are then presented as number of spots per million incubated PBMC throughout.

Data were log transformed to normalize distributions. Geometric means with 95% confidence intervals (by Stata 8™, Stata coorp) are used to describe groups. To avoid multiple comparisons, ANOVA was performed to test for heterogeneity of results. Where ANOVA results showed $p < 0.05$, factorized linear regression was used to identify significantly different groups.

In examining for either cross reactivity between different effector responses, or correlations between memory and effector responses, results were paired according to the region of CS/TRAP and volunteer. Each individual contributes 3 points, leading to an overestimate of significance. The cluster subcommand of Stata 8™ (Stata coorp) was therefore used to adjust p values upwards.

4.3   Results

The peak effector responses seen on *ex vivo* ELISpot were compared, using samples taken one week after each vaccination.

4.3.1 ME-TRAP was more immunogenic than CS

Single vaccination with FP9 ME-TRAP raised T cell responses significantly above baseline (p<0.0005), to a geometric mean of 57 spots (95% CI 28-112) from a mean of 8 spots at baseline (95% CI 4-14). FP9 CS alone was not significantly immunogenic. Geometric mean responses after FP9 CS were 40 spots per million (95% CI 15-100) compared to 17 spots per million (95% CI 7-48) at baseline, p=0.11.

However, for regimes encoding both ME-TRAP and CS, the "boosting" immunisation with MVA expanded T cell responses further. Geometric mean responses to ME-TRAP were 185 spots per million PBMC after FM, and 389 spots after FFM. Responses to CS were 72 spots per million after vaccination with FM and 204 spots per million after FFM. The expansion was significant for FFM ME-TRAP (p<0.005), FM ME-TRAP (p<0.0005) and FFM CS (p=0.014) but not FM CS (p=0.2).

Thus, for each vector combination, regimens encoding ME-TRAP were of greater immunogenicity than regimens encoding CS. An estimate of overall significance was made using a linear model to compare CS encoding regimens with ME-TRAP regimens, adjusting for the sequence of vectors. Immunogenicity was higher for ME-TRAP (p=0.001), and further study focused on ME-TRAP regimens. Median responses for each regimen are plotted in Figure 4.1.

Figure 4.1. Peak effector (*ex vivo* ELISpot) responses.

Results are shown for *ex vivo* ELISpot assays taken one week after the vaccination regimen displayed on the category axis. Spot numbers per million PBMC are plotted along the y axis (log scale). F is used for FP9, and M for MVA. 2 or 3 dose regimes are described by the sequence of vaccinations. For instance, FFM refers to two sequential FP9 vaccinations followed by a single MVA vaccination. Boxes show the distribution of results, the resting line indicating median response, upper and lower box boundaries showing 25th and 75th centiles, and the whiskers show adjacent values. The circles show outlier results. Single vector immunizations were immunogenic for ME-TRAP ($p < 0.0005$) but not for CS. Overall, immunogenicity was higher for ME-TRAP than for CS ($p = 0.001$), and FFM was significantly more immunogenic than both M and FM ($p = 0.018$).
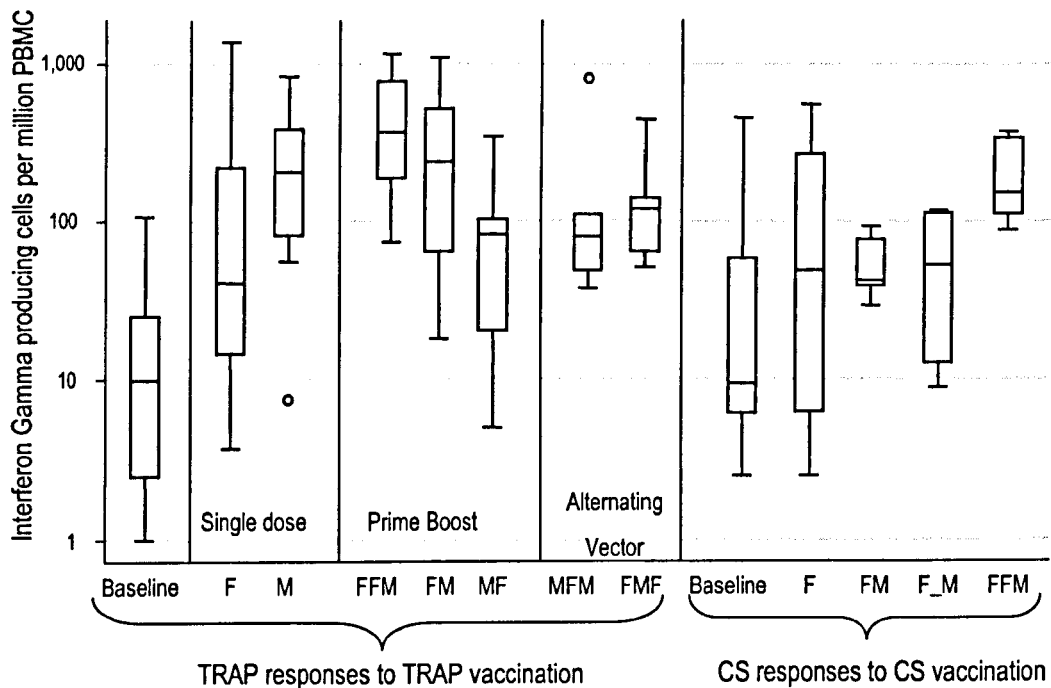
4.3.2 Multiple Priming enhances immunogenicity

The responses following MVA ME-TRAP given without priming (M) were compared with single priming (FM) and double priming (FFM). The geometric mean response after single dose MVA ME-TRAP was 150 spots per million PBMC (95% CI 45-510). This compares with 185 spots, (95% CI 96-360) when previously primed once (i.e. FM ME-TRAP), and 389 spots per million (95% CI 190-800) if primed twice (i.e. FFM ME-TRAP). MVA CS was not used alone, but responses were higher when primed twice (responses after FFM CS were 204 spots, CI 75-325) than when primed once, whether the interval between vaccinations was 4 weeks (responses were 50 spots per million, CI 30-78, after FM CS) or eight weeks (40 spots, CI 12-130, after F_M CS).

A second linear model was then used to compare M, FM and FFM, adjusting for the two different antigen inserts. There was significant variation by ANOVA (i.e. FFM, FM and M raised significantly different responses), p=0.037. By individual analysis, FFM was significantly more immunogenic than both M and FM (p=0.018).

Peak responses following alternating vector vaccinations (MFM or FMF ME-TRAP) were similar to M alone (105 spots per million, 95% CI 23-470 and 110 spots, 95% CI 38-316 respectively).

4.3.3 Alternating vector vaccinations generate more memory then Prime Boost

Whereas the response after prime boost (FFM or FM ME-TRAP) fell to 5% of the peak response after 270 days (fig 4.2), alternating vectors (MFM or FMF) maintained 84% of

the peak response after 270 days. Thus, although the alternating vector responses were significantly lower than prime boost (FFM or FM), p=0.04, at 7 days and similar at 56 days (p=0.3), alternating vector responses were higher (p<0.0005) at 270 days. 4 prime boost vaccinees were immunised concurrently with alternating vector vaccinees. The geometric mean response at 56 days among prime boost vaccinees one year previously was 123 spots per million PBMC (range 0-500). Of the 4 prime boost vaccinees that were concurrent with alternating vector vaccinations, one response was above the mean (295 spots), one response below the mean (18 spots) and two were non-responders (0 spots).

Figure 4.2. Timecourse of effector (ex vivo ELISpot) responses.

The durability of effector cells was measured by *ex vivo* ELISpots on blood samples drawn at 7, 56 and 270 days after the final immunization. This is indicated on the category axis, grouped according to vaccination regimen. Prime Boost describes both FM and FFM immunisations, Alternating vector describes FMF and MFM immunisations. Subjects receiving MVA ME-TRAP immunisation without priming or FP9 ME-TRAP went on to complete full vaccination regimes (MFM or FM/FFM/FMF), and so time course data is not available. The response to prime boost immunization fell to 5% of the peak response at 270 days, but alternating vectors (MFM or FMF) maintained 84% of their peak response.
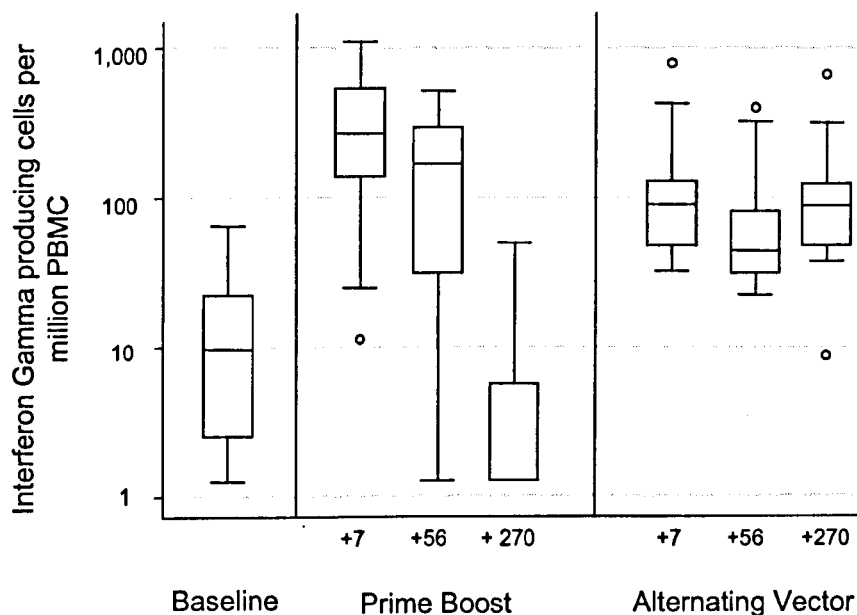
A similar pattern was seen for cultured ELISpot responses, which detect resting memory cells. Prime Boost TRAP encoding regimes provoked an 8 fold rise in geometric mean resting memory cell responses (Fig 4.3, p=0.02), from 120 spots (95% CI 55-255) to 810

spots per million (CI 470-1150), and Prime Boost CS regimes provoked only a 2 fold rise of borderline significance, from 230 spots (95% CI 80-685) to 633 (CI 250-1580), p=0.08. However, the mean resting memory response was 1580 spots (CI 1000-2488) after alternating vectors, considerably higher than for prime boost (FFM or FM) (p<0.0005). The resting memory population generated by prime boost vaccination fell to 33% of the peak over 270 days, but the resting memory generated by alternating vectors did not diminish.

Figure 4.3 Timecourse of resting memory (cultured ELISpot) responses.

The durability of resting memory cells was measured by cultured ELISpot. The resting memory was measured by cultured ELISpot on blood samples drawn 7, 56 and 270 days after the final immunization. This is indicated on the category axis, grouped according to vaccination regimen. Prime Boost describes both FM and FFM immunisations. Alternating vector describes FMF and MFM immunisations. The induction of resting memory cells was significant after prime boost TRAP encoding regimes (p=0.02), but not after prime boost CS regimes (p=0.08). Alternating vector regimes induced more resting memory responses than prime boost (p<0.0005), and the response was sustained.

70
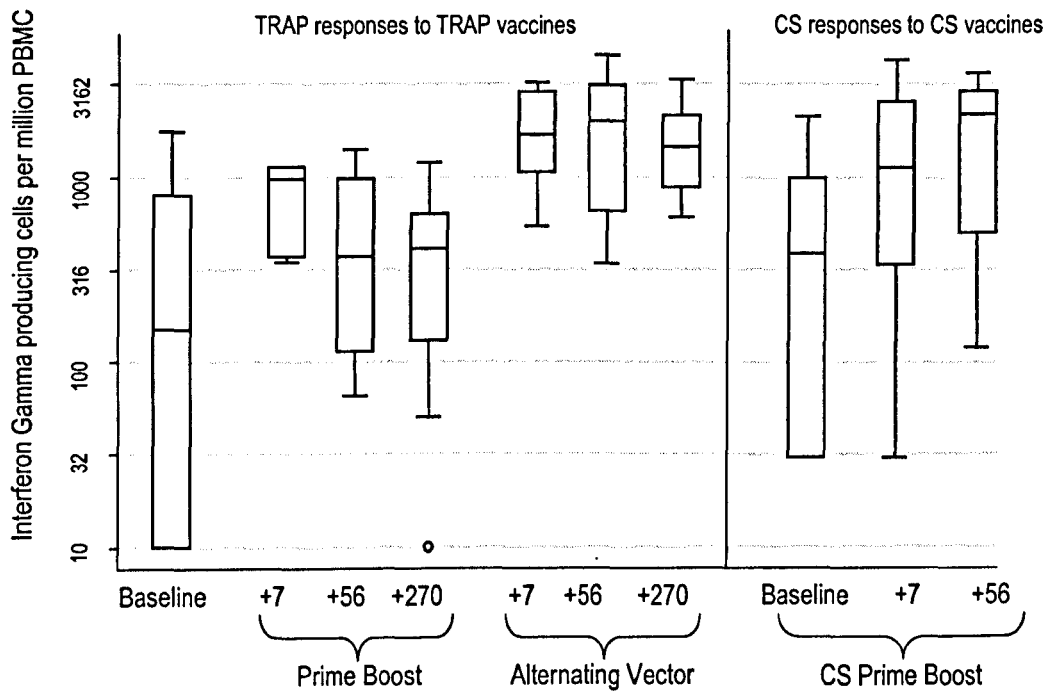
4.3.4 TRAP and CS responses to vaccination are not limited to the vaccination strain

TRAP responses at baseline appeared cross-reactive ($r=0.46$, $p=0.0001$, figure 4.4), and those raised by immunisation were more so ($r=0.75$, $p<0.0005$). However, T cell responses to CS at baseline were reactive to either 3D7 strain or 7G8 strain, but not both (excepting one response out of 14). However, immunisation with either FP9 CS or MVA CS produced cross reactive responses ($r=0.54$, $p=0.001$ or $r=0.75$, $p<0.0005$ respectively). There was a bias towards responses against the vaccine strain after vaccination. Responses to the vaccine strain were 3 fold higher (95% CI 1.5-6 fold) after MVA CS, and 1.3 fold higher (95% CI 1-1.7 fold) after MVA ME-TRAP.

Figure 4.4 Strain cross reactivity.

Results from *ex vivo* ELISpots 7 days after vaccination are plotted. The numbers of cells per million PBMC responding to the vaccine strain are on the x axis, against numbers responding to variant peptides on the y axis. A point in the far right corner of a graph would then represent a high response to both the vaccine strain peptides and the variant peptides, suggesting cross reactivity. Points closer to either axis represent responses specific to either the vaccine strain (if close to the x axis) or specific to variant peptides (close to the y axis). The top three panels demonstrate TRAP responses, the lower three CS responses. For each peptide group, the left panel shows responses prior to vaccination, the central panel after FP9, the right panel responses after MVA. TRAP responses appeared cross-reactive at baseline (r=0.46, p=0.0001) and after immunisation (r=0.75, p<0.0005). Responses to CS at baseline were not cross reactive at baseline (r=0.04), but were after vaccination (r=0.75, p<0.0005).

TRAP variant peptides
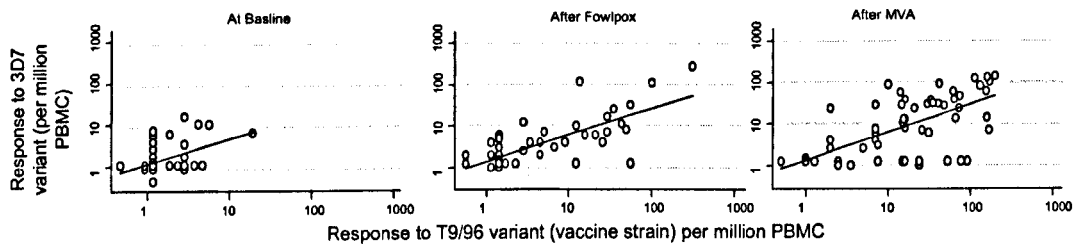
Circumsporozoite: TH2R/TH3R regions
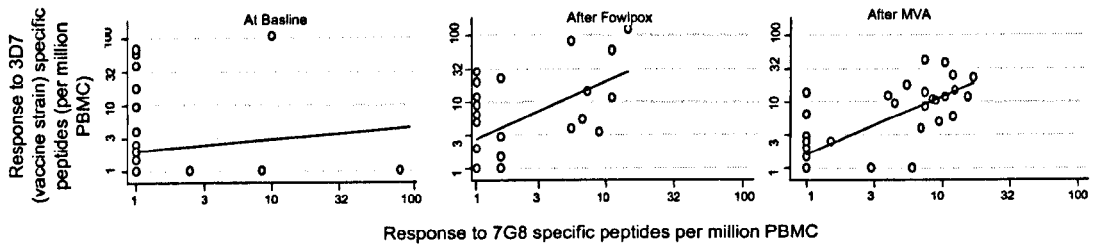
4.3.5 Batches of FP9 ME-TRAP have different immunogenicity (Figure 4.5)

Two different batches of FP9 ME-TRAP were used. In most cases, this did not vary within regimen (i.e. batch 040803 for FFM, batch 020602 for FMF/MFM), but among the FM regimen, eight subjects received batch 040803, and eight 020602. One MVA ME-TRAP batch was used throughout. Batch 020602 FP9 ME-TRAP was immunogenic given alone (p=0.002, 120 spots per million), but responses after batch 040803 were not different to those present at baseline (p=0.45). Similarly, batch 020602 was more effective at priming the subsequent MVA ME-TRAP vaccination. Geometric mean responses were 331 spots per million after batch 020602, compared to 110 after batch 040803, p=0.0054. Responses were also more long lived using batch 020602. Mean responses were 64 spots per million at 56 days, compared to 17 spots with batch 040803 (p=0.037). However, the anti-vector immunity raised by the two FP9 ME-TRAP batches were similar, at 22 spots (CI 13-45) for 020602 and 20 (CI 5-62) for 040803.

Figure 4.5 Comparison of FP9 ME-TRAP batches (*ex vivo* ELISpot).

Subjects who received FM vaccination regimes were divided according to the batch of FP9 ME-TRAP vaccination they received (all received the same MVA ME-TRAP batch). Responses are shown before immunization (baseline) and then 7 days after the first FP9 ME-TRAP vaccination (F+7). 14 days after the FP9 ME-TRAP vaccination, MVA ME-TRAP was given. Then responses after a further 7 days (FM+7) and 56 days (FM+56) are displayed. At each timepoint, responses are split by the batch of FP9 ME-TRAP used in the overall vaccination regimen. Batch 020602 FP9 ME-TRAP was immunogenic given alone (p=0.002), as priming the later MVA ME-TRAP vaccination (p=0.0054), and generated more long-lived responses (p=0.037).
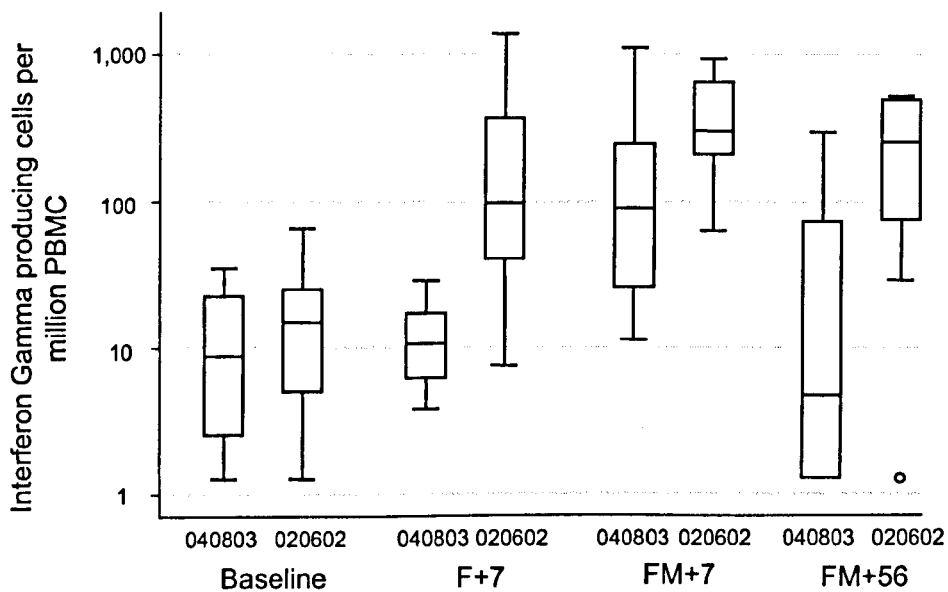
4.3.6 Pre-vaccination memory predicts post-vaccination response for single vector immunisations

The resting memory responses present before vaccination predicted the post vaccination effector response (r=0.21, p=0.021, Figure 4.6). This correlation was entirely due to subjects receiving single immunisations encoding TRAP (i.e. F or M alone). When this group was analysed alone, r=0.49, p=0.0003, but for the other prime boost regimes, r=0.02, p=0.8. This could be better expressed by testing for an interaction between pre-vaccination memory and vaccination regimen in predicted outcome (i.e. effector response to vaccination). A model allowing this interaction between vaccination group (single vaccination or multiple vaccinations) and pre-existing resting memory was significantly better (p=0.011) than a model without interaction. This further confirms that the correlation between prevaccination memory and post vaccination response was specific to one vaccination group (single dose vaccination).

Figure 4.6 Resting Memory responses pre-vaccination against effector responses post-vaccination.

The cultured ELISpot responses found prior to vaccination are plotted at the x axis. The subsequent *ex vivo* ELISpot result 7 days after vaccination is plotted on the y axis. The left graph shows responses following prime boost vaccination regimes. The right graph shows responses following single vaccinations (i.e. either FP9 ME-TRAP or MVA ME-TRAP alone). Prior resting memory responses predicted post vaccination response (r=0.21, p=0.021). For single immunisations encoding TRAP (i.e. F or M alone), r=0.49, p=0.0003, but r=0.02 for prime boost regimes. This suggests that for single vaccinations (but not for prime boost regimes) immunogenicity depends on prior memory responses. Pre-vaccination effector responses were not significantly above background (p=0.17), and so were not analysed here.
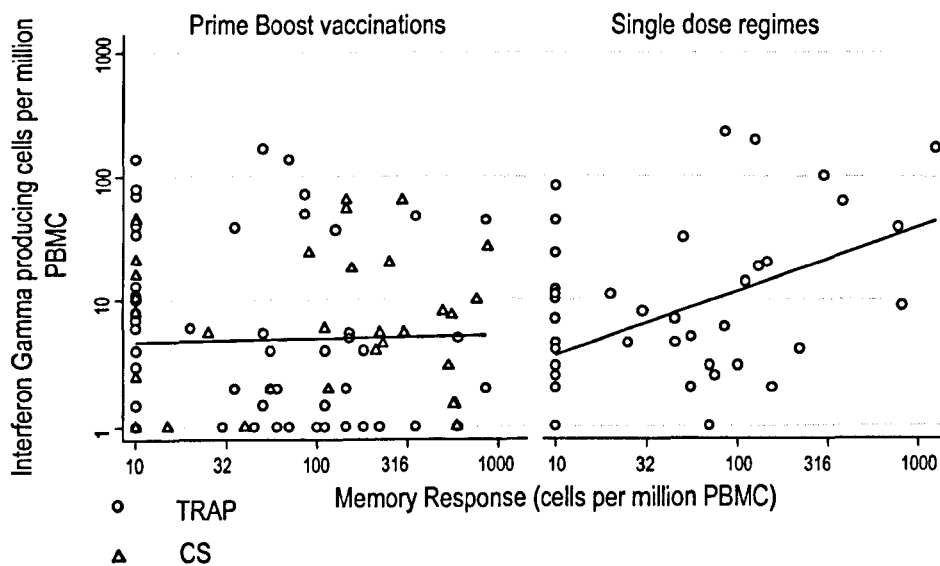
Concurrent ex-vivo effector responses and resting memory cells after vaccination were quite strongly associated (r=0.41, p=0.003), although there was no correlation prior to vaccination (r=-0.12 p=0.56).

4.3.7 Anti Vector Immunity

When Modified Vaccinia Ankara was used to infect PBMC in the ELISpot assay, high levels of reactivity were seen in all samples, whether immunised or not (data not shown). However, non specific reactivity was not seen with wild type vaccinia (WR), and so responses to wild type vaccinia are presented here, since the non-specific reactivity seen with MVA was not considered informative. Responses to vaccinia averaged 7 spots per million at baseline (ranging from 2.5 to 144), but were higher after FP9 ME-TRAP, at 20 spots per million (range 2.5 to 450), p=0.002.

Since there was a wide range of anti-vector specific immunity, and there appeared to be cross-reactivity between FP9 and MVA, the potential for these responses to impair subsequent immunogenicity was compared. The anti vector immunity measured by *ex vivo* ELISpot responses to vaccinia at baseline was plotted against the subsequent response to FP9 ME-TRAP, and the anti vector immunity detected one week after FP9 ME-TRAP was plotted against the subsequent response to MVA ME-TRAP (Figure 4.7).

Figure 4.7. Pre vaccination anti-vector immunity against post vaccination insert response in adults.

The *ex vivo* response to vaccinia found before vaccination is plotted on the x axis. The subsequent *ex vivo* response to the antigen insert is shown on the y axis. Responses are split into two graphs. The left panel relates vector responses at screening to antigen insert responses immediately after FP9 vaccination, the right panel relates vector responses after FP9 vaccination to antigen insert responses immediately after MVA vaccination. Negative correlations of r=-0.35, p=0.09 for FP9 ME-TRAP and r=-0.59 p=0.0058 for MVA ME-TRAP were seen.
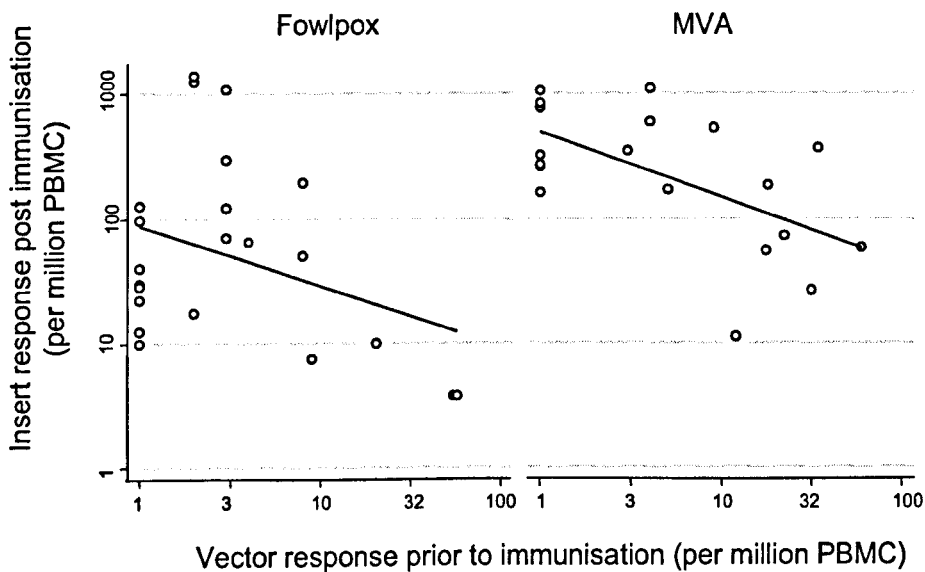
Negative correlations of r=-0.35, p=0.09 for FP9 ME-TRAP and r=-0.59 p=0.0058 for MVA ME-TRAP were seen. This gave a negative correlation of r=-0.32, p=0.03 overall, and a coefficient of -0.5, p=0.002 when adjusted for vaccination. The least squares regression line was positioned differently for FP9 and MVA. This was expected, since

MVA was more immunogenic than FP9, and was given after FP9, when anti-vector responses had already been induced. This suggests that even a weak anti-vaccinia response prior to either FP9 ME-TRAP or MVA ME-TRAP immunization attenuates the response induced to the insert.

4.3.8 Immunisation of children

The first four children to be immunised received only single priming immunisations with FP9 ME-TRAP followed by MVA ME-TRAP, but subsequently children were immunised with two priming vaccinations with FP9 ME-TRAP, followed by boosting with MVA ME-TRAP. Different doses of FP9 ME-TRAP were compared, to examine whether a lower dose would result in less anti-vector immunity, and so improve the immunogenicity of the final vaccination with MVA ME-TRAP. Different doses of MVA ME-TRAP were also compared to examine whether the full dose was required for strong immunogenicity.

4.3.9 FP9 ME-TRAP and MVA ME-TRAP were immunogenic in children

Both the first FP9 ME-TRAP vaccination and the final vaccination with MVA ME-TRAP raised responses above baseline (Figure 4.8). On *ex vivo* ELISpot, the geometric mean response was 6 spots per million at baseline (95% CI 2-12), rising to 13 (95% CI 8-21) spots per million (p=0.10) after FP9 ME-TRAP, and rising further to 113 per million (p<0.0005) after MVA ME-TRAP. By cultured ELISpot, geometric mean resting memory responses were 35 per million at baseline, 245 per million after FP9 ME-TRAP (p=0.006), and 780 per million after MVA ME-TRAP (p<0.0005). *Ex vivo* responses

after the second FP9 ME-TRAP vaccination were not higher than baseline (p=0.82), and cultured responses were not measured. Responses 3 months post vaccination were still elevated above baseline for both *ex vivo* (17 spots, p=0.014) and cultured ELISpot (150 spots, p=0.014).

Figure 4.8. *ex vivo* and cultured ELISpot responses in children over time.

Samples were drawn at screening, one week after each vaccination, and 90 days after completing the regimen. Spot numbers per million PBMC are plotted (log scale) on the y axis. The time points are described by the sequence of vaccinations. For instance, FF+7 refers to the timepoint one week after the second of two sequential FP9 vaccinations. Boxes show the distribution of results, the resting line indicating median response, upper and lower box boundaries showing 25th and 75th centiles, and the whiskers show 5th and 95th centiles where there are outliers, or minimum and maximum results where there are no outliers. The circles show outlier results. Both the first FP9 ME-TRAP vaccination and the final vaccination with MVA ME-TRAP raised responses above baseline. Responses above baseline were seen after FP9 ME-TRAP (p=0.1 for *ex vivo*, p=0.006 for cultured ELISpot), after MVA ME-TRAP (p<0.0005 for both assays) and at 90 days (p=0.014 for *ex vivo*, and 0.014 for cultured). n=18 at all time points.
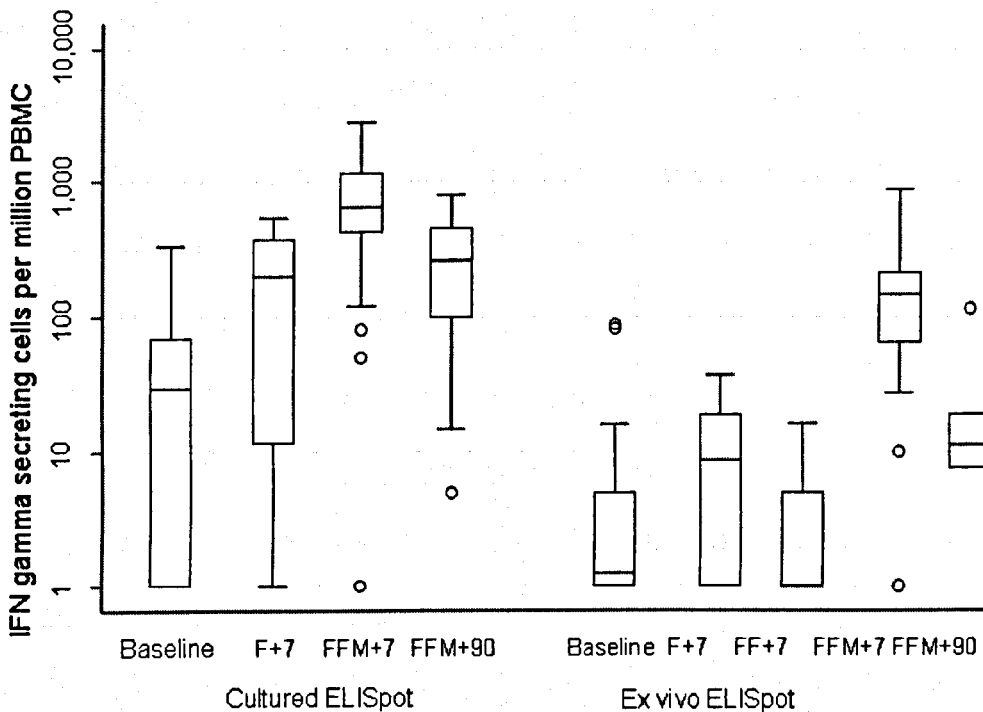
4.3.10 Half dose FP9 ME-TRAP priming was more immunogenic than full dose.

After the regimen using full dose FP9 and full dose MVA, the geometric mean response on *ex vivo* ELISpot was 70 (95% CI 20-260). Geometric mean responses were higher for the regimes that used half dose FP9, at 197 (CI 164-240) spots per million for half dose FP9 then full dose MVA and at 94 spots (CI 23-320) for half dose FP9 then half dose MVA, p=0.034 (Figure 4.9).

Figure 4.9. _Ex vivo_ and cultured ELISpot responses in children by vaccination regimen.

Responses for cultured and _ex vivo_ ELISpots seven days after final vaccination are shown by regimen, which vary according to the doses of component vaccinations used. f refers to a half dose FP9 immunisation, i.e. $5x10^7$ plaque forming units (pfu) FP9 ME-TRAP. m refers to half dose MVA immunisation, i.e. $7.5x10^7$ pfu MVA ME-TRAP, F to full dose FP9 immunisation, i.e. $1x10^8$ pfu FP9 ME-TRAP and M refers to full dose MVA immunisation, i.e. $1.5x10^8$ pfu MVA ME-TRAP. These abbreviations are then used to describe the sequence of vaccinations in each regimen, so that ffM refers to 2 half doses of FP9 (given sequentially) followed by a full dose of MVA. Means according to FP9 dose are shown by the dotted lines. The very first immunisations given to children only used a single FP9 dose. 2 children received fm and 2 children FM. These data are also shown. Immunogenicity was lower with full dose FP9, both for _ex vivo_ (p=0.034) and cultured ELISpot (p=0.058).
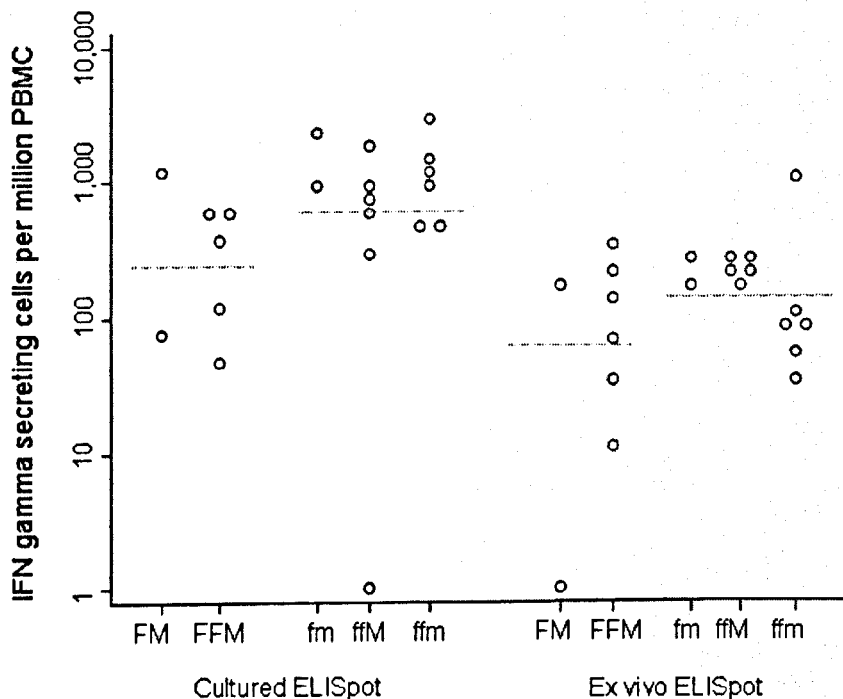
A similar pattern was seen on cultured ELISpot. After full dose FP9, then full dose MVA the geometric mean response was 246 (CI 61-980). However, when half dose FP9 was used to prime the later MVA vaccination, mean responses were 755, CI 335-1700 (after full dose MVA) and 1220, CI 300-2100 (after half dose MVA), p=0.058.

4.3.11 Half dose MVA ME-TRAP compared with full dose.

Although MVA dose made no difference to responses seen on *ex vivo* ELISpot (p=0.46), half doses appeared to be more immunogenic by cultured ELISpot. The geometric mean response of all those given half dose MVA (i.e. ffm and fm) was 1110 (CI 650-1900), compared to 410 (CI 200-830) after full dose MVA (i.e. FFM, FM and ffM), p=0.05. However, half dose FP9 had been shown to be more immunogenic than full dose. When only half dose FP9 regimens were compared (i.e. ffm and fm compared with ffM), the responses were 1096 spots per million at low dose MVA (95% CI 630-2000) compared with 741 (95% CI 320-1600) at high dose, p=0.16.

4.3.12 There was no relationship between pre-vaccination anti-vector immunity and immunogenicity in children.

Less PBMC were available for study in children, and anti-vector immunity was only measured seven days after the second FP9 vaccination. No correlation was found between pre-vaccination anti-vector immunity and the immunogenicity of the boosting vaccination with MVA ME-TRAP (r=0, after excluding the single outlying result, Figure 4.10). However, there was very little anti vector response evident after the second FP9

immunisation. The geometric mean was 2.5 spots per million, and the highest response 18 spots per million. It appeared that in children, vaccination with FP9 did not induce cross reactive responses to vaccinia.

Figure 4.10 Pre vaccination anti-vector immunity against post vaccination insert response in children.

The *ex vivo* response to vaccinia found before vaccination with MVA ME-TRAP is plotted on the x axis. The subsequent *ex vivo* response to the antigen insert is shown on the y axis. No correlation is seen (r=0 after excluding the single outlier).
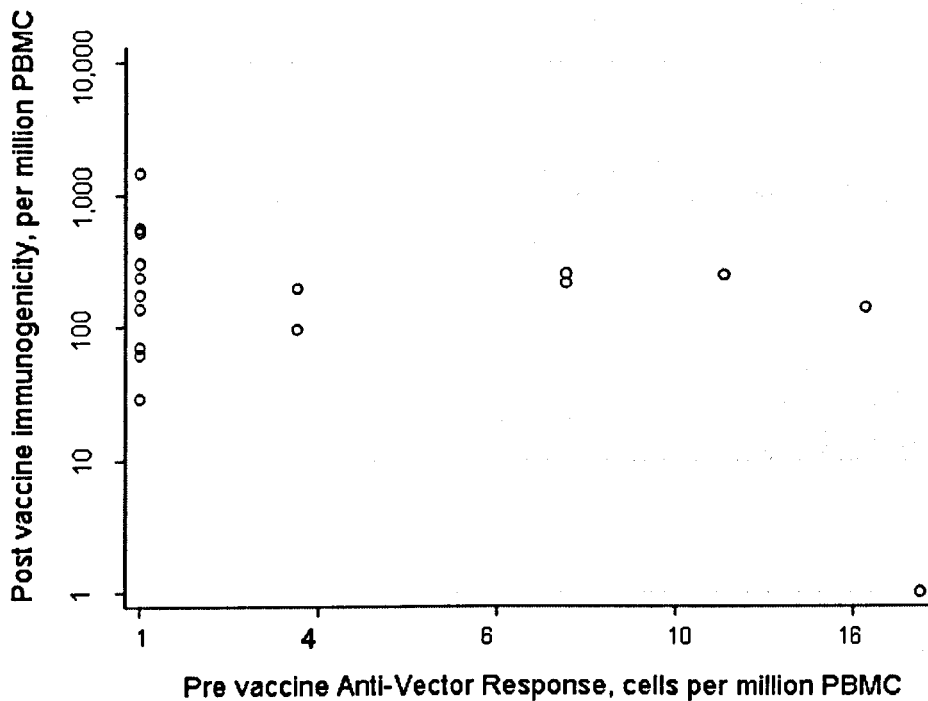
4.3.13 To further examine the determinants of vector cross reactivity, adult responses were analysed by prior smallpox vaccination status.

Responses at baseline to vaccinia virus were higher in those adults with scars suggestive of prior smallpox immunization. Geometric means were 4.5 (95% CI 2-10) without a scar, but 10 (CI 5-25) with a scar, p=0.01 (Figure 4.11). FP9 immunisations did not provoke a rise in vaccinia reactivity in subjects without previous smallpox

immunisations, among whom the geometric mean was 6 spots per million PBMC (CI 3-10). However, in subjects with scars, FP9 induced a significant response, with a geometric mean of 45 spots per million (CI 18-122). This was significantly higher than baseline responses to vaccinia (p=0.021) and significantly higher than responses to vaccinia after FP9 in subjects without scars (p=0.027). Following subsequent immunisation with MVA, both subjects with and without scars had similar anti-vaccinia responses, at 94 spots (CI 35-225) with prior smallpox immunisation, and 89 (CI 42-176) without.

Figure 4.11 Comparison of anti-vector response by smallpox vaccination scar status (*ex vivo* ELISpot).

Ex vivo ELISpots using vaccinia rather than peptides representing the antigen insert were conducted. Responses are shown before immunization (baseline) and then 7 days after the first FP9 ME-TRAP or FP9 CS vaccination (F), or 7 days after MVA ME-TRAP or MVA CS was given (M). At each timepoint, responses are split according to the presence or absence of a scar from previous smallpox vaccination. Subjects with smallpox vaccination scars had higher responses at baseline (p=0.01) and after FP9 (p=0.0001).
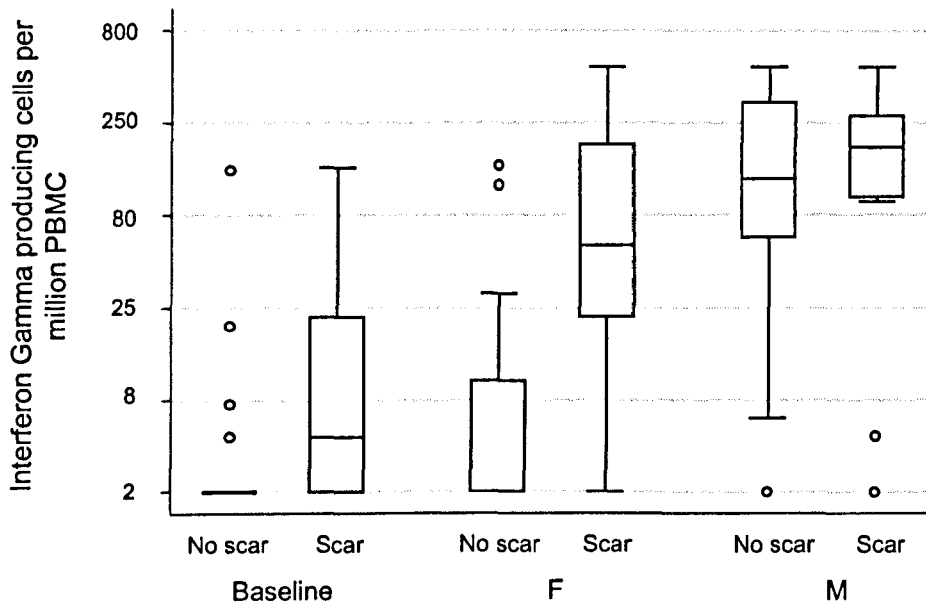
4.4 Discussion

Alternating vector regimes (MFM or FMF) generated more persistent T cell responses than heterologous prime boost regimes (FFM or FM). Over 270 days the effector T cell response to alternating vector regimes was well sustained (84% of the peak response), but fell to pre-vaccination levels after heterologous prime boost vaccinations. The alternating vector regimes also raised stronger resting memory T cell responses, which did not fall over 270 days, whereas resting memory T cell responses to prime boost immunizations (FFM or FM) decayed to 33% of peak levels.

Results from cultured ELISpot assay may not have a linear relationship with the numbers of resting memory cells at the start of the assay. Culture conditions may disproportionately limit the proliferation of very high numbers of cells. It is possible that the limited proliferation of very high cell numbers masked a loss of resting memory cells following alternating vector immunization. However, if analysis was restricted to responses below 1500 cells per million, there was still no detectable fall in resting memory responses over 270 days. The mean response for this restricted analysis was very similar to that following prime boost vaccination (790 vs. 720 spots), for which a fall in responses over time was seen.

This is an important finding in vaccine development for two reasons. Firstly the longevity of response is critical to efficacy in the field, and such sustained effector responses have not been previously described for T cell inducing vaccinations. Secondly, in both sporozoite challenge studies in non-endemic areas (Keating et al. 2005) and field trials in endemic areas (Reece et al. 2004), the resting memory population of T cells correlated with protection against malaria.

4.4.1 Heterologous versus homologous boosting.

Previous phase 1 studies in non-endemic areas demonstrated that multiple priming (i.e. two sequential FP9 ME-TRAP or DNA ME-TRAP immunizations before boosting with MVA ME-TRAP rather than one FP9 ME-TRAP or DNA ME-TRAP) increases the immunogenicity of viral vector vaccination regimes. However, neither multiple priming nor multiple boosting resulted in similarly maintained effector responses (McConkey et al. 2003). Memory cells begin to differentiate following the initial expansion of peak effector cells. In mouse models, T cell contraction is governed by early inflammation (Badovinac et al. 2004), but accelerated by dendritic cell stimulation (Badovinac et al. 2005), perhaps by prolonged contact with the antigen. Repeated homologous vector boosting may not deliver the antigen because of significant anti vector immunity. However, alternating the vector might allow effective antigen delivery and expand the memory population, provided that strong anti-vector immunity is relatively short lived (as suggested in studies of MVA re-boosting in West Africa (Moorthy et al. 2004a)). The effector population may be refractory to further stimulation at this stage (Corbin & Harty 2005), and alternating vectors seems to improve memory but not increase the peak effector response.

4.4.2 Pre-vaccination resting memory

The responsiveness of the resting memory population prior to vaccination correlated with *in vivo* effector response generated after single dose regimes (FP9 ME-TRAP or MVA ME-TRAP), suggesting that the resting memory population are the pool of cells from which effector cells differentiate *in vivo* following a second antigen exposure, and this probably underlies the correlations seen with protection (Keating et al.

2005; Reece et al. 2004). Similar memory populations are not identified in malaria naïve subjects (Keating et al. 2005), for whom single dose regimes are not immunogenic. However, primed boost regimes do not depend on pre-vaccination resting memory populations. There was no correlation between prior resting memory and effector response when considering prime boost regimes in this study, and those regimes were immunogenic in naïve subjects in previous studies (Webster et al. 2005). This was unlikely to represent a chance finding of subgroup analysis. A model with pre-vaccination memory and vaccination group as interacting terms explaining post vaccination response was significantly better than a model without the interaction.

4.4.3 CS vaccinations were less immunogenic

It is unclear why CS vaccinations should be less immunogenic. It is unlikely that the lower immunogenicity is caused by natural exposure, since immunogenicity was also low in non-endemic areas (Walther M et al, in preparation). It is possible that the GPI anchor motif included in the CS insert is responsible for this reduced immunogenicity (Bruna-Romero et al. 2004), although both immunogenicity and efficacy was observed when CS was delivered by the adjuvanted protein particulate vaccine RTS,S (Alonso et al. 2004; Lalvani et al. 1999).

FFM was identified as more immunogenic than FM, and spacing prime and boost by 8 weeks instead of 4 did not confer an advantage. Findings with DNA prime and MVA boost in naïve subjects were similar (McConkey et al. 2003). Antibody responses have often been higher when longer intervals between vaccinations were used (Pittman et al. 2000). This may not be the case for T cell inducing vaccinations.

4.4.4 Batch variability

Surprisingly, different batches of the same vaccine produced different immunogenicity. Both batches were prepared by GMP from the same master seed virus. FP9 ME-TRAP batch 020602 was more immunogenic as a single immunization and as priming for boosting with MVA. Repeated titrations of plaque forming units excluded an error in dosing, and titrations of viable virus from vials stored in Kilifi confirmed stability. Potency testing in mice was satisfactory for both batches.

The less immunogenic batch (040803) was used in half the FM immunizations, and all the alternating vector immunizations. Although less inflammation has been associated with lower effector responses and stronger memory responses (Badovinac et al. 2004), this was not the case here, since the less immunogenic batch for effector responses (040803) was associated with greater cutaneous inflammation (see section 3.3.10). Furthermore, this cannot explain the increased longevity seen with alternating vectors, since similar longevity was not a feature of prime boost FM immunizations using batch 040803. However, the use of batch 040803 for alternating vectors might well explain the lower peak effector response.

4.4.5 Strain Cross-reactivity

T cell responses to the insert after both vaccinations were crossreactive (figure 4.4). This was demonstrable by pooling variant and synonymous peptides separately. Had they been pooled together, it would have been difficult to distinguish cross reactivity

because of responses to shared peptides from cross reactivity between polymorphic peptides.

Responses to the circumsporozoite protein raised by natural exposure were almost entirely strain specific, but following immunisation marked strain cross reactivity was generated (figure 4.4). Responses at baseline favored 3D7. However 3D7 and 7G8 responses were similar after MVA CS, despite the vaccine encoding 3D7 strain CS. Altered peptide ligands have been identified among the polymorphic regions of CS, and can render T cells capable of responding to one variant only (Good et al. 1993). Repeated contact with various altered peptide ligands during natural exposure might "switch off" the T cell response to some peptides, and avoid repeated boosting of responses. The cytokine milieu following vaccination with viral vectors may reverse that process, and produce cross-reactive responses. Responses were strain specific after immunizations with RTS,S (Dunachie et al. 2006; Lalvani et al. 1999). Further studies are required to assess whether strain cross reactivity is a particular feature of delivery with viral vectors.

These data from a malaria endemic area provide evidence that alternating vector regimes enhance memory over multiple boosting or multiple priming regimes, possibly by prolonging antigen exposure in the host. Increasing numbers of heterologous prime boost regimes are being developed to vaccinate against infectious pathogens such as HIV, HBV and M. tuberculosis and to treat chronic infections and cancer. These data suggest that alternating vector regimes should be assessed in the clinical development of such products.

4.4.6 Vaccination of children

Vaccination regimes that primed with FP9 ME-TRAP and boosted with MVA ME-TRAP were immunogenic in children. Responses were still evident 3 months after immunisation. Both immediate effector (seen on *ex vivo* ELISpot) and resting memory (seen by cultured ELISpot) T cells were significantly induced. However, the immunogenicity was slightly lower in children compared to adults. On *ex vivo* ELISpot, the geometric mean responses after FFM ME-TRAP were 113 spots per million (CI 70-180) among the children compared to 389 spots per million (95% CI 190-800) in adults. By cultured ELISpot, mean responses were 623 spots per million (390-1000) in children, compared with 810 spots per million (CI 470-1150) among adults.

4.4.7 Vector cross-reactivity

The data from adult vaccinations suggested that FP9 induces some cross reactive anti-vector immunity against MVA in subjects who had previously received smallpox vaccination, and that this anti-vector immunity impairs the immunogenicity of subsequent vaccination with MVA ME-TRAP. This suggests that strategies to reduce the anti-vector responses raised by the priming might enhance the immunogenicity of the overall regimen.

Immunisations of children were designed to compare regimes using half or full doses of FP9. Half dose priming with FP9 was associated with a marginal trend towards

increased immunogenicity for the malaria insert (p=0.035). The varying immunogenicity with FP9 and MVA doses should be interpreted with caution at this stage, since the sample size was relatively small (22 children), the spread of results quite wide, and little anti-vector immunity was identified after FP9 vaccination in children. However, cultured ELISpot responses to the vector were not measured, and these might have explained the lower immunogenicity seen with full dose FP9 priming.

In adults, anti-vector immunity was measured after the first FP9 ME-TRAP immunization. In children limited PBMC were available, so anti-vector immunity was only measured after the second FP9 ME-TRAP. However, since only infrequent anti-vaccinia responses were seen after the second FP9 ME-TRAP vaccination in children, this is unlikely to represent a critical difference.

4.4.8   Smallpox vaccination

T cell responses to vaccinia virus were higher among those adults previously given smallpox vaccine, even though smallpox vaccinations were given more than 20 years ago. Smallpox vaccination ceased in Kenya in 1980, and so none of the children could have been immunised. FP9 provoked cross reactive anti-vaccinia responses in adults volunteers with scars suggestive of previous smallpox vaccine, but anti-vaccinia responses were absent in adult volunteers without scars. This suggests an "original antigenic sin", i.e. prior memory cell populations reactive to vaccinia differentiate to effector phenotype specific to vaccinia on exposure to FP9. However, those without prior immunization develop primary responses to FP9, which do not cross react with vaccinia.

Original antigenic sin has also been observed in humans for T cell responses in chronic HIV and hepatitis B infection, when virus mutants emerge *in vivo* with variant T cell epitopes (Bertoletti et al. 1994). The subsequent host response may continue to be directed against the initial epitope rather than the new variant, allowing the virus to evade host immunity. The data presented here relate to a defined exposure and demonstrate the skewing of responses consequent on prior exposure to smallpox virus, despite the long time intervals involved. Since FP9 and MVA are highly attenuated, host immunity is not required to limit viral replication (Carroll & Moss 1997). No greater reactogenicity was observed in subjects with vaccinia scars, or the immunosuppressed subjects with HIV infection who received single dose immunizations to assess safety, described in section 3.3.6.

Prime boost regimes should ideally use vectors with minimal pre-existing immunity and vector cross reactivity. The former has particular relevance for adenovirus vectors. Recombinant adenovirus immunizations are increasingly being developed as vaccines in humans to induce strong T cell responses, and immunity against many serotypes is widespread. Natural exposure to adenovirus is high, particularly in malaria endemic areas. The data here suggest anti-vector immunity does limit immunogenicity in adults, and so efforts to genetically modify human adenovirus, or identify adenovirus from another species to limit anti-vector immunity are important. Similarly, cross-reactive immunity between different pox virus vectors chosen in prime boost combinations may limit the immunogenicity of the boosting vector, and these data suggest vectors with less cross-reactivity will be more immunogenic.

4.4.9   Vaccine Dose

Higher doses of MVA have been associated with greater immunogenicity by *ex vivo* ELISpot (Moorthy et al. 2003a), but this was not so here (p=0.46). Unexpectedly, half dose MVA was instead associated with higher cultured ELISpot responses (p=0.05). Higher doses of MVA cause a greater local inflammatory reaction (section 3.3.9) and in mouse models this has been associated with greater contraction of T cells post vaccination (Badovinac et al. 2004). The lesser inflammatory reaction from half dose MVA may have resulted in less T cell contraction, and therefore the higher resting memory responses seen here.

This work emphasizes the need for dose-finding studies in children with new vectored vaccines, and the potential for enhanced immunogenicity with half doses of priming vectors warrants further investigation. Since heterologous prime boost immunization regimes using FP9 ME-TRAP and MVA ME-TRAP were immunogenic in children, larger studies to determine protection against malaria were conducted.

5 Phase 2 in Kenya: Efficacy.

A Randomized Controlled Trial to Measure Efficacy
of the Candidate Malaria Vaccines FP9 ME-TRAP
and MVA ME-TRAP in Children Aged 1-6 Years
Old in a Malaria Endemic Area.

Immunogenicity and safety data were acquired for adults and then children in Phase 1
studies in Kenya before a Phase 2 study was undertaken (sections 3 and 4), including the
FFM ME-TRAP regimen (i.e. two sequential FP9 ME-TRAP vaccinations followed by
MVA ME-TRAP). Although single vector immunisations and alternating vector regimens
were compared in phase 1 studies in adults, the FFM ME-TRAP regimen had
demonstrated the greatest peak responses, and there was evidence of protection for this
regimen in sporozoite challenge in naïve volunteers (Webster et al. 2005). The aim of the
Phase 2b trial was to evaluate safety, immunogenicity and efficacy of the vaccination in
the target population (i.e. children in a malaria endemic area), using mild febrile malaria
as an endpoint.

5.1   Methods.

5.1.1   Study Design

The study was randomised, controlled and double blind. Ethical approval was obtained from the Kenyan Medical Research Institute National Ethics Committee, the Central Oxford Research Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee. An independent Data Safety Monitoring Board and a Local Safety Monitor were appointed. The DSMB reviewed all serious adverse events as they occurred, approved the selection of vaccine dose based on previous phase 1 studies, and reviewed the analysis plan. Research was conducted in accordance with the Helsinki Declaration of 1975 (revised 1983). The trial was conducted according to Good Clinical Practice. Oxford University, acting as trial sponsor, arranged external monitoring and oversaw the conduct of the trial.

405 children were immunised with at least one dose of vaccine. Blood tests for immunology, safety, and cross-sectional assessments of malaria parasitaemia were conducted pre-vaccination, at screening, one week after the third vaccination, then at 3 months and at 9 months. Monitoring for solicited adverse events was conducted for one week after each vaccination. Unsolicited adverse events and episodes of malaria were monitored throughout the 1 year study duration. Children were screened in February 2005, immunised between March 2005 and May 2005, and followed up until February 2006. Monitoring is continuing in a further study.

5.1.2 Location

The study was carried out in Junju sublocation in Kilifi District, on the Kenyan coast. Junju contains a group of 5 closely related villages within the Chonyi area of Kilifi district. Junju lies between Kilifi and Mombasa, 14 km inland from the coastal road. Kilifi is malaria endemic, with all year round transmission and two high transmission

seasons (Mwangi et al. 2005). The transmission intensity is 22-53 infective bites per year (Mbogo et al. 2003). Junju is served by a local government dispensary, which has an active ITN distribution programme. Inpatient care is provided at Kilifi District Hospital.

5.1.3 Study Participants

The participating children were aged 1-6 years old (inclusive), healthy, and resident in the study area. After a series of public meetings and individual discussions, a screening date was set at which study information was repeated, and consent was sought before proceeding. Subjects were not immunized until at least one week after their parents signed consent, to allow the parents time to consider their decision.

Children were screened by history, examination and blood tests (full blood count, creatinine, alanine transaminase). Subjects with clinically significant illness were excluded. Clinically evident immunsuppression was one of the criteria for exclusion, but no children were excluded on this basis. HIV testing was not conducted. Abnormal alanine transaminase or creatinine levels were exclusion criteria, but anaemia without clinically significant signs and symptoms was not (although iron supplementation was given). Recent blood transfusions (2 months), current participation in another clinical trial, or receipt of another experimental vaccine were also exclusion criteria. Eligible children were invited to attend vaccination in the order in which they were screened.

5.1.4 Vaccines

The antigen insert used was TRAP, joined to a multiple epitope (ME) string from six *P. falciparum* pre-erythrocytic antigens (Gilbert et al. 1997). The ME string contains 14 pre-erythrocytic MHC class I epitopes from six *P falciparum* pre-erythrocytic antigens, three class II epitopes, two pre-erythrocytic B cell epitopes and pb9 (a *Plasmodium berghei* T-cell epitope that allows pre-clinical potency and stability testing).

The vectors used were an attenuated fowlpox virus, FP9, and modified vaccinia virus Ankara. Recombinant vaccine stock was supplied to contract manufacturer IDT (Rosslau, Germany), who produced clinical lots under GMP conditions. Single batches of FP9 ME-TRAP and MVA ME-TRAP were used throughout (both batch 051204).

The trial vaccination regimen was two sequential FP9 ME-TRAP vaccinations (5×10^7 plaque forming units) followed by MVA ME-TRAP vaccination (1.5×10^8 plaque forming units), given intradermally. The control was rabies vaccine (Aventis Pasteur, WISTAR strain), administered according to the same timings. Rabies was also given intradermally, at 0.25 IU. Vaccinations were spaced 4 weeks apart (acceptable range 3-5 weeks).

5.1.5   Randomization and Vaccination

Vaccines were shipped to Kenya on dry ice with logged temperature monitoring, stored at -80°C, and transported to the field in cool boxes for use within 6 hours. Vaccines were given intradermally over the deltoid area of the non-dominant arm, using a

27 gauge needle to raise a visible intradermal bleb. Volunteers were observed for 1 hour with resuscitation facilities available for advanced life support. Children received vitamin A supplements with each vaccination, as per Government of Kenya guidelines, and parents were given two doses of paracetamol syrup for use if the child developed fever at night.

Eligible children were assigned a randomization number, and vaccine group allocated using restricted randomization in blocks of 10 after sorting the list of eligible subjects by age and village. Randomization envelopes were prepared and sealed in the UK, without the involvement of any investigators involved in enrolling or assessing children at the study site. Each child was assigned the sealed opaque envelope bearing their study number, which was opened when they attended for the first vaccination. The nurses who administered vaccinations did not take part in any other trial related procedure, and were subsequently based in Kilifi District Hospital rather than the trial site. They drew up vaccinations according to the instructions in the randomisation envelope, and documented the vaccination in notes that were not available to the investigators until after unblinding, after follow up was completed for all children. The randomisation envelopes were re-sealed after each vaccination, and stored in a locked office for re-use at subsequent vaccinations. Neither parents nor investigators were told of the vaccination allocation, and the investigators did not enter the vaccination room while vaccinations were conducted.

5.1.6   Assessment of safety.

Field workers visited subjects daily for the first three days after vaccination and at one week after vaccination. Solicited adverse events were recorded, and the diameters of

102

skin discolouration, and blistering measured. Loss of the epidermis or upper part of the dermis was described as a deroofed blister. If unsolicited adverse events were reported, these were assessed and documented by a medically qualified investigator. Blood tests for routine biochemistry (plasma alanine transaminase and creatinine) and haematology (full blood counts) were conducted 7 days after the final vaccination, then at 3 months and 9 months.

5.1.7 ELISpots

ELISpots were conducted and analysed as described previously (section 4.2.1 and 4.2.2)

5.1.8 Monitoring for malaria episodes.

The primary endpoint was a clinical episode of malaria, defined as an axillary temperature greater than 37.5 degrees centigrade, with a *Plasmodium falciparum* parasitaemia greater than 2,500 parasites per μl. The presence of any falciparum parasitaemia with fever above 37.5 degrees was a secondary endpoint.

Estimating a 50% incidence of malaria, the study was designed to detect 35% efficacy with 80% power by enrolling 400 children. In fact there was less malaria transmission than expected (as in most of East Africa in 2005) and only a 25% incidence was recorded, and so the actual trial was only powered to detect 50% efficacy.

Asymptomatic parasitaemia is common in children under endemic conditions (70% of children in this study). If a child with asymptomatic parasitaemia is evaluated

during an unrelated viral illness, they will be wrongly ascertained as a case of febrile malaria (Schellenberg et al. 1994). An endpoint with low specificity leads to a misleading estimate of efficacy (Scott et al. 2005), and I therefore sought to minimise the effect of asymptomatic, chronic parasitaemia in three ways. A previously defined threshold parasitaemia was used for the diagnosis of malaria (Mwangi et al. 2005), all children received curative treatment for malaria before monitoring began, and only children where fever was confirmed by measurement (rather than simply reported) had blood films made. This latter measure is important since subjective reporting of fever by parents is not specific for objective fever (Mwangi et al. 2003).

The curative treatment was given one week after the final vaccination, using 7 days of directly observed dihydroartemisinin monotherapy (2mg per kg on the first day, followed by 1mg per kg for 6 days). Blood films were taken to confirm children were parasite-negative one week after the end of treatment.

Children were then visited every week by fieldworkers. The mother was asked whether she thought the child was hot, and the axillary temperature was also measured. When the temperature was greater than 37.5 degrees, a blood film was made and a rapid near-patient test for malaria conducted. The blood film was reviewed within 24-48 hours, but treatment decisions were based on the rapid test result.

When the mother reported the child was hot, but an objectively elevated temperature was not identified, blood films and rapid testing was not performed, but the field worker returned to the child a further three times in the next 24 hours. Rapid testing and blood films were performed if the temperature was elevated on any of these visits. Parents could bring their children for assessment in between regular weekly visits if they

thought the child had developed fever, and the child was assessed as above. Field workers were recruited from the villages in which the study was conducted, and so were readily accessible to the parents. Treatment for episodes of malaria was with the Government of Kenya recommended first line treatment, artemether-lumefantrine.

During blood tests for safety and T cell response, blood films were made on all children. The results of this testing was not available for several weeks, during which monitoring of febrile illnesses continued. Asymptomatic parasitaemia was therefore not treated unless the child developed a fever. All blood films (from well children and febrile children) were counted in duplicate by two microscopists, and a third count conducted if they were discrepant.

5.1.9 Analysis

No interim analyses were planned or conducted, and the analysis plan was approved by the DSMB before unblinding. The primary analysis was a log rank test comparing the time to the first or only episode of malaria (defined as fever with parasitaemia above 2500/μL) between the vaccination groups, stratified by age group, ITN (insecticide treated net) use and village, for both ATP and ITT subjects. The hazard ratio and 95% confidence interval was estimate by Cox's regression adjusted for the same covariates. Age group was a categorical variable with three levels (1-2 years old, 2-5 years old, 5-6 years old). Village had 5 levels. ITN use was defined as sleeping under a treated net every night, which had less than three holes into which a finger could comfortably fit.

Poisson regression was used to estimate the incidence rate ratio taking into account all malaria episodes, adjusted for the same covariates. A period of 28 days after each malaria episode was deducted from the person time at risk, since individuals were assumed not to be at risk of malaria during this period. In secondary analyses, malaria was defined as fever with parasitaemia at any density. ELISpot results were log transformed, substituting one spot per million for negative results (half the lower limit of detection), and then Students T tests used to compare results. To examine the effect of ELISpot results on episodes of malaria, the responses were divided into tertiles, and the tertile was then used as a categorical variable.

The analysis plan specified that monitoring of malaria episodes would continue for a further 9 months, after which a second analysis of episodes would be conducted.

5.2 Results.

5.2.1 Trial profile

530 children were screened. Eligible children were invited to attend for vaccination in the order in which they were screened. Vaccination continued until at least 400 children were immunised (on the last day of vaccination, the total had reached 405). The trial participants were balanced between treatment allocation groups in both demographic characteristics at baseline and progress through the trial (Table 8). 275 of 386 children (71%) were parasitaemic at baseline (data for parasitaemia was missing for one child at baseline). 73 children received anti-malarial treatment for intercurrent febrile malaria during vaccinations. Blood films were not taken on the days of vaccination, but 179 of 374 children (52%) were parasitaemic one week following final vaccination, before receiving anti-malarials at the start of monitoring.

PAGE HAS NO CONTENT

Table 5.1. Baseline covariates for children enrolled in the Phase 2b trial.

Covariate	FFM ME-TRAP	Rabies
Village		
Gongoni	36	40
Junju	50	50
Kolewa	50	59
Mapawa	39	32
Mwembe Tsungu	15	16
Age Category (years)		
1-2	36	41
2-5	107	108
5-7	47	48
ITN* (baseline)		
Without	135	140
With	54	54
ITN (end)		
Without	75	70
With	113	123
Parasitaemia at screening visit	134	141
Without parasitaemia	55	56

* ITN use was defined as sleeping under an insecticide treated net every night with less than three holes that could comfortably admit a finger. ITN data is missing for 4 children at baseline and 6 at the end. Data on parasitaemia at screening was missing for 1 child.

The Intention To Treat (ITT) cohort included all children who received any vaccinations where data was available for at least one week's surveillance for episodes of malaria. The According To Protocol (ATP) cohort included only children where immunisations and pre-monitoring anti-malarial therapy were given as planned. In total 346 children were vaccinated According To Protocol, and an additional 41 children were included only in the Intention To Treat analyses (Figure 5.1). 15 of the original 405 vaccinees did not complete all three vaccinations, and 1 child moved out of the area after the third vaccination but before monitoring for malaria episodes began. The 41 children included in ITT analysis but not ATP had received all three vaccinations, but 28 had not complied with observed anti-malarial therapy, and 17 had incorrect timings of vaccinations. Nevertheless, all subjects had received the correct doses of the correct vaccination. Of the 28 who had not complied with treatment, blood films were available from only 8, of whom 7 were positive for malaria parasites. The distribution of ATP and ITT groups by vaccination is given in Figure 5.1. After screening for eligibility, parents were invited to bring their children back to the dispensary for immunisation. Children were randomized on attending for vaccination.

<u>**Figure 5.1 The Trial Profile for phase 2b efficacy trial..**</u>

Trial Profile

```
                    ┌──────────────────────┐
                    │ Assessed for eligibility │
                    │ (n=530)               │
                    └──────────┬───────────┘
                               │          ┐
                               ▼           ┌──────────────────────────────┐
              ┌──────────────────────┐     │ Excluded on eligibility (n=5)  │
              │ Randomized (n=405)    │     │ Did not attend vaccination (n=120) │
              └──────────┬───────────┘     └──────────────────────────────┘
```

Allocated to placebo (n=204)	Allocated to FFM ME-TRAP (n=201)
First Dose (n=204)	First Dose n=201
Last Dose (n=198)	Last Dose n=190
Pre-surveillance treatment (n=182)	Pre-surveillance treatment (178)

At least one follow up visit (n=197)	At least one follow up visit n=190
9 months follow up (n=192)	9 months follow up n=188

Analyzed ATP n=171	Analyzed ATP n=175
Analyzed ITT n=197	Analyzed ITT n=190

5.2.2 Safety

The vaccine was well tolerated. 10 serious adverse events occurred. 4 occurred after rabies vaccination (severe malaria, gastroenteritis, trauma and asthma) and 6 after FFM ME-TRAP (severe malaria, an abdominal skin infection distant to the vaccination site, trauma, gastroenteritis and multiple seizures). The serious adverse events identified were not unexpected events given the study population, were not closely related to vaccination in timing, and were judged unlikely to be linked to vaccination by the investigators and the Data Safety Monitoring Board. Local cutaneous discolouration was frequent (seen after 72% of FP9 ME-TRAP vaccinations and 64% of MVA ME-TRAP vaccinations). Cutaneous blistering occurred after 16% and 19% of FP9 and MVA vaccinations respectively, but blistering with a diameter greater than 0.5cm occurred at a

110

rate of 1-4%. No blisters were greater than 1cm in diameter, or associated with reports of marked pain. Marked pain was reported by the children's parents after 0.5% of vaccinations. Keloid formation or hypertrophic scars were not seen. Systemic adverse events and limitations of normal daily activities (such as appetite and play) were rare, and distributed evenly between the control (Rabies) vaccine recipients and the poxvirus vaccine recipients. (Table 5.1). 94% of those receiving a first vaccination returned to complete the course of 3 vaccinations. The range of routine haematology and biochemistry results, and frequency of abnormal results, was similar for the two groups. No abnormal laboratory results were attributed to vaccination. Very long term, passive follow up for safety is possible because of the Demographic Surveillance Study (DSS) well established in the area.

Table 5.2. Solicited adverse events: Frequencies of children experiencing the named adverse events at any time during the first week of monitoring after vaccinations given in the Phase 2b trial.

Vaccination	Adverse event	Freq.	Percent	Confidence interval
1st FP9 ME-TRAP	Any blister	32	15.9%	(10-20%)
2nd FP9 ME-TRAP	Any blister	31	16.1%	(10-21%)
MVA ME-TRAP	Any blister	36	19.0%	(13-24%)
Rabies	Any blister	13	2.2%	(0-3%)
1st FP9 ME-TRAP	Blister >0.5 cm diameter	8	4.0%	(1-6%)
2nd FP9 ME-TRAP	Blister >0.5 cm diameter	2	1.0%	(0-2%)
MVA ME-TRAP	Blister >0.5 cm diameter	7	3.7%	(1-6%)
Rabies	Blister >0.5 cm diameter	2	0.3%	(0-1%)
1st FP9 ME-TRAP	Marked local pain and reduced activities	0	0.0%	(0-1%)
2nd FP9 ME-TRAP	Marked local pain and reduced activities	0	0.0%	(0-1%)
MVA ME-TRAP	Marked local pain and reduced activities	1	0.5%	(0-1%)
Rabies	Marked local pain and reduced activities	0	0.0%	(0-1%)
1st FP9 ME-TRAP	Marked local pain without impact on activities	1	0.5%	(0-1%)
2nd FP9 ME-TRAP	Marked local pain without impact on activities	0	0.0%	(0-1%)
MVA ME-TRAP	Marked local pain without impact on activities	2	1.1%	(0-2%)
Rabies	Marked local pain without impact on activities	0	0.0%	(0-1%)

1st FP9 ME-TRAP	Temp>39.0 degrees	2	1.0%	(0-2%)
2nd FP9 ME-TRAP	Temp>39.0 degrees	0	0.0%	(0-1%)
MVA ME-TRAP	Temp>39.0 degrees	2	1.1%	(0-2%)
Rabies	Temp>39.0 degrees	1	0.2%	(0-1%)

Freq. refers to the number of children experiencing the adverse event listed. 'Reduced activities' refers to children whose parents stated they were not eating or not playing after receiving the vaccine. 'Marked pain' refers to the report given by parents, who were offered the options 'none', "a little" or "a lot" of pain experienced by the child after vaccination (excluding the immunisation itself). Adverse events for rabies vaccinations are given by aggregate for all vaccinations.

5.2.3 Immunogenicity

The vaccine was immunogenic (Figure 5.2). T cell responses were measured using both the ex-vivo and the cultured ELISpot, and both assays detected a response to vaccination. This was significantly greater than the response measured pre-vaccination ($p<0.0005$), and greater in FFM ME-TRAP vaccinees than children receiving control vaccinations ($p<0.0005$). _Ex vivo_ T cell responses rose from 30 spots per million before vaccination (95% confidence interval 21-40) to 107 spots per million (95% CI 88-127) after vaccination. Cultured responses rose from 123 spots at baseline (95% CI 104-142) to 407 (95% CI 349-464). 9 months post vaccination, the mean response among FFM ME-TRAP vaccinees remained above the control group for both _ex vivo_ (44 spots among FFM ME-TRAP vaccines, 95% CI 32-57, compared with 27 spots among controls,

95%CI 21-32) and cultured ELISpot (464 spots, CI 371-557 compared with 206 spots, CI

156-256), p=0.015 for *ex vivo*, p<0.0005 for cultured ELISpot.

Figure 5.2. T cell responses to vaccination identified by both _ex vivo_ and cultured ELISpot, are displayed by time. Median, 25th and 75th quartile, 5th and 95% quartile and outlying results are given by box and whisker plots. Data was available for 400 children at screening (i.e. pre-vaccination), for 379 seven days after the last vaccination, for 345 at three months and 304 at nine months. T cell numbers were similar at baseline for _ex vivo_ (p=0.4) and cultured (p=0.91) responses. _Ex vivo_ responses were higher among FFM vaccinees at one week (p<0.00005), three months (p=0.27) and nine months (p=0.015), as were cultured responses (p<0.00005, p=0.0007 and p<0.00005 respectively).

Immunogenicity: Ex vivo and cultured ELISpots
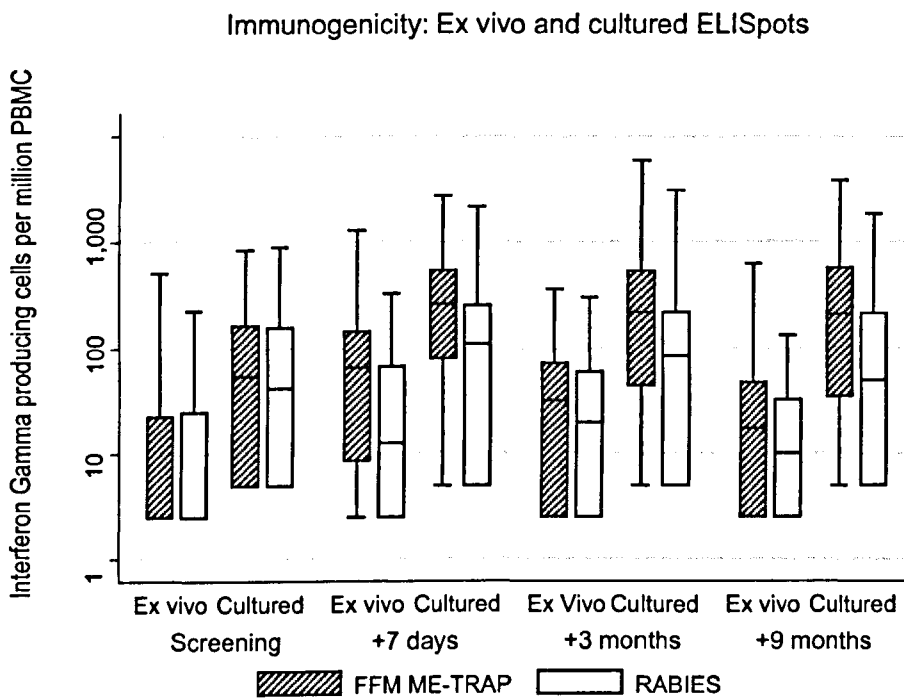
5.2.4 Efficacy

The cumulative incidence of malaria (fever with parasitaemia >2500/μL) was 52/190 children in the malaria vaccine group and 40/197 in the control group, (stratified log rank test χ^2=2.2, P=0.14), with a hazard ratio of 1.5, 95% confidence interval 1.0 to 2.3. 346 children were vaccinated according to protocol and followed up, of these 40/175 had malaria in the malaria vaccine group compared to 36/171 in the control group,

115

(stratified log rank test χ^2=0.36, P=0.55; hazard ratio 1.3, 95%CI 0.83 to 2.1). Kaplan-Meier plots are shown in figure 5.3.

In a secondary analysis, the number of malaria cases (malaria defined as fever with parasitaemia at any density) was 69/190 in the malaria vaccine group and 54/197 in the controls, stratified logrank test χ^2=2.79, P=0.095, hazard ratio 1.5 (95%CI 1.0 to 2.1), and among those vaccinated according to protocol, 58/175 and 48/171 respectively, logrank test χ^2=1.26, P=0.26.

Figure 5.3. The probability of remaining free of clinical malaria is plotted over the 9 months of monitoring (the primary analysis). Numbers of children at risk are given below the Kaplan Meier plots. Both plots use an endpoint of >2,500 parasites per microlitre and fever, plot a) describes the Intention To Treat (ITT) group, plot b) describes According To Protocol (ATP). P=0.55 for plot a), 0.14 for plot b).

Kaplan Meier plots of the probability of remaining free of clinical malaria
(fever plus parasitaemia >2,500 per ul).

Intention to treat

Time (days)	0	50	100	150	200	250
Number at risk: Placebo	194	181	167	162	161	156
FFM	193	166	153	150	145	139

According to protocol

Time (days)	0	50	100	150	200	250
Number at risk: Placebo	175	160	148	145	144	138
FFM	171	155	141	139	135	130
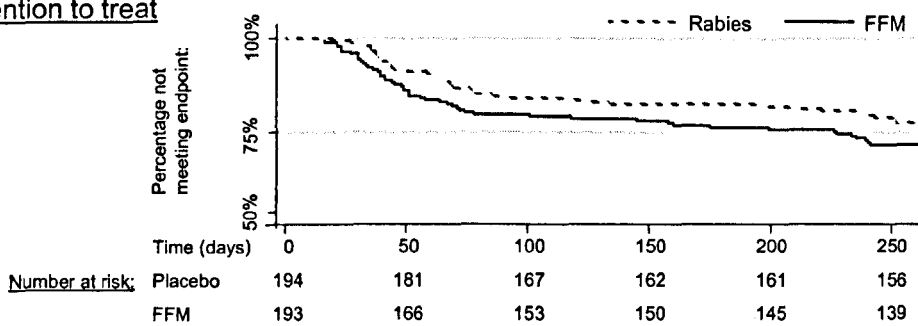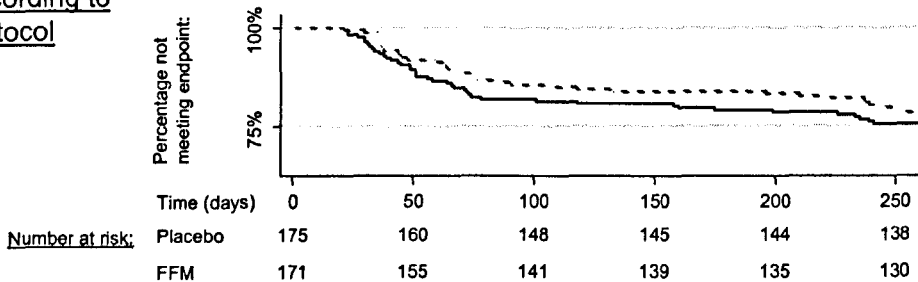
Multiple episodes were modelled by Poisson regression, adjusted for the same covariates. There were 0.46 episodes of malaria (fever with parasitaemia >2500/μL) per person year among the FFM ME-TRAP vaccinees, and 0.36 episodes per person year among the control group (incident rate ratio 1.6, 95%CI 1.1 to 2.3, P=0.017). Among those vaccinated according to protocol, the rate ratio was 1.4 (95%CI 0.89 to 2.1, P=0.16). When these analyses were repeated using malaria definition of fever with any parasitaemia, the rate ratios were 1.5 (1.1 to 2.0), P=0.013 (ITT) and 1.4 (0.96 to 1.9), P=0.08 (ATP). Incidence rate ratios are plotted in figure 5.4.

117

Only two episodes of malaria required hospital admission (one in each vaccination group), and neither of these met accepted criteria for severe malaria.

5.2.5   Covariates

Age, village and ITN use were specified as covariates. Older age was significantly protective on both Cox survival analysis for time of first episode (HR=0.24, CI 0.1-0.54, p=0.001) and poisson regression for number of episodes (Incidence Rate Ratio=0.2, CI 0.09-0.42, p<0.0005). Village was a significant factor in both analyses (p=0.0093 in Cox, p=0.012 by poisson). There was a tendency towards a protective effect of Insecticide Treated Net use on Cox (HR=0.84, CI 0.47-1.48 p=0.55) and poisson regression for multiple episodes (IRR=0.6, CI 0.36-1.04, p=0.07). However, analysis of ITN use was complicated by the increased prevalence of use from 28% at baseline to 62% by the end of the study.

5.2.6   Secondary Analysis by Sub-group

The possibility that susceptibility might vary by T cell response to vaccination (either by *ex vivo* or cultured ELISpot) was considered. Vaccinees were divided into tertiles according to the ex-vivo or cultured ELISpot responses measured one week after vaccination, and each tertile was compared with the control group (Fig 5.4). No clear trend for differential susceptibility according to immunogenicity was seen, and a log likelihood ratio test suggested the two models using immunogenicity (*ex vivo* and cultured) were not significantly better than using covariates alone (Log likelihood testing gave p=0.4 for *ex vivo* and p=0.24 for cultured). However, owing to the overall low

immunogenicity seen in this trial, the arithmetic mean was only 228 spots per million on *ex vivo* ELISpot in the highest tertile. Exploratory analysis divided vaccinees into 7 groups of 28 children per group (on average), according to *ex vivo* T cell response. The highest responders were at a mean of 325 spots per million, but still had more frequent episodes of malaria than the control group (Hazard Ratio=2.39, 95% CI 1.1-5.9). The highest 27 responders by cultured ELISpot were at mean response of 1,130 spots per million, and the Hazard Ratio was 1.76, 95% CI 0.78-4 compared with controls.

Cultured and *ex vivo* ELISpots were also conducted at 3 months and 9 months post vaccination. Data for number of episodes of malaria were split into 3 three month blocks, and T cell responses related to each block divided into tertiles for comparison with control vaccinations as above. As with the survival analysis, there was no indication that immune response predicted susceptibility to malaria (p=0.4, p=0.29 for log likelihood).

Other endpoints were haemoglobin and parasitaemia detected at cross-sectional surveys at 3 and 9 months. These were not different between vaccination groups, with the prevalence of parasitaemia at 29% and 26% at 3 months and 33% and 33% at 9 months for FFM ME-TRAP and the control group respectively. Mean haemoglobin was 11.1 g/dl before monitoring began, and 10.1 (95% CI 9.8-10.5) in both the FFM ME-TRAP and control group.

Figure 5.4 The results from secondary analyses of malaria episodes are shown.
These multiple analyses were generated by varying the case definition and statistical methodology, and are shown for both ATP and ITT. The more rigorous case definition (>2500 parasites per μl and fever) is the first in each group of comparisons. Cox regression was used to estimate hazard ratios for time to first episode (panel a) and poisson regression was used to estimate incidence rate ratios for the frequency of episodes (panel b). Models were adjusted for age, village and ITN use. The two groups of 3 points to the right of each panel show the hazard ratios and incidence rate ratios for subgroups FFM ME-TRAP vaccinated subjects. Subjects were divided into tertiles based on either *ex vivo* or cultured ELISpot responses. Hazard ratios and incidence rate ratios for each tertile relative to the control group are shown, using a case definition of parasitaemia >2500 and fever.

Hazard ratios (a) and Incidence Rate Ratios (b) for the effect of vaccination according to population and endpoint, and for T cell responses by tertile.
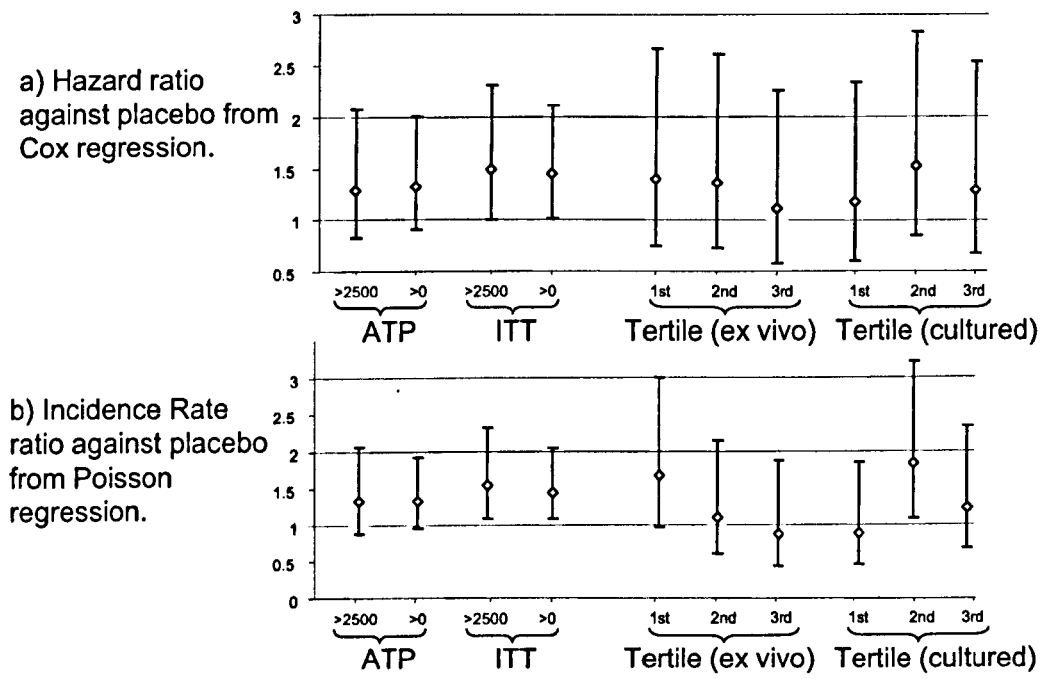
5.3 Discussion.

Apart from BCG trials, no other vaccines that primarily induce T cells responses have been studied for efficacy in children. I report here a phase 2b evaluation of one of very few vaccines shown to protect against malaria in challenge studies in human volunteers.

FFM ME-TRAP was safe when given to 1-6 year old children in an endemic area. It was also immunogenic, but not as immunogenic as in malaria naïve (Webster et al. 2005) or semi-immune adults (Moorthy et al. 2004a), and was not protective against episodes of clinical malaria. Although more malaria episodes were observed in the malaria vaccine group, the difference was not significant by primary analysis.

5.3.1 Blinding

Intradermal poxvirus vaccinations are associated with skin discolouration and blistering (see 3.3.4). This was unlikely to have compromised blinding, since children were routinely assessed by fieldworkers who were unaware of an expected difference in local reactogenicity. Furthermore, 16% of intradermal rabies vaccinations in the trial were associated with skin discolouration.

5.3.2 Adverse T cell responses

It is theoretically possible that TRAP responses induced by vaccination might be anti-inflammatory, and so reduce naturally-acquired protective responses (Flanagan et al.

121

2006). Altered peptide ligand effects of vaccination could increase susceptibility to malaria (Gilbert et al. 1998), and high levels of TGF beta release after vaccination might promote parasite growth (Walther et al. 2005b). However, there was no indication in our study that higher T cell responses predicted greater susceptibility to malaria within the vaccination group (either cultured or *ex vivo*). Furthermore, naturally acquired responses were not associated with protection among the control group in this study (as in a previous study of natural immunity (Flanagan et al. 2003)). It therefore seems unlikely that vaccine induced responses could lead to greater susceptibility by suppressing naturally acquired responses. The DNA-MVA regimen tested in The Gambia induced stronger T cell responses to TRAP than seen here, but there was no evidence of enhanced susceptibility in vaccinees (Moorthy et al. 2004b). There is therefore no obvious hypothesis to link vaccine induced responses with enhanced susceptibility, and the significance testing by primary analysis suggests that this was a chance finding.

5.3.3 Moderate immunogenicity.

FFM ME-TRAP was only moderately immunogenic in this population, despite the strong immunogenicity observed previously. In particular, stronger immunogenicity was seen in previous phase 1 studies in adults and children in Kilifi. Arithmetic mean responses by *ex vivo* ELISpot were 610 spots per million PBMC in malaria naive adults (Webster et al. 2005) but 350 spots per million in semi-immune adults in The Gambia and 360 spots per million in Kenya (see 4.3.2). The arithmetic mean T cell response during phase 1 trials in children in Vipingo, Kilifi was 200 spots per million, and in the Phase 2b study in Junju reported here, arithmetic mean responses were 107 spots per million. Vipingo, the site of the earlier phase 1 trial (4.3.8), lies only 3-4km from the main coastal road and has an annual EIR of 1, compared to the EIR of 22-53 in Junju

(Mbogo et al. 2003). It seems that immunogenicity is moderately reduced for semi-immune adults compared with malaria naive adults, and reduced further among children in a malaria endemic area. The children immunised in the phase 1 trials lived on a plantation in Kilifi, where there is slightly lower transmission intensity than among the children immunised in the phase 2b trial in Junju.

5.3.4   Batch variability

It has recently been shown that different batches of FP9 ME-TRAP can be associated with different immunogenicity (4.3.5), and work to assess factors that may underlie this variability is in progress. However, studies on semi-immune and naive adults used the same batch of vaccine, and preliminary studies in the same group of semi-immune adults suggested that the batches used for phase 1 and phase 2b trials in children had similar immunogenicity. It is therefore unlikely that most of the observed variation in immunogenicity is accounted for by batch variation.

5.3.5   Immunosuppression

Several studies have shown dendritic cell function to be impaired by malaria and other chronic infections (Wilson et al. 2006), and placental malaria induces T regulatory responses at birth (Brustoski et al. 2006). The children in Junju had greater malaria exposure than the children from the plantation in phase 1 studies. Semi-immune adults have less frequent blood stage malaria infection than children, but more than naive volunteers. The association between poor immunogenicity and more frequent malaria might be causal. There may also be other relevant factors, such as infection with other

parasites (Nookala et al. 2004; Sabin et al. 1996) or nutrition, and these will be explored later (section 6.3).

The *ex vivo* T cell responses previously associated with protection of naïve volunteers in sporozoite challenge were at a mean of over 500 per million PBMC. It has previously been demonstrated that the much lower responses (mean 44 spots per million PBMC) are not protective in the field (Flanagan et al. 2003). In the trial presented here, the mean response to vaccination was 110 spots per million PBMC, and was not protective. Furthermore, the mean response in the upper tertile of responders was 230 spots per million, and there was no tendency towards protection against episodes of malaria (HR 1.26, CI 0.68-2.33) although the confidence intervals in this analysis are now very wide. Similarly, in an exploratory analysis examining the 30 highest responders, among whom the mean response was 325 spots per million, the Hazard Ratio was 2.39 (CI 1.1-5.18).

Data from sporozoite challenge studies suggests that cultured ELISpot responses to examine resting memory T cells (Keating et al. 2005) may be a better correlate of protection than *ex vivo* responses. The mean cultured response in the present study was 407 spots per million, compared with previous studies showing 810 spots per million for adults and 880 per million for children in Kilifi (4.3.3 and 4.3.9), and 500 per million in adults in the UK (Keating et al. 2005). The cultured responses in the present study were durable (Figure 5.1), but not associated with protection (Figure 5.3).

5.3.6 Conclusions

There was thus no tendency towards protection with higher *ex vivo* or cultured ELISpot responses. This suggests that similar T cell inducing vaccinations designed for malaria endemic conditions should demonstrate considerably greater immunogenicity in phase 1 studies in the target population before progressing to phase 2 studies. However, it might be possible to induce qualitatively different T cell responses that are more protective in sporozoite challenge studies. For instance FP9 MVA regimes induce more CD8 responses than DNA MVA regimes, and these are more protective in animal models (Anderson et al. 2004).

It is unclear whether similar reductions in liver parasites to those seen in sporozoite challenge (section 2.3.1) might have occurred in this trial without a reduction in clinical episodes, but these reductions might be expected among children with the highest T cell responses. These data, together with the Phase 2a and 2b data for RTS,S/AS02 (Alonso et al. 2004; Stoute et al. 1997) suggest that greater than 90% reductions in liver parasites are required for protection in the field.

This trial reports a novel design for field trials of interventions against malaria. The method of monitoring described here identifies a clinically relevant endpoint (febrile malaria) with a uniform case definition, and without the unpredictable element of health seeking behaviour involved in passive case detection. This approach will be suitable for further phase 2b field trials without requiring the larger sample sizes involved in passive case detection studies. This trial also suggests a threshold of T cell responses that pre-erythrocytic vaccines will need to exceed in order to provide measurable efficacy in

children, and raises the possibility that T cell inducing vaccines may show lower immunogenicity in higher transmission settings.

6 Malaria and Immunosuppression:

(The induction and persistence of T cell responses after natural exposure and vaccination is reduced by prior exposure to *Plasmodium falciparum* parasitaemia.)

6.1 Background

The vaccination regimen FFM ME-TRAP was not protective among children in the field, and the immunogenicity of vaccination appeared lower than seen previously (5.2.3). Some epidemiological observations suggest that cellular responses may be suppressed in malaria endemic areas (Floyd et al. 2002; Greenwood & Armstrong 1991; Greenwood et al. 1972), but it is not clear why. Malaria infected erythrocytes modulate dendritic cell function *in vitro*, inhibiting T helper cell induction (Urban et al. 1999; Wilson et al. 2006) and stimulating T regulatory cells (Riley et al. 2006), but the evidence that associates malaria with immunosuppression *in vivo* is limited. Routine childhood antibody inducing vaccinations appear to be effective in malaria endemic areas, and mostly unaffected by chemoprophylaxis (Bradley-Moore et al. 1985). There may be a transient suppression of cellular responses during acute infection in Thai adults (Ho et al. 1986; Walsh et al. 1995). Acute infection has also been associated with a loss of T cell inhibition of Epstein-Barr virus infected cells (Whittle et al. 1984), and there is a transient

suppression of vaccine induced antibody responses after inpatient treatment for malaria (Williamson & Greenwood 1978). However, when malaria naïve volunteers were exposed to very low parasitaemia, they rapidly acquired cellular responses (Pombo et al. 2002), although subsequent data has suggested that a drug effect may have accounted for the protection against malaria seen in association with these responses (Edstein et al. 2005). It may be that exposures of either longer duration or higher parasitaemia would instead suppress cellular responses. Longitudinal studies of the kinetics of cellular responses with individual data on malaria exposure are lacking.

However, other factors might also suppress cellular responses in malaria endemic areas. Severely malnourished children have a suppressed cellular response to mycobacterial antigens (Liebeschuetz et al. 2004). Severe malnutrition is rare, but mild malnutrition is common in malaria endemic areas, and might also lead to depressed cellular immunity. Concurrent helminth infections suppress or alter T cell responses by inducing a TH2 cytokine profile (Ramalingam et al. 2005). T cell responses to tetanus toxoid (Cooper et al. 1998; Nookala et al. 2004; Sabin et al. 1996) and BCG (Malhotra et al. 1999) may be inhibited by helminth infection.

A further analysis was designed to examine factors that might influence natural or vaccine induced acquisition of T cell responses, and factors influencing the subsequent attrition of those responses. Responses to the pre-erythrocytic antigen TRAP were measured in both actively vaccinated and control groups. Exposure to malaria and other parasites was studied throughout the year of follow up. In order to account for the influence of prior exposure, the ELISpot data was adjusted for ELISpot results from earlier time points.

6.2 Methods

6.2.1 Analysis.

ELISpot wells were assayed in duplicate, taking the mean and subtracting the negative control well result. Assays were discounted if the positive control was less than 50 spots, or the negative control greater than 20 spots for *ex vivo* ELISpots, or 40 spots for cultured ELISpots. Pools were summed to calculate total response. Results are presented per million incubated PBMC. Geometric means and 95% confidence intervals are reported, substituting 1 spot per well above background for blank wells (the lower limit of detection). T cell responses are compared using Students T test on log transformed results.

Multiple regression analysis used log transformed spot numbers. Peptide pool, previous ELISpot response, village and age as a categorical variable were first examined as explanatory variables. Where peptide pool, village and prior response were consistently significant predictors, all subsequent analysis of additional factors was adjusted by them. The additional factors nutritional status, gastrointestinal helminth infection, urinary schistosomiasis, eosinophilia (as a categorical variable with three levels), malaria parasitaemia (concurrent and previous) and episodes of febrile malaria (binary variables, present or absent) were examined. The coefficients derived from analysis of log transformed spot numbers were then multiplied by 100 to give the percentage change in response.

6.2.2 ELISpots and monitoring

ELISpot assays were described in 5.1.7, and monitoring for episodes of malaria was described in 5.1.8.

6.3 Results

6.3.1 Kinetics of responses

Vaccination with FFM ME-TRAP induced both *ex vivo* ELISpot measured responses (from a geometric mean of 9 spots per million, CI 7-11 to 55 per million, CI 45-67, p<0.0005) and cultured ELISpot measured responses (from a geometric mean of 55 spots per million, CI 44-68 to 240 per million, CI 200-290, p<0.0005). These vaccine induced responses constitute the first data set analysed (Figure 6.1)

There was also a significant rise in T cell responses during the malaria season among placebo vaccinees. *Ex vivo* responses rose from 10 spots per million (95% CI 8-12) before the malaria season (January 2005), to 23 spots per million (95% CI 18-28) in the malaria season (August 2005) p=0.0005. Resting memory (cultured) responses also rose significantly during the malaria season, from 53 per million (CI 42-66) in January 2005, to 97 per million (CI 79-120) in August 2005, p=0.0001. These naturally acquired responses make up the second data set.

Responses that had been induced by vaccination then fell over the following 9 months. *Ex vivo* responses fell from a geometric mean of 55 spots per million (CI 43-66) in early May 2005 (one week after vaccination) to 19 spots per million (CI 16-24) in January 2006 (9 months after vaccination), p<0.00005. However, resting memory responses were

preserved, from 234 spots per million in May 2005 (CI 194-281) to 213 (CI 169-269) in

January 2006, p=0.51. The attrition of responses during the 9 months after vaccination is

shown in Figure 6.1, data set 3.

Figure 6.1. T cell responses to vaccination identified by both *ex vivo* and cultured ELISpot, are displayed over time for each data set analysed.

Median, 25th and 75th quartile, 5th and 95% quartile and outlying results are given by box and whisker plots. Vaccination with FFM ME-TRAP induced *ex vivo* ELISpot and cultured responses (p<0.0005, p<0.0005, Data set 1). There was also a significant rise in T cell responses during the malaria season among placebo vaccinees (Data set 2, p<0.0005, p=0.001 for *ex vivo* and cultured responses respectively). Vaccine induced *ex vivo* response fell over the next 9 months. (Data set 3, p< 0.0005), but cultured responses were sustained (p=0.51).
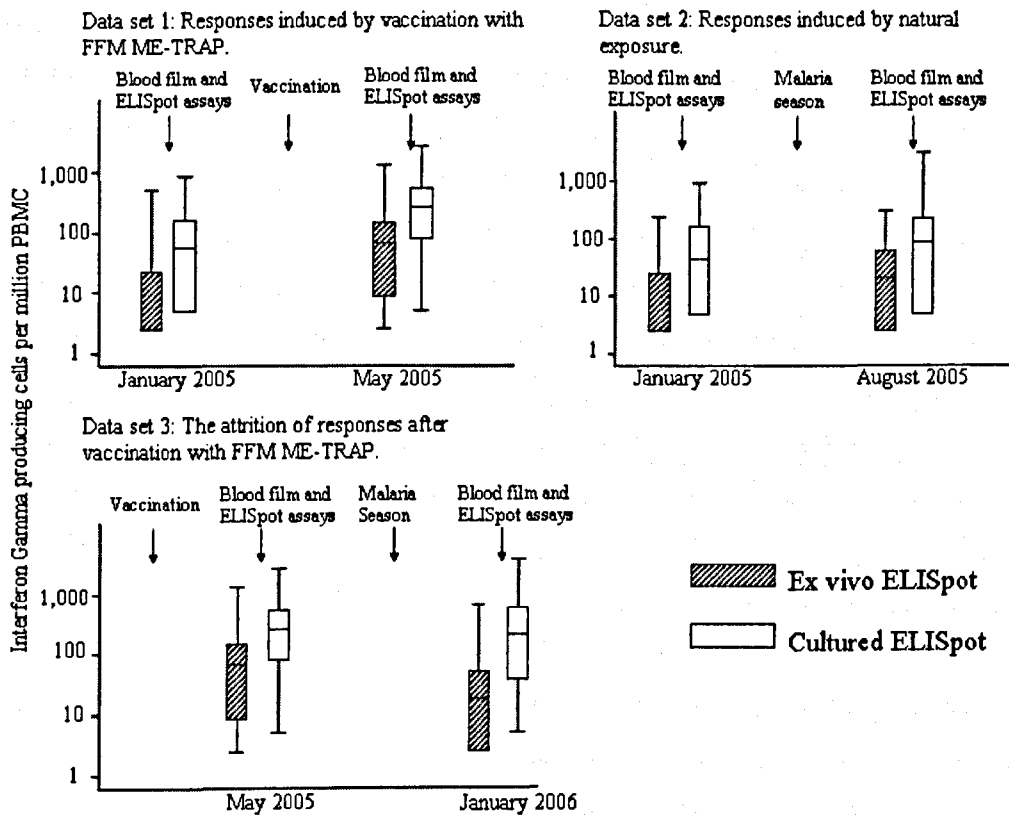
6.3.2 Multiple regression models to determine factors associated with variation of responses.

The responses at the second time point for each of the three data sets are modelled as outcomes, using the earlier T cell responses and blood film results at both time points as explanatory variables. The frequencies of other covariates in the population are given in Table 6.1, and the results of multiple regression models to identify explanatory variables for T cell responses are shown in Table 6.2(a-c).

Table 6.1: The frequencies of explanatory factors among study subjects. Percentages are given within each variable.

Variable	Factor	Data set 1 (vaccine induced responses)	Data set 2 (responses induced by natural exposure)	Data set 3 (responses 9 months after vaccination)
Village	Gongoni	34 (18%)	31 (18%)	35 (20%)
	Junju	47 (25%)	39 (23%)	40 (23%)
	Kolewa	51 (27%)	50 (30%)	44 (26%)
	Mapawa	37 (20%)	31 (18%)	35 (20%)
	Mwembe Tsungu	14 (7%)	15 (9%)	14 (8%)
MUAC	1st tertile	44 (24%)	45 (27%)	38 (22%)
	2nd tertile	54 (29%)	62 (37%)	53 (31%)
	3rd tertile	85 (46%)	59 (35%)	77 (45%)
GI worm	Infected	34 (23%)	41 (29%)	32 (24%)
	Uninfected	111 (77%)	96 (71%)	103 (76%)
Schist.	Infected	12 (8%)	7 (5%)	10 (7%)
	Uninfected	127 (92%)	117 (95%)	119 (93%)
Eosinophils	Norm	88 (48%)	79 (47%)	81 (48%)
	Mod.	53 (28%)	48 (28%)	54 (32%)
	High	25 (13%)	33 (19%)	27 (16%)
Febrile malaria	≥1 Episode.	24 (11%)	30 (20%)	38 (25%)
	No Episodes	194 (89%)	120 (80%)	114 (75%)
Previous blood film	Positive	123 (70%)	79 (50%)	72 (43%)
	Negative	52 (30%)	79 (50%)	72 (57%)
Current Blood film	Positive	78 (43%)	38 (23%)	47 (28%)
	Negative	102 (57%)	123 (77%)	117 (72%)

Abbreviations: Age cat.= age category; M. Tsungu = Mwembe Tsungu, MUAC = Mid Upper Arm Circumference, GI worm = Microscopy of Stool positive for helminth infection, Schist = Microscopy of urine positive for schistosomiasis, Mod.= eosinophil count above 0.5×10^6 per ml, High = above 1×10^6 per ml. Prev film = thick film positive for malaria parasites previously, Current Film= thick film positive for malaria parasites

currently, Feb Mal = Febrile Malaria = at least one episode of febrile malaria during surveillance previously.

6.3.3 Covariates

The pool of TRAP peptides that were tested, the child's village and prior immunity were all significant factors in each data set. Neither vaccine induced nor naturally acquired responses examined in data sets 1 and 2 varied by age. 9 months after vaccination, *ex vivo* responses in data set 3 were significantly better sustained among older children (2-7 year olds) compared with 1-2 year old children, but cultured responses did not vary.

The *ex vivo* T cell responses measured prior to vaccination predicted the *ex vivo* responses after vaccination (p=0.012). Cultured responses 9 months after vaccination were predicted by cultured responses one week after vaccination (p<0.0005), and *ex vivo* responses 9 months after vaccination were predicted by *ex vivo* responses one week after vaccination (p=0.009). However, neither cultured nor *ex vivo* responses during the dry season predicted naturally acquired responses seen during the rainy season.

Analysis for further factors was then adjusted for the covariates peptide pool, prior ELISpot results and village, but age category was not used as a covariate in further analysis.

6.3.4   Nutrition and helminth infection

T cell responses did not vary by nutritional status (as determined by Mid Upper Arm Circumference in January 2005). Parasitic infection was determined by stool and urine microscopy, and peripheral blood eosinophil counts (conducted in January 2005). There were no associations between T cell responses and microscopy results for urinary schistosomiasis or gastro-intestinal helminth infections, but eosinophilia was strongly associated with a greater attrition of *ex vivo* ELISpot responses, examined in data set 3 (p=0.006 by log likelihood ratio testing, Table 6.2c). Responses in eosinophilic subjects were 69-79% of the magnitude of responses without eosinophilia, Table 6.2c. However, cultured responses did not vary by eosinophilia, and eosinophilia did not alter vaccine induction of responses (Table 6.2a) or naturally acquired responses (Table 6.2b), examined by data sets 1 and 2 respectively.

6.3.5   Parasitaemia and malaria infection

Asymptomatic malaria parasitaemia as vaccination began was associated with a reduction in post vaccination T cell responses (i.e. data set 1) to 85% of the magnitude of non-parasitaemic children (p=0.09) by *ex vivo* assays and 79% (p=0.043) by cultured ELISpot assays (Table 7.2a). Natural acquired responses (data set 2) were also suppressed by previous parasitaemia, to 75% (p<0.0005) by *ex vivo* assays and 75% (p=0.032) by cultured assays (Table 7.2b). However, parasitaemia at the time blood was drawn for ELISpot assays did not reduce T cell responses for either vaccine induced or naturally acquired responses.

ex vivo responses to vaccination with FFM ME-TRAP were less well sustained among children previously parasitaemic (at 81%, p=0.002), with concurrent parasitaemia (69%, p=0.014) and with episodes of febrile malaria prior to the ELISpot assays (79% p=0.014). Cultured responses did not vary by asymptomatic parasitaemia, but were reduced to 72% by episodes of febrile malaria (p=0.04).

Further sub-group analysis (data not shown) did not suggest that higher parasitaemias were associated with greater reductions in T cell responses.

Figure 6.2 shows the frequencies of T cell responses by previous exposure to malaria parasitaemia, displayed for each peptide pool and for each data set studied.

Figure 6.2: Interferon gamma producing T cells are compared by prior exposure to malaria parasitaemia (paired box and whisker plots).

Responses to each of the three peptide pools are shown within each panel. *Ex vivo* assay results are shown in the upper row, cultured assay results in the lower row. The left column displays immediate vaccine induced responses left, naturally acquired responses are centre and sustained vaccine induced responses right. Prior exposure to malaria parasitaemia was associated with 15% (p=0.09) and 21% (p=0.043) respective reductions in *ex vivo* and cultured ELISpot immediate responses to vaccination, 25% (p<0.0005) and 25% (p=0.032) reductions following natural exposure and 19% (p=0.018) and no reduction (p=0.48) in sustained responses after vaccination.

138
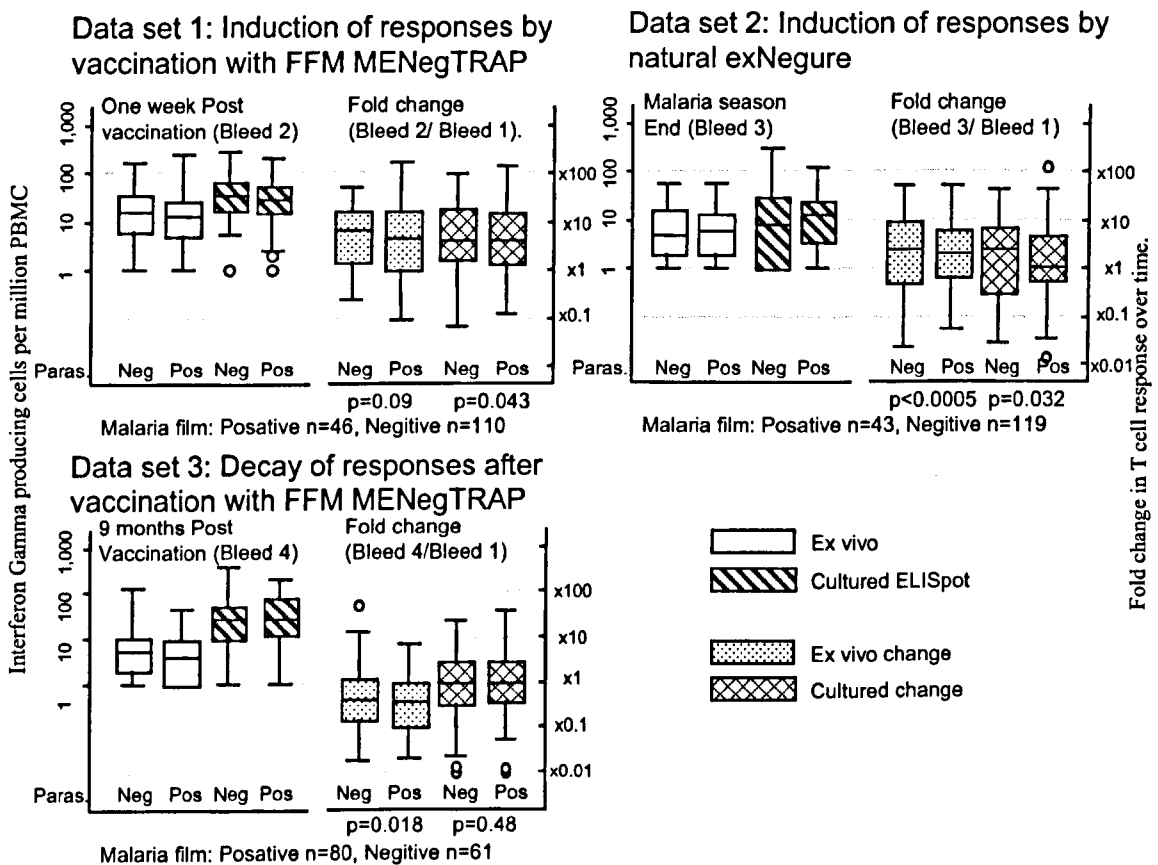
6.3.6 Combined multiple regression analysis for sustained ex vivo responses.

In data sets 1 and 2, only one factor significantly altered responses after adjusting for peptide pool, previous ELISpot results and village. However, several additional factors were predictors of the attrition of *ex vivo* responses after vaccination (Table 6.2c, data set 3), and so were entered into a further multiple regression analysis. The effect of eosinophilia was still highly significant (p=0.0005, with reductions in response to 63% and 79% respectively for moderate and high eosinophilia). Concurrent parasitaemia (74%, p=0.001), previous parasitaemia (85%, p=0.048) and episodes of febrile malaria (81%, p=0.033) were also independently associated with *ex vivo* ELISpot results.

6.3.7 Protection against episodes of malaria

Vaccination with FFM ME-TRAP was not protective, and neither *ex vivo* nor cultured ELISpot results predicted protection within FFM ME-TRAP vaccinees (5.2.4). The naturally acquired T cell responses were not protective, either for *ex vivo* (IRR=1.17 for each 10 fold rise in T cell numbers, 95% CI 0.7-2) or cultured ELISpot (IRR=0.84, 95% CI 0.5-1.3, p=0.48).

6.3.8 Relationship of ex vivo to cultured responses.

Although both cultured and *ex vivo* responses did not correlate among unvaccinated children (r=-0.017, p=0.71), there was a significant association between cultured and *ex vivo* responses one week after vaccination, in May 2005 (r=0.18, p<0.00005 unadjusted, coefficient=0.14 p=0.006 when adjusted for peptide pool and village).

139

Table 6.2a Results of multiple regression model to examine factors influencing the acquisition of T cell responses after vaccination (Data set 1). The average effect associated with each factor is given as a percentage relative to individuals without that factor for each variable. (For instance, responses to the middle region of the TRAP molecule are, on average, 60% of the responses seen to the N terminal portion). Where more than two options exist for a given factor, a log likelihood ratio test (LR test) is given for the overall significance of that factor in the model.

Factor	Ex vivo Coefficient	p value	LR test	Cultured Coefficient	p value	LR test
Peptides						
N terminal	100%		<0.00005	100%		<0.00005
Middle	60% (48-75%)	<0.0005		50% (38-64%)	<0.0005	
C terminal	91% (74-112%)	0.4		74% (56-56%)	0.016	
Age cat.						
1-2 years	100%		0.2	100%		0.1
2-5 years	109% (87-138%)	0.41		79% (6-97%)	<0.076	
5-7 years	125% (97-165%)	0.086		97% (7-128%)	0.79	
Prev. ex vivo						
(January 2005)	141% (108-189%)	0.012		75% (56-102%)	0.09	
Prev. cult.	109% (89-134%)	0.362		117% (91-147%)	0.22	
Village						
Gongoni	100%		0.01	100%		<0.00005
Junju	91% (69-12%)	0.51		93% (69-127%)	0.66	
Kolewa	72% (54-93%)	0.013		69% (51-95%)	0.024	
Mapawa	72% (53-95%)	0.019		47% (34-67%)	0.0005	
M. Tsungu	104% (72-154%)	0.8		138% (91-213%)	0.123	
MUAC						
1st tertile	100%		0.59	100%		0.38
2nd tertile	109% (71-141%)	0.45		83% (63-109%)	0.2	
3rd tertile	112% (89-141%)	0.32		95% (74-123%)	0.73	
GI worm	117% (57-151%)	0.136		81% (61-107%)	0.13	
Schist.	151% (103-218%)	0.03		85% (56-125%)	0.42	
Eosin.						
Normal	100%		0.12	100%		0.48
Mod.	134% (99-181%)	0.06		114% (83-162%)	0.38	
High	112% (85-147%)	0.39		12% (89-162%)	0.23	
Prev. Film (January 2005)	85% (69-102%)	0.09		79% (63-99%)	0.043	
Current film (May 2005)	95% (79-112%)	0.57		99% (79-117%)	0.7	
Febrile malaria (Jan-May 2005)	102% (77-134%)	0.86		99% (72-134%)	0.97	

Table 6.2b Results of multiple regression model to examine factors influencing the acquisition of T cell responses after natural exposure (Data set 2). The average response associated with each factor is given as a percentage relative to the comparison for each variable. Where more than two options exist for a given factor, a log likelihood ratio test (LR test) is given for the factors addition to the model.

Factor	*Ex vivo* Coefficient	p value	LR test	Cultured Coefficient	p value	LR test
Peptides						
N terminal	100%		0.0028	100%		0.11
Middle	75% (63-91%)	0.005		74% (57-98%)	0.038	
C terminal	125% (85-123%)	0.74		89% (67-115%)	0.357	
Age cat.						
1-2 years	100%		0.09	100%		0.88
2-5 years	95% (79-119%)	0.73		98% (74-128%)	0.88	
5-7 years	79% (61-101%)	0.064		93% (67-128%)	0.63	
Prev. *ex vivo* (January 2005)	75% (56-102%)	0.09		117% (85-158%)	0.34	
Prev. cult.	117% (91-147%)	0.22		125% (97-162%)	0.079	
Village						
Gongoni	100%		<0.00005	100%		<0.00005
Junju	75% (6-93%)	0.01		98% (74-131%)	0.89	
Kolewa	151% (123-19%)	<0.0005		43% (32-58%)	<0.0005	
Mapawa	141% (109-181%)	0.007		37% (26-51%)	<0.0005	
M. Tsungu	112% (83-151%)	0.43		77% (5-12%)	0.255	
MUAC						
1st tertile	100%		0.27	100%		0.11
2nd tertile	109% (93-128%)	0.27		77% (58-99%)	0.046	
3rd tertile	97% (83-114%)	0.71		93% (7-121%)	0.59	
GI worm	98% (12-117%)	0.86		83% (64-107%)	0.16	
Schist.	109% (75-154%)	0.62		107% (64-173%)	0.78	
Eosinophils						
Normal	100%			100%		
Mod.	107% (85-134%)	0.53	0.81	89% (66-123%)	0.478	0.32
High	128% (83-125%)	0.8		81% (61-107%)	0.14	
Prev. Film (May 2005)	75% (64-87%)	<0.0005		75% (57-97%)	0.032	
Current film (August 2005)	95% (79-114%)	0.62		91% (72-117%)	0.465	
Febrile malaria (May-Aug 2005)	102% (83-125%)	0.828		91% (7-12%)	0.51	

Table 6.2c Results of multiple regression model to examine factors influencing the attrition of T cell responses after vaccination (Data set 3). The average response associated with each factor is given as a percentage relative to the comparison for each variable. Where more than two options exist for a given factor, a log likelihood ratio test (LR test) is given for the factors addition to the model.

Factor	*Ex vivo* Coefficient	p value	LR test	Cultured Coefficient	p value	LR test
Peptides						
N terminal	100%			100%		
Middle	66% (53-81%)	<0.0005	0.0003	69% (48-66%)	0.028	0.038
C terminal	21% (69-104%)	0.134		69% (48-64%)	0.027	
Age cat.						
1-2 years	100%			100%		
2-5 years	128% (91-144%)	0.198	0.12	169% (74-151%)	0.775	0.019
5-7 years	97% (74-123%)	0.776		165% (108-251%)	0.02	
Prev. *ex vivo* (May 2005)	138% (114-165%)	<0.0005		154% (39-169%)	0.155	
Prev. Cult.	93% (81-108%)	0.384		141% (109-181%)	0.009	
Village						
Gongoni	100%			100%		
Junju	103% (14-129%)	0.78	<0.00005	151% (102-223%)	0.039	0.0006
Kolewa	154% (123-198%)	<0.0005		89% (57-134%)	0.59	
Mapawa	199% (157-263%)	<0.0005		69% (44-102%)	0.075	
M. Tsungu	144% (104-199%)	0.025		74% (39-134%)	0.3	
MUAC						
1st tertile	100%			100%		
2nd tertile	112% (89-141%)	0.31	0.5	114% (79-169%)	0.45	0.44
3rd tertile	101% (82-125%)	0.89		93% (66-128%)	0.67	
GI worm	95% (77-117%)	0.66		113% (77-165%)	0.51	
Schist.	120% (89-165%)	0.228		77% (42-144%)	0.43	
Eosinophils						
Normal	100%			100%		
Mod.	69% (53-87%)	0.002	0.006	75% (50-120%)	0.26	0.337
High	79% (63-99%)	0.043		70% (48-112%)	0.15	
Prev. Film (Aug 2005)	81% (69-95%)	0.018		111% (85-147%)	0.48	
Current film time assay (Jan 2006)	69% (58-83%)	<0.0005		112% (91-165%)	0.2	
Febrile malaria (May 2005-Jan 2006)	79% (66-95%)	0.014		72% (52-98%)	0.04	

Peptides give the different peptide pools, grouped by region of TRAP from which they were derived. Abbreviations: Age cat.= age category; M. Tsungu = Mwembe Tsungu, MUAC = Mid Upper Arm Circumference, GI worm = Stool microscopy positive for helminth infection, Schist = Urine microscopy positive for schistosome ova, Mod.= eosinophil count above $0.5x10^6$ per ml, High = above $1x10^6$ per ml. Prev film = thick film positive for malaria parasites previously, Current Film= thick film positive for malaria parasites currently, Febrile malaria = at least one episode of febrile malaria during surveillance previously, Prev *ex vivo* = T cell responses detected by *ex vivo* ELISpot on prior blood tests, Prev Cult = T cell responses detected by cultured ELISpot on prior blood test.

6.4   Discussion

Parasitaemia at the start of monitoring, but not parasitaemia at the time of the ELISpot assay inhibited the acquisition of T cell responses in data set 1 (responses acquired after vaccination) and data set 2 (responses acquired after natural infection). The data sets are demonstrated in figure 6.1, and the effect of parasitaemia in figure 6.2. This might reflect an effect of parasitaemia *per se* at the time point immediately prior to vaccination, or simply that more highly exposed children are both parasitaemic and immunosuppressed. However, concurrent parasitaemia would be equally strongly linked to overall exposure, but did not influence T cell responses. Furthermore malaria episodes during the time of monitoring were not associated with lower T cell responses in data sets 1 and 2.

6.4.1   Potential mechanisms

By what mechanism might early parasitaemia but not parasitaemia at the time of the assay for cellular responses influence T cell induction? Children received priming vaccinations 2 weeks after the earlier blood films, and boosting vaccinations one week prior to the concurrent blood films. In the cohort studied for natural exposure, malaria transmission was high throughout the interval between blood film examinations. Parasitaemia might inhibit T cell priming (in both vaccination and natural exposure), but not the subsequent boosting or recall of responses.

However, concurrent parasitaemia, earlier parasitaemia and episodes of febrile malaria were all independently associated with a more rapid attrition of *ex vivo* T cell responses (data set 3). Eosinophilia was not associated with altered vaccine immunogenicity or the acquisition of natural immunity, but was strongly associated with greater attrition of *ex vivo* ELISpot responses after vaccination (cultured ELISpot responses were not altered). Furthermore, although village significantly influenced all three data sets, the effects were not in consistent directions for the induction (Data sets 1 and 2) and the attrition of responses (data set 3). Taken together, these data suggest that T cell priming and memory are suppressed by different factors.

The priming of T cells responses depends on dendritic cells, whereas subsequent memory appears to be independent of dendritic cells (Kassiotis & Stockinger 2004), but is dependent on cytokines such as IL 7 and IL 15 (Geginat et al. 2001; Kaech et al. 2003). Previous parasitaemia may act on dendritic cells to inhibit T cell priming, but intercurrent febrile malaria, parasitaemia, and the TH2 responses associated with eosinophilia may

alter the cytokine milieu required to support memory cells. The inflammatory cytokine response during malaria infection in mice depletes memory cells (Xu et al. 2002).

6.4.2 Accuracy of estimates

The results are not due to confounding by T cell responses protecting against infection. Previous studies have demonstrated that the *ex vivo* responses to TRAP raised by natural infection are not associated with protection, and in this study neither cultured nor *ex vivo* detected responses were protective. Malaria exposure led to subsequently reduced T cell responses, but T cell responses were not prospectively associated with a lower malaria risk.

The 15-30% reductions in T cell responses in parasitaemic children are likely to be an underestimate of the overall immunsuppression caused by malaria in endemic areas. Parasitaemia below the level of blood film detection is frequent in endemic areas (Ndao et al. 2004), and some children may have cleared their parasitaemia soon after the film was taken. It is likely that the majority of children in the study (film negative and positive) were exposed to malaria, but those with positive blood films had a greater suppression of cellular responses.

The ELISpot assay allows accurate, reproducible and high through-put measurement of T cell responses (Tassignon et al. 2005). Many of the quite low frequencies of T cell identified in this study would not have been above background reactivity in standard intracellular cytokine staining assays for use with FACS. Although I did not confirm the phenotype of responding cells, previous work has shown that both natural infection

(Flanagan et al. 2003) and vaccination (Moorthy et al. 2004a) induce primarily CD4 responses.

6.4.3 Helminth infections

Neither gastrointestinal helminth infection nor urinary schistosomiasis was associated with altered cellular immunity. However, only single specimens of stool and urine were examined, and children with lower parasite burdens might have been identified by repeated specimens or concentration techniques. 50% of children had either moderate or high eosinophilia, compared with a prevalence of 7% for urinary schistosomiasis and 25% for gastro-intestinal helminth infection. The higher prevalence may reflect infections missed by microscopy, but also could be due to filarial infection (night time blood films were not performed). The only common cause of eosinophilia among children in Africa is helminth infection, and eosinophilia may be a more discriminatory marker for altered immunity, since it is more closely associated with the TH2 cytokine responses than the presence of absence of parasites (Akdis et al. 2003). Since eosinophilia was associated with greater attrition of cellular responses, it is very likely that helminths do play a role, and more intensive examination might identify the parasites involved.

6.4.4 Conclusions

Malaria parasitaemia and eosinophilia should be considered in assessing the immunogenicity and efficacy of experimental and routine (i.e. BCG) T cell inducing vaccinations. T cell inducing vaccines are in development for diverse pathogens, including HIV (Mwau et al. 2004) and TB (McShane et al. 2004). Efficacy may vary

according to malaria endemicity, and clinical development plans should include trials in high transmission areas. Although vaccine efficacy was seen only in children not pre-treated with anti-malarial drugs in one trial (Genton et al. 2002) , the influence of parasitaemia on efficacy in a particular trial design may not correlate with immunogenicity. I observed that the immunogenicity of prime-boost vaccination was lower in a population with more frequent blood stage malaria infection (5.2.3). The inhibition of T helper cells might also explain the poorly sustained antibody responses to blood stage antigens seen among children in malaria endemic areas (Kinyanjui et al. 2003). Since Intermittent Presumptive Treatment with antimalarials in infants may become public health policy, the interaction between antimalarial use and vaccine immunogenicity and efficacy may soon be of practical importance.

7 Concluding Remarks

Although there were encouraging indications that the vaccination regimen FFM ME-TRAP was partially protective in experimental studies of sporozoite challenge in the UK (section 2.3.1), and that it was safe and immunogenic in phase 1 studies in Kenya, the regimen did not subsequently protect 1-6 year old children against clinical disease. Since the immunogenicity of vaccination in the efficacy study was lower than that seen previously (5.2.3), it is unclear whether similar reductions in liver parasites to those seen in sporozoite challenge might have occurred without a reduction in clinical episodes, but similar reductions might have been expected among children with the highest T cell responses in the efficacy study. However, taking these findings together with data from comparable sporozoite challenge studies and field efficacy studies for RTS,S/AS02 (Alonso et al. 2004; Stoute et al. 1997), it is probable that vaccines will need to achieve greater than 90% reductions in pre-erythrocytic parasites in order to see protection in the field. There is evidence that the reduction in pre-erythrocytic parasites in sporozoite challenge studies is proportional to immunogenicity (Webster et al. 2005). Given that 90% reductions in pre-erythrocytic parasites have already been seen, it is possible that even modest increases in immunogenicity will result in more frequent fully protected volunteers.

7.1 Immunogenicity

Immunogenicity also needs to be documented in the target population before conducting a larger trial to establish efficacy. The immunogenicity was lower among children in Kenya than had been seen in naïve volunteers, and the data presented in section 6.3.5 suggests that malaria infection may have contributed to this. In the development plan of future vaccination regimens, immunogenicity should be documented under different malaria transmission intensities, and in children with and without asymptomatic parasitaemia.

In phase 1 trials, I showed that novel, alternating vector vaccinations result in greater memory responses than classic prime-boost vaccinations. The longevity of protection is critical to the utility of any vaccination. Since prime boost vaccinations are in clinical development for other pathogens such as TB (McShane et al. 2004), HIV (Mwau et al. 2004) and malignancy (Greiner et al. 2002), further study should investigate whether the enhanced memory can be achieved with other vector and antigen combinations.

7.2 Batch variability

I showed a variation in immunogenicity and reactogenicity for one batch of FP9 ME-TRAP. The reasons for variability were not related to vaccine titre or loss of potency over time. Further *in vitro* characterisation of pox virus preparations is underway. A single seed lot of virus had been used to generate the batches of FP9 ME-TRAP used in this study, and the growth time to produce the virus required in these studies was not of

sufficient length to allow significant genetic variation of the virus, as has been when very large scale production is conducted (Sauerbrei et al. 2006). Although electron microscopy studies did not identify significant differences in the batches of FP9 ME-TRAP used here, the technique examines only a few virus particles. Studies to measure virus particle agglutination using a novel adaption of flow cytometry will allow a more thorough characterisation. Clinically insignificant variation between lots of commercially licensed attenuated vaccines has been identified (Lieberman et al. 2006). There is no evidence of variable immunogenicity or reactogenicity by batch of MVA vaccines. Batch variability may be confined to FP9, but data are limited. The clinical development plans of new virally vectored vaccine regimens will need to consider the possibility of significant batch variability at an appropriate stage.

7.3 Other pre-erythrocytic vaccinations in development

7.3.1 Adenovirus

Similar viral vectored regimens have been used to induce T cells reactive to HIV antigens (Mwau et al. 2004). However, regimens using adenovirus appear to have induced the strongest T cell responses thus far (Duerr et al. 2006). Wild type adenovirus is pathogenic in humans. Instead of the multiple passage used to attenuate MVA, specific genes were deleted to render the virus replication defective (Santra et al. 2005). Adenovirus vectored regimens are more immunogenic when given after a DNA prime (Xin et al. 2005).

However, immunity to adenovirus from natural exposure is common, particularly in Africa (Nwanegbo et al. 2004). Antibodies to adenovirus limit the vector's

immunogenicity in animal models (Lemckert et al. 2005), and I have presented data here showing the immunogenicity of recombinant MVA was reduced by prior anti-vector immunity. Studies in progress in humans using adenovirus vectored vaccinations have selected subjects with low antibody titres to the vector. However, there are a large number of simian adenoviruses, with varying degrees of sequence homology (Avvakumov et al. 2004). Commercial development of these vectors is underway. Since there is no evidence of cross-reactivity between adenovirus and MVA, these vectors would be suitable for prime boost vaccinations. This approach may further improve immunogenicity.

7.3.2 RTS,S

The other approach to pre-erythrocytic vaccination against malaria in field trials uses the adjuvanted particulate protein vaccine, RTS,S (GlaxoSmithKline Biologicals, Rixensart, Belgium). This comprises the carboxyl terminal (a.a. 207-395) of the 3D7 circumsporozoite protein fused to the hepatitis B surface antigen, co-expressed in yeast with the non-fused hepatitis B surface antigen. When given with a proprietary adjuvant AS02A, an oil-in-water emulsion using the two immunostimulants QS21 and MPL, both antibody and cellular responses were induced (Lalvani et al. 1999). It is unclear whether antibodies, cellular responses, or both mediate protection in sporozoite challenge studies (Stoute et al. 1997; Sun et al. 2003). Field trials have demonstrated short-lived efficacy in adults (Bojang et al. 2001), and 30% efficacy against febrile malaria in children (Alonso et al. 2004). The efficacy appears to be durable (Alonso et al. 2005), and further studies are in progress to optimise the adjuvant used (Stewart et al. 2006). Further phase 2b field trials with the new adjuvant are planned, and these are likely to follow similar methodology in defining cases of febrile malaria to that used in section 5.1.8. It is likely this will lead to a phase 3 trial in the near future.

7.4 In conclusion

Although the regimen FFM ME-TRAP that I tested was not protective against febrile malaria in children, the data acquired in the course of phase 1 and 2 studies will inform the development of the next generation of T cell inducing vaccinations against malaria. This is likely to involve more immunogenic vectors, studies examining batch variability, single vector and alternating vector regimens, and careful attention to the immunogenicity of vaccination in the field.

8   References

Aidoo M, Lalvani A, Allsopp CE, Plebanski M, Meisner SJ, Krausa P, Browning M, Morris-Jones S, Gotch F, Fidock DA, et al. Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. **Lancet 1995**; Vol.345 (8956), pp1003-7.

Akdis CA, Kussebi F, Pulendran B, Akdis M, Lauener RP, Schmidt-Weber CB, Klunker S, Isitmangil G, Hansjee N, Wynn TA, Dillon S, Erb P, Baschang G, Blaser K, Alkan SS. Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. **Eur J Immunol 2003**; Vol.33 (10), pp2717-26.

Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, Sigauque B, Milman J, Mandomando I, Bassat Q, Guinovart C, Espasa M, Corachan S, Lievens M, Navia MM, Dubois MC, Menendez C, Dubovsky F, Cohen J, Thompson R, Ballou WR. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. **Lancet 2005**; Vol.366 (9502), pp2012-8.

Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, Mandomando I, Spiessens B, Guinovart C, Espasa M, Bassat Q, Aide P, Ofori-Anyinam O, Navia MM, Corachan S, Ceuppens M, Dubois MC, Demoitie MA, Dubovsky F, Menendez C, Tornieporth N, Ballou WR, Thompson R, Cohen J. Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. **Lancet 2004**; Vol.364 (9443), pp1411-20.

Anderson RJ, Hannan CM, Gilbert SC, Laidlaw SM, Sheu EG, Korten S, Sinden R, Butcher GA, Skinner MA, Hill AV. Enhanced CD8+ T cell immune responses and protection elicited against Plasmodium berghei malaria by prime boost immunization regimens using a novel attenuated fowlpox virus. **J Immunol 2004**; Vol.172 (5), pp3094-100.

Andrews L, Andersen RF, Webster D, Dunachie S, Walther RM, Bejon P, Hunt-Cooke A, Bergson G, Sanderson F, Hill AV, Gilbert SC. Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials. **Am J Trop Med Hyg 2005**; Vol.73 (1), pp191-8.

Appawu M, Owusu-Agyei S, Dadzie S, Asoala V, Anto F, Koram K, Rogers W, Nkrumah F, Hoffman SL, Fryauff DJ. Malaria transmission dynamics at a site in northern Ghana proposed for testing malaria vaccines. **Trop Med Int Health 2004**; Vol.9 (1), pp164-70.

Avvakumov N, Kajon AE, Hoeben RC, Mymryk JS. Comprehensive sequence analysis of the E1A proteins of human and simian adenoviruses. **Virology 2004**; Vol.329 (2), pp477-92.

Badovinac VP, Messingham KA, Jabbari A, Haring JS, Harty JT. Accelerated CD8(+) T-cell memory and prime-boost response after dendritic-cell vaccination. **Nat Med 2005**; Vol.11 (7), pp748-56.

Badovinac VP, Porter BB, Harty JT. CD8+ T cell contraction is controlled by early inflammation. **Nat Immunol 2004**; Vol.5 (8), pp809-17.

Baird JK, Owusu Agyei S, Utz GC, Koram K, Barcus MJ, Jones TR, Fryauff DJ, Binka FN, Hoffman SL, Nkrumah FN. Seasonal malaria attack rates in infants and young children in northern Ghana. **Am J Trop Med Hyg 2002**; Vol.66 (3), pp280-6.

Beier JC, Beier MS, Vaughan JA, Pumpuni CB, Davis JR, Noden BH. Sporozoite transmission by Anopheles freeborni and Anopheles gambiae experimentally infected with Plasmodium falciparum. **J Am Mosq Control Assoc 1992**; Vol.8 (4), pp404-8.

Bertoletti A, Costanzo A, Chisari FV, Levrero M, Artini M, Sette A, Penna A, Giuberti T, Fiaccadori F, Ferrari C. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. **J Exp Med 1994**; Vol.180 (3), pp933-43.

Birkett A, Lyons K, Schmidt A, Boyd D, Oliveira GA, Siddique A, Nussenzweig R, Calvo-Calle JM, Nardin E. A modified hepatitis B virus core particle containing multiple epitopes of the Plasmodium falciparum circumsporozoite protein provides a highly immunogenic malaria vaccine in preclinical analyses in rodent and primate hosts. **Infect Immun 2002**; Vol.70 (12), pp6860-70.

Blanchard TJ, Alcami A, Andrea P, Smith GL. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. **J Gen Virol 1998**; Vol.79 (Pt 5), pp1159-67.

Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, Ballou WR, Conway DJ, Reece WH, Gothard P, Yamuah L, Delchambre M, Voss G, Greenwood BM, Hill A, McAdam KP, Tornieporth N, Cohen JD, Doherty T. Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. **Lancet 2001**; Vol.358 (9297), pp1927-34.

Bradley-Moore AM, Greenwood BM, Bradley AK, Bartlett A, Bidwell DE, Voller A, Craske J, Kirkwood BR, Gilles HM. Malaria chemoprophylaxis with chloroquine in young Nigerian children. II. Effect on the immune response to vaccination. **Ann Trop Med Parasitol 1985**; Vol.79 (6), pp563-73.

Brichacek B, Swindells S, Janoff EN, Pirruccello S, Stevenson M. Increased plasma human immunodeficiency virus type 1 burden following antigenic challenge with pneumococcal vaccine. **J Infect Dis 1996**; Vol.174 (6), pp1191-9.

Bruna-Romero O, Rocha CD, Tsuji M, Gazzinelli RT. Enhanced protective immunity against malaria by vaccination with a recombinant adenovirus encoding the circumsporozoite protein of Plasmodium lacking the GPI-anchoring motif. **Vaccine 2004**; Vol.22 (27-28), pp3575-84.

Brustoski K, Moller U, Kramer M, Hartgers FC, Kremsner PG, Krzych U, Luty AJ. Reduced cord blood immune effector-cell responsiveness mediated by CD4+ cells induced in utero as a consequence of placental Plasmodium falciparum infection. **J Infect Dis 2006**; Vol.193 (1), pp146-54.

Burton P, Gurrin L, Sly P. Extending the simple linear regression model to account for correlated responses: an introduction to generalized estimating equations and multi-level mixed modelling. **Stat Med 1998**; Vol.17 (11), pp1261-91.

Carroll MW, Moss B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. **Virology 1997**; Vol.238 (2), pp198-211.

Casey CG, Iskander JK, Roper MH, Mast EE, Wen XJ, Torok TJ, Chapman LE, Swerdlow DL, Morgan J, Heffelfinger JD, Vitek C, Reef SE, Hasbrouck LM, Damon I, Neff L, Vellozzi C, McCauley M, Strikas RA, Mootrey G. Adverse events associated with smallpox vaccination in the United States, January-October 2003. **Jama 2005**; Vol.294 (21), pp2734-43.

Cavacini LA, Duval M, Eder JP, Posner MR. Evidence of determinant spreading in the antibody responses to prostate cell surface antigens in patients immunized with prostate-specific antigen. **Clin Cancer Res 2002**; Vol.8 (2), pp368-73.

Cavanagh DR, Dodoo D, Hviid L, Kurtzhals JA, Theander TG, Akanmori BD, Polley S, Conway DJ, Koram K, McBride JS. Antibodies to the N-terminal block 2 of Plasmodium falciparum merozoite surface protein 1 are associated with protection against clinical malaria. **Infect Immun 2004**; Vol.72 (11), pp6492-502.

Cheng Q, Lawrence G, Reed C, Stowers A, Ranford-Cartwright L, Creasey A, Carter R, Saul A. Measurement of Plasmodium falciparum growth rates in vivo: a test of malaria vaccines. **Am J Trop Med Hyg 1997**; Vol.57 (4), pp495-500.

Cooper PJ, Espinel I, Paredes W, Guderian RH, Nutman TB. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10. **J Infect Dis 1998**; Vol.178 (4), pp1133-8.

Corbin GA, Harty JT. T cells undergo rapid ON/OFF but not ON/OFF/ON cycling of cytokine production in response to antigen. **J Immunol 2005**; Vol.174 (2), pp718-26.

Daubersies P, Thomas AW, Millet P, Brahimi K, Langermans JA, Ollomo B, BenMohamed L, Slierendregt B, Eling W, Van Belkum A, Dubreuil G, Meis JF, Guerin-Marchand C, Cayphas S, Cohen J, Gras-Masse H, Druilhe P, Mohamed LB. Protection against Plasmodium falciparum malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3. **Nat Med 2000**; Vol.6 (11), pp1258-63.

Doolan DL, Hoffman SL. Pre-erythrocytic-stage immune effector mechanisms in Plasmodium spp. infections. **Philos Trans R Soc Lond B Biol Sci 1997**; Vol.352 (1359), pp1361-7.

Druilhe P SF, Corradin G, Audran R, Singh S, Bouzidi A. Safety and Immunogenicity of the Merozoite surface Protein 3 antigen in phase 1 trials and assessment of the biological effect of the antibodies induced. **MIM conference, 2002 2002.**

Druilhe P, Spertini F, Soesoe D, Corradin G, Mejia P, Singh S, Audran R, Bouzidi A, Oeuvray C, Roussilhon C. A malaria vaccine that elicits in humans antibodies able to kill Plasmodium falciparum. **PLoS Med 2005**; Vol.2 (11), ppe344.

Duerr A, Wasserheit JN, Corey L. HIV vaccines: new frontiers in vaccine development. **Clin Infect Dis 2006**; Vol.43 (4), pp500-11.

Dunachie SJ, Walther M, Vuola JM, Webster DP, Keating SM, Berthoud T, Andrews L, Bejon P, Poulton I, Butcher G, Watkins K, Sinden RE, Leach A, Moris P, Tornieporth N, Schneider J, Dubovsky F, Tierney E, Williams J, Gray Heppner D, Jr., Gilbert SC, Cohen J, Hill AV. A clinical trial of prime-boost immunisation with the candidate malaria vaccines RTS,S/AS02A and MVA-CS. **Vaccine 2006**; Vol.24 (15), pp2850-9.

Edstein MD, Kotecka BM, Anderson KL, Pombo DJ, Kyle DE, Rieckmann KH, Good MF. Lengthy antimalarial activity of atovaquone in human plasma following atovaquone-proguanil administration. **Antimicrob Agents Chemother 2005**; Vol.49 (10), pp4421-2.

Egan JE, Weber JL, Ballou WR, Hollingdale MR, Majarian WR, Gordon DM, Maloy WL, Hoffman SL, Wirtz RA, Schneider I, et al. Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. **Science 1987**; Vol.236 (4800), pp453-6.

Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, Takala A, Kayhty H, Karma P, Kohberger R, Siber G, Makela PH. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. **N Engl J Med 2001**; Vol.344 (6), pp403-9.

Flanagan KL, Lee EA, Gravenor MB, Reece WH, Urban BC, Doherty T, Bojang KA, Pinder M, Hill AV, Plebanski M. Unique T cell effector functions elicited by Plasmodium falciparum epitopes in malaria-exposed Africans tested by three T cell assays. **J Immunol 2001**; Vol.167 (8), pp4729-37.

Flanagan KL, Mwangi T, Plebanski M, Odhiambo K, Ross A, Sheu E, Kortok M, Lowe B, Marsh K, Hill AV. Ex vivo interferon-gamma immune response to thrombospondin-related adhesive protein in coastal Kenyans: longevity and risk of Plasmodium falciparum infection. **Am J Trop Med Hyg 2003**; Vol.68 (4), pp421-30.

Flanagan KL, Plebanski M, Akinwunmi P, Lee EA, Reece WH, Robson KJ, Hill AV, Pinder M. Broadly distributed T cell reactivity, with no immunodominant loci, to the pre-erythrocytic antigen thrombospondin-related adhesive protein of Plasmodium falciparum in West Africans. **Eur J Immunol 1999**; Vol.29 (6), pp1943-54.

Flanagan KL, Plebanski M, Odhiambo K, Sheu E, Mwangi T, Gelder C, Hart K, Kortok M, Lowe B, Robson KJ, Marsh K, Hill AV. Cellular reactivity to the P. falciparum protein TRAP in adult Kenyans: novel epitopes, complex cytokine patterns, and the impact of natural antigenic variation. **Am J Trop Med Hyg 2006**; Vol.74 (3), pp367-75.

Floyd S, Ponnighaus JM, Bliss L, Nkhosa P, Sichali L, Msiska G, Fine PE. Kinetics of delayed-type hypersensitivity to tuberculin induced by bacille Calmette-Guerin vaccination in northern Malawi. **J Infect Dis 2002**; Vol.186 (6), pp807-14.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B. Genome sequence of the human malaria parasite Plasmodium falciparum. **Nature 2002**; Vol.419 (6906), pp498-511.

Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. **J Exp Med 2001**; Vol.194 (12), pp1711-9.

Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D, Irving DO, Smith TA, Beck HP, Alpers MP. A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. **J Infect Dis 2002**; Vol.185 (6), pp820-7.

Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, Kwiatkowski D, Greenwood BM, Whittle HC, Hill AV. Association of malaria parasite population structure, HLA, and immunological antagonism. **Science 1998**; Vol.279 (5354), pp1173-7.

Gilbert SC, Plebanski M, Harris SJ, Allsopp CE, Thomas R, Layton GT, Hill AV. A protein particle vaccine containing multiple malaria epitopes. **Nat Biotechnol 1997**; Vol.15 (12), pp1280-4.

Godkin AJ, Thomas HC, Openshaw PJ. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. **J Immunol 2002**; Vol.169 (4), pp2210-4.

Good MF, Zevering Y, Currier J, Bilsborough J. 'Original antigenic sin', T cell memory, and malaria sporozoite immunity: an hypothesis for immune evasion. **Parasite Immunol 1993**; Vol.15 (4), pp187-93.

Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, Heppner DG, Yuan G, Hollingdale M, Slaoui M, et al. Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine. **J Infect Dis 1995**; Vol.171 (6), pp1576-85.

Graves P, Gelband H. Vaccines for preventing malaria. **Cochrane Database Syst Rev 2003**; (1), ppCD000129.

Greenwood BM, Armstrong JR. Comparison of two simple methods for determining malaria parasite density. **Trans R Soc Trop Med Hyg 1991**; Vol.85 (2), pp186-8.

Greenwood BM, Bradley-Moore AM, Bryceson AD, Palit A. Immunosuppression in children with malaria. **Lancet 1972**; Vol.1 (7743), pp169-72.

Greiner JW, Zeytin H, Anver MR, Schlom J. Vaccine-based therapy directed against carcinoembryonic antigen demonstrates antitumor activity on spontaneous intestinal tumors in the absence of autoimmunity. **Cancer Res 2002**; Vol.62 (23), pp6944-51.

Gurvich EB. The age-dependent risk of postvaccination complications in vaccinees with smallpox vaccine. **Vaccine 1992**; Vol.10 (2), pp96-7.

Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, Florens L, Janssen CS, Pain A, Christophides GK, James K, Rutherford K, Harris B, Harris D, Churcher C, Quail MA, Ormond D, Doggett J, Trueman HE, Mendoza J, Bidwell SL, Rajandream MA, Carucci DJ, Yates JR, 3rd, Kafatos FC, Janse CJ, Barrell B, Turner CM, Waters AP, Sinden RE. A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. **Science 2005**; Vol.307 (5706), pp82-6.

Harrop R, Ryan MG, Golding H, Redchenko I, Carroll MW. Monitoring of human immunological responses to vaccinia virus. **Methods Mol Biol 2004**; Vol.269, pp243-66.

Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW. The global distribution and population at risk of malaria: past, present, and future. **Lancet Infect Dis 2004**; Vol.4 (6), pp327-36.

Hermsen CC, Telgt DS, Linders EH, van de Locht LA, Eling WM, Mensink EJ, Sauerwein RW. Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR. **Mol Biochem Parasitol 2001**; Vol.118 (2), pp247-51.

Herrington D, Davis J, Nardin E, Beier M, Cortese J, Eddy H, Losonsky G, Hollingdale M, Sztein M, Levine M, et al. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. **Am J Trop Med Hyg 1991**; Vol.45 (5), pp539-47.

Herrington DA, Clyde DF, Losonsky G, Cortesia M, Murphy JR, Davis J, Baqar S, Felix AM, Heimer EP, Gillessen D, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. **Nature 1987**; Vol.328 (6127), pp257-9.

Herrington DA, Clyde DF, Murphy JR, Baqar S, Levine MM, do Rosario V, Hollingdale MR. A model for Plasmodium falciparum sporozoite challenge and very early therapy of parasitaemia for efficacy studies of sporozoite vaccines. **Trop Geogr Med 1988**; Vol.40 (2), pp124-7.

Hill AV. The immunogenetics of resistance to malaria. **Proc Assoc Am Physicians 1999**; Vol.111 (4), pp272-7.

Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, Greenwood BM. Common west African HLA antigens are associated with protection from severe malaria. **Nature 1991**; Vol.352 (6336), pp595-600.

Hill AV, Elvin J, Willis AC, Aidoo M, Allsopp CE, Gotch FM, Gao XM, Takiguchi M, Greenwood BM, Townsend AR, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. **Nature 1992**; Vol.360 (6403), pp434-9.

Ho M, Webster HK, Looareesuwan S, Supanaranond W, Phillips RE, Chanthavanich P, Warrell DA. Antigen-specific immunosuppression in human malaria due to Plasmodium falciparum. **J Infect Dis 1986**; Vol.153 (4), pp763-71.

Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, Sacci J, de la Vega P, Dowler M, Paul C, Gordon DM, Stoute JA, Church LW, Sedegah M, Heppner DG, Ballou WR, Richie TL. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. **J Infect Dis 2002**; Vol.185 (8), pp1155-64.

Jerome KR, Sloan DD, Aubert M. Measurement of CTL-induced cytotoxicity: the caspase 3 assay. **Apoptosis 2003**; Vol.8 (6), pp563-71.

John CC, Moormann AM, Pregibon DC, Sumba PO, McHugh MM, Narum DL, Lanar DE, Schluchter MD, Kazura JW. Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection. **Am J Trop Med Hyg 2005**; Vol.73 (1), pp222-8.

John CC, O'Donnell RA, Sumba PO, Moormann AM, de Koning-Ward TF, King CL, Kazura JW, Crabb BS. Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage Plasmodium falciparum infection in individuals in a malaria endemic area of Africa. **J Immunol 2004**; Vol.173 (1), pp666-72.

Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. **Nat Immunol 2003**; Vol.4 (12), pp1191-8.

Kassiotis G, Stockinger B. Anatomical heterogeneity of memory CD4+ T cells due to reversible adaptation to the microenvironment. **J Immunol 2004**; Vol.173 (12), pp7292-8.

Keating SM, Bejon P, Berthoud T, Vuola JM, Todryk S, Webster DP, Dunachie SJ, Moorthy VS, McConkey SJ, Gilbert SC, Hill AV. Durable Human Memory T Cells Quantifiable by Cultured Enzyme-Linked Immunospot Assays Are Induced by Heterologous Prime Boost Immunization and Correlate with Protection against Malaria. **J Immunol 2005**; Vol.175 (9), pp5675-80.

Kinyanjui SM, Bull P, Newbold CI, Marsh K. Kinetics of antibody responses to Plasmodium falciparum-infected erythrocyte variant surface antigens. **J Infect Dis 2003**; Vol.187 (4), pp667-74.

Laidlaw SM, Skinner MA. Comparison of the genome sequence of FP9, an attenuated, tissue culture-adapted European strain of Fowlpox virus, with those of virulent American and European viruses. **J Gen Virol 2004**; Vol.85 (Pt 2), pp305-22.

Lalvani A, Moris P, Voss G, Pathan AA, Kester KE, Brookes R, Lee E, Koutsoukos M, Plebanski M, Delchambre M, Flanagan KL, Carton C, Slaoui M, Van Hoecke C, Ballou WR, Hill AVS, Cohen J. Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine. **J Infect Dis 1999**; Vol.180 (5), pp1656-64.

Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, Lyon JA. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. **J Immunol 1997**; Vol.159 (12), pp6112-9.

Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM, Nanda A, Ewald BA, Gorgone DA, Lifton MA, Goudsmit J, Havenga MJ, Barouch DH. Immunogenicity of heterologous prime-boost regimens involving recombinant

adenovirus serotype 11 (ad11) and ad35 vaccine vectors in the presence of anti-ad5 immunity. **J Virol 2005**; Vol.79 (15), pp9694-701.

Lengeler C. Insecticide-treated bednets and curtains for preventing malaria (Cochrane Review). **Cochrane Database Syst Rev 2004**; (1).

Li S, Rodrigues M, Rodriguez D, Rodriguez JR, Esteban M, Palese P, Nussenzweig RS, Zavala F. Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. **Proc Natl Acad Sci U S A 1993**; Vol.90 (11), pp5214-8.

Lieberman JM, Williams WR, Miller JM, Black S, Shinefield H, Henderson F, Marchant CD, Werzberger A, Halperin S, Hartzel J, Klopfer S, Schodel F, Kuter BJ. The safety and immunogenicity of a quadrivalent measles, mumps, rubella and varicella vaccine in healthy children: a study of manufacturing consistency and persistence of antibody. **Pediatr Infect Dis J 2006**; Vol.25 (7), pp615-22.

Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. **Lancet 2004**; Vol.364 (9452), pp2196-203.

Malhotra I, Mungai P, Wamachi A, Kioko J, Ouma JH, Kazura JW, King CL. Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. **J Immunol 1999**; Vol.162 (11), pp6843-8.

Malkin E, Dubovsky F, Moree M. Progress towards the development of malaria vaccines. **Trends Parasitol 2006**; Vol.22 (7), pp292-5.

Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. **Parasite Immunol 2006**; Vol.28 (1-2), pp51-60.

Mayr A, Baljer G, Wagner C, Sailer J. [Development of a numerically additive combined vaccine against tetanus and smallpox]. **Zentralbl Bakteriol Mikrobiol Hyg [A] 1985**; Vol.259 (2), pp206-18.

Mayr A, Stickl H, Muller HK, Danner K, Singer H. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]. **Zentralbl Bakteriol [B] 1978**; Vol.167 (5-6), pp375-90.

Mbogo CM, Mwangangi JM, Nzovu J, Gu W, Yan G, Gunter JT, Swalm C, Keating J, Regens JL, Shililu JI, Githure JI, Beier JC. Spatial and temporal heterogeneity of Anopheles mosquitoes and Plasmodium falciparum transmission along the Kenyan coast. **Am J Trop Med Hyg 2003**; Vol.68 (6), pp734-42.

McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, Vuola JM, Blanchard TJ, Gothard P, Watkins K, Hannan CM, Everaere S, Brown K, Kester KE, Cummings J, Williams J, Heppner DG, Pathan A, Flanagan K, Arulanantham N, Roberts MT, Roy M, Smith GL, Schneider J, Peto T, Sinden RE, Gilbert SC, Hill AV. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. **Nat Med 2003**; Vol.9 (6), pp729-35.

McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, Fletcher HA, Hill AV. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. **Nat Med 2004**; Vol.10 (11), pp1240-4.

Meis JF, Verhave JP, Meuwissen JH, Jap PH, Princen HM, Yap SH. Fine structure of Plasmodium berghei exoerythrocytic forms in cultured primary rat hepatocytes. **Cell Biol Int Rep 1984**; Vol.8 (9), pp755-65.

Meis JFRPJ, Verhave J.P., Ponnudurai T., Hollingdale M.R., Smith J. E., Sinden R.E., Jap P.H., Meuwissen J.H., Yap S.H. Fine structure of the malaria parasite *Plasmodium falicparum* in human hepatocytes *in vitro*. **Cell Tissue Res. 1986**; Vol.244 (2), pp345-50.

Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Druilhe P. Protection against malaria induced by irradiated sporozoites. **Lancet 1990**; Vol.335 (8691), pp721.

Mitchell GH, Butcher GA, Cohen S. A merozoite vaccine effective against Plasmodium knowlesi malaria. **Nature 1974**; Vol.252 (5481), pp311-3.

Moore AC, Hill AV. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. **Immunol Rev 2004**; Vol.199, pp126-43.

Moorthy VS, Imoukhuede EB, Keating S, Pinder M, Webster D, Skinner MA, Gilbert SC, Walraven G, Hill AV. Phase 1 evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month reboosting vaccination, for malaria vaccination in Gambian men. **J Infect Dis 2004a**; Vol.189 (12), pp2213-9.

Moorthy VS, Imoukhuede EB, Milligan P, Bojang K, Keating S, Kaye P, Pinder M, Gilbert SC, Walraven G, Greenwood BM, Hill AS. A Randomised, Double-Blind, Controlled Vaccine Efficacy Trial of DNA/MVA ME-TRAP Against Malaria Infection in Gambian Adults. **Plos Med 2004b**; Vol.1 (2), ppe33.

Moorthy VS, McConkey S, Roberts M, Gothard P, Arulanantham N, Degano P, Schneider J, Hannan C, Roy M, Gilbert SC, Peto TE, Hill AV. Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage P. falciparum malaria in non-immune volunteers. **Vaccine 2003a**; Vol.21 (17-18), pp1995-2002.

Moorthy VS, Pinder M, Reece WH, Watkins K, Atabani S, Hannan C, Bojang K, McAdam KP, Schneider J, Gilbert S, Hill AV. Safety and immunogenicity of DNA/modified vaccinia virus ankara malaria vaccination in African adults. **J Infect Dis 2003b**; Vol.188 (8), pp1239-44.

Mwangi TW, Ross A, Marsh K, Snow RW. The effects of untreated bednets on malaria infection and morbidity on the Kenyan coast. **Trans R Soc Trop Med Hyg 2003**; Vol.97 (4), pp369-72.

Mwangi TW, Ross A, Snow RW, Marsh K. Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. **J Infect Dis 2005**; Vol.191 (11), pp1932-9.

Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EG, Beattie T, Chen YH, Dorrell L, McShane H, Schmidt C, Brooks M, Patel S, Roberts J, Conlon C, Rowland-Jones SL, Bwayo JJ, McMichael AJ, Hanke T. A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. **J Gen Virol 2004**; Vol.85 (Pt 4), pp911-9.

Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, MacLean JD, Ward BJ. Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. **J Clin Microbiol 2004**; Vol.42 (6), pp2694-700.

Nookala S, Srinivasan S, Kaliraj P, Narayanan RB, Nutman TB. Impairment of tetanus-specific cellular and humoral responses following tetanus vaccination in human lymphatic filariasis. **Infect Immun 2004**; Vol.72 (5), pp2598-604.

Nussenzweig V, Nussenzweig RS. Circumsporozoite proteins of malaria parasites. **Cell 1985**; Vol.42 (2), pp401-3.

Nwanegbo E, Vardas E, Gao W, Whittle H, Sun H, Rowe D, Robbins PD, Gambotto A. Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. **Clin Diagn Lab Immunol 2004**; Vol.11 (2), pp351-7.

Owusu-Agyei S, Koram KA, Baird JK, Utz GC, Binka FN, Nkrumah FK, Fryauff DJ, Hoffman SL. Incidence of symptomatic and asymptomatic Plasmodium falciparum infection following curative therapy in adult residents of northern Ghana. **Am J Trop Med Hyg 2001**; Vol.65 (3), pp197-203.

Peetermans J. Production, quality control and characterization of an inactivated hepatitis A vaccine. **Vaccine 1992**; Vol.10 Suppl 1, ppS99-101.

Peetermans JH. Specifications and quality control of a yeast-derived hepatitis B vaccine. **Postgrad Med J 1987;** Vol.63 Suppl 2, pp97-100.

Pittman PR, Mangiafico JA, Rossi CA, Cannon TL, Gibbs PH, Parker GW, Friedlander AM. Anthrax vaccine: increasing intervals between the first two doses enhances antibody response in humans. **Vaccine 2000;** Vol.19 (2-3), pp213-6.

Polley SD, Conway DJ, Cavanagh DR, McBride JS, Lowe BS, Williams TN, Mwangi TW, Marsh K. High levels of serum antibodies to merozoite surface protein 2 of Plasmodium falciparum are associated with reduced risk of clinical malaria in coastal Kenya. **Vaccine 2006;** Vol.24 (19), pp4233-46.

Polley SD, Mwangi T, Kocken CH, Thomas AW, Dutta S, Lanar DE, Remarque E, Ross A, Williams TN, Mwambingu G, Lowe B, Conway DJ, Marsh K. Human antibodies to recombinant protein constructs of Plasmodium falciparum Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria. **Vaccine 2004;** Vol.23 (5), pp718-28.

Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, Bojang KA, Conway DJ. Plasmodium falciparum merozoite surface protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection. **J Infect Dis 2007;** Vol.195 (2), pp279-87.

Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, Cloonan N, Anderson K, Mahakunkijcharoen Y, Martin LB, Wilson D, Elliott S, Eisen DP, Weinberg JB, Saul A, Good MF. Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. **Lancet 2002;** Vol.360 (9333), pp610-7.

Ramalingam TR, Reiman RM, Wynn TA. Exploiting worm and allergy models to understand Th2 cytokine biology. **Curr Opin Allergy Clin Immunol 2005;** Vol.5 (5), pp392-8.

Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, Plebanski M, Akinwunmi P, Everaere S, Watkins KR, Voss G, Tornieporth N, Allouche A, Greenwood BM, Kester KE, McAdam KP, Cohen J, Hill AV. A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease. **Nat Med 2004**; Vol.10 (4), pp406-10.

Riley EM, Wahl S, Perkins DJ, Schofield L. Regulating immunity to malaria. **Parasite Immunol 2006**; Vol.28 (1-2), pp35-49.

Robson KJ, Frevert U, Reckmann I, Cowan G, Beier J, Scragg IG, Takehara K, Bishop DH, Pradel G, Sinden R, et al. Thrombospondin-related adhesive protein (TRAP) of Plasmodium falciparum: expression during sporozoite ontogeny and binding to human hepatocytes. **Embo J 1995**; Vol.14 (16), pp3883-94.

Robson KJ, Hall JR, Jennings MW, Harris TJ, Marsh K, Newbold CI, Tate VE, Weatherall DJ. A highly conserved amino-acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. **Nature 1988**; Vol.335 (6185), pp79-82.

Rogers WO, Weiss WR, Kumar A, Aguiar JC, Tine JA, Gwadz R, Harre JG, Gowda K, Rathore D, Kumar S, Hoffman SL. Protection of rhesus macaques against lethal Plasmodium knowlesi malaria by a heterologous DNA priming and poxvirus boosting immunization regimen. **Infect Immun 2002**; Vol.70 (8), pp4329-35.

Romero P, Maryanski JL, Corradin G, Nussenzweig RS, Nussenzweig V, Zavala F. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. **Nature 1989**; Vol.341 (6240), pp323-6.

Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Topalian SL, Sherry RM, Restifo NP, Wunderlich JR, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, Gritz L, Panicali DL, White DE. Recombinant fowlpox viruses encoding the anchor-modified

gp100 melanoma antigen can generate antitumor immune responses in patients with metastatic melanoma. **Clin Cancer Res 2003**; Vol.9 (8), pp2973-80.

Roth A, Sodemann M, Jensen H, Poulsen A, Gustafson P, Gomes J, Djana Q, Jakobsen M, Garly ML, Rodrigues A, Aaby P. Vaccination technique, PPD reaction and BCG scarring in a cohort of children born in Guinea-Bissau 2000-2002. **Vaccine 2005**; Vol.23 (30), pp3991-8.

Saber AA, Aboolian A, LaRaja RD, Baron H, Hanna K. HIV/AIDS and the risk of deep vein thrombosis: a study of 45 patients with lower extremity involvement. **Am Surg 2001**; Vol.67 (7), pp645-7.

Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with Schistosoma mansoni. **J Infect Dis 1996**; Vol.173 (1), pp269-72.

Sagara I, Sangare D, Dolo G, Guindo A, Sissoko M, Sogoba M, Niambele MB, Yalcoue D, Kaslow DC, Dicko A, Klion AD, Diallo D, Miller LH, Toure Y, Doumbo O. A high malaria reinfection rate in children and young adults living under a low entomological inoculation rate in a periurban area of Bamako, Mali. **Am J Trop Med Hyg 2002**; Vol.66 (3), pp310-3.

Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. **Nature 1999**; Vol.401 (6754), pp708-12.

Santra S, Seaman MS, Xu L, Barouch DH, Lord CI, Lifton MA, Gorgone DA, Beaudry KR, Svehla K, Welcher B, Chakrabarti BK, Huang Y, Yang ZY, Mascola JR, Nabel GJ, Letvin NL. Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. **J Virol 2005**; Vol.79 (10), pp6516-22.

Satchidanandam V, Zavala F, Moss B. Studies using a recombinant vaccinia virus expressing the circumsporozoite protein of Plasmodium berghei. **Mol Biochem Parasitol 1991**; Vol.48 (1), pp89-99.

Sauerbrei A, Zell R, Harder M, Wutzler P. Genotyping of different varicella vaccine strains. **J Clin Virol 2006**; Vol.37 (2), pp109-17.

Sauerwein R TD, Verhage D, Theisen M. Assessment of the Safety of a Long Synthetic Peptide derived from *Plasmodium falciparum* GLURP in a Phase 1 clinical trial. **MIM conference 2002**.

Scheibenbogen C, Romero P, Rivoltini L, Herr W, Schmittel A, Cerottini JC, Woelfel T, Eggermont AM, Keilholz U. Quantitation of antigen-reactive T cells in peripheral blood by IFNgamma-ELISPOT assay and chromium-release assay: a four-centre comparative trial. **J Immunol Methods 2000**; Vol.244 (1-2), pp81-9.

Schellenberg JR, Smith T, Alonso PL, Hayes RJ. What is clinical malaria? Finding case definitions for field research in highly endemic areas. **Parasitol Today 1994**; Vol.10 (11), pp439-42.

Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, Becker M, Sinden R, Smith GL, Hill AV. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. **Nat Med 1998**; Vol.4 (4), pp397-402.

Schneider J, Langermans JA, Gilbert SC, Blanchard TJ, Twigg S, Naitza S, Hannan CM, Aidoo M, Crisanti A, Robson KJ, Smith GL, Hill AV, Thomas AW. A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the Plasmodium falciparum TRAP antigen in chimpanzees. **Vaccine 2001**; Vol.19 (32), pp4595-602.

Scott JA, Mlacha Z, Nyiro J, Njenga S, Lewa P, Obiero J, Otieno H, Sampson JS, Carlone GM. Diagnosis of invasive pneumococcal disease among children in Kenya with

enzyme-linked immunosorbent assay for immunoglobulin G antibodies to pneumococcal surface adhesin A. **Clin Diagn Lab Immunol 2005**; Vol.12 (10), pp1195-201.

Sedegah M, Hedstrom R, Hobart P, Hoffman SL. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. **Proc Natl Acad Sci U S A 1994**; Vol.91 (21), pp9866-70.

Simpson JA, Aarons L, Collins WE, Jeffery GM, White NJ. Population dynamics of untreated Plasmodium falciparum malaria within the adult human host during the expansion phase of the infection. **Parasitology 2002**; Vol.124 (Pt 3), pp247-63.

Smith T, Killeen G, Lengeler C, Tanner M. Relationships between the outcome of Plasmodium falciparum infection and the intensity of transmission in Africa. **Am J Trop Med Hyg 2004**; Vol.71 (2 Suppl), pp80-6.

Snow RW, Craig M, Deichmann U, Marsh K. Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. **Bull World Health Organ 1999**; Vol.77 (8), pp624-40.

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. **Nature 2005**; Vol.434 (7030), pp214-7.

Staprans SI, Hamilton BL, Follansbee SE, Elbeik T, Barbosa P, Grant RM, Feinberg MB. Activation of virus replication after vaccination of HIV-1-infected individuals. **J Exp Med 1995**; Vol.182 (6), pp1727-37.

Stewart VA, McGrath SM, Walsh DS, Davis S, Hess AS, Ware LA, Kester KE, Cummings JF, Burge JR, Voss G, Delchambre M, Garcon N, Tang DB, Cohen JD, Heppner DG, Jr. Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A. **Vaccine 2006**; Vol.24 (42-43), pp6483-92.

Stoute JA, Gombe J, Withers MR, Siangla J, McKinney D, Onyango M, Cummings JF, Milman J, Tucker K, Soisson L, Stewart VA, Lyon JA, Angov E, Leach A, Cohen J,

Kester KE, Ockenhouse CF, Holland CA, Diggs CL, Wittes J, Heppner DG, Jr. Phase 1 randomized double-blind safety and immunogenicity trial of Plasmodium falciparum malaria merozoite surface protein FMP1 vaccine, adjuvanted with AS02A, in adults in western Kenya. **Vaccine 2007**; Vol.25 (1), pp176-84.

Stoute JA, Kester KE, Krzych U, Wellde BT, Hall T, White K, Glenn G, Ockenhouse CF, Garcon N, Schwenk R, Lanar DE, Sun P, Momin P, Wirtz RA, Golenda C, Slaoui M, Wortmann G, Holland C, Dowler M, Cohen J, Ballou WR. Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. **J Infect Dis 1998**; Vol.178 (4), pp1139-44.

Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, Wellde BT, Garcon N, Krzych U, Marchand M. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. **N Engl J Med 1997**; Vol.336 (2), pp86-91.

Stowers AW, Keister DB, Muratova O, Kaslow DC. A region of Plasmodium falciparum antigen Pfs25 that is the target of highly potent transmission-blocking antibodies. **Infect Immun 2000**; Vol.68 (10), pp5530-8.

Sun P, Schwenk R, White K, Stoute JA, Cohen J, Ballou WR, Voss G, Kester KE, Heppner DG, Krzych U. Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma. **J Immunol 2003**; Vol.171 (12), pp6961-7.

Tassignon J, Burny W, Dahmani S, Zhou L, Stordeur P, Byl B, De Groote D. Monitoring of cellular responses after vaccination against tetanus toxoid: comparison of the measurement of IFN-gamma production by ELISA, ELISPOT, flow cytometry and real-time PCR. **J Immunol Methods 2005**; Vol.305 (2), pp188-98.

Taylor J, Paoletti E. Fowlpox virus as a vector in non-avian species. **Vaccine 1988**; Vol.6 (6), pp466-8.

Taylor J, Weinberg R, Languet B, Desmettre P, Paoletti E. Recombinant fowlpox virus inducing protective immunity in non-avian species. **Vaccine 1988**; Vol.6 (6), pp497-503.

Urban BC, Ferguson DJ, Pain A, Willcox N, Plebanski M, Austyn JM, Roberts DJ. Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. **Nature 1999**; Vol.400 (6739), pp73-7.

Walsh DS, Looareesuwan S, Vaniganonta S, Viravan C, Webster HK. Cutaneous delayed-type hypersensitivity responsiveness in patients during and after Plasmodium falciparum and Plasmodium vivax infections. **Clin Immunol Immunopathol 1995**; Vol.77 (1), pp89-94.

Walther M, Dunachie S, Keating S, Vuola JM, Berthoud T, Schmidt A, Maier C, Andrews L, Andersen RF, Gilbert S, Poulton I, Webster D, Dubovsky F, Tierney E, Sarpotdar P, Correa S, Huntcooke A, Butcher G, Williams J, Sinden RE, Thornton GB, Hill AV. Safety, immunogenicity and efficacy of a pre-erythrocytic malaria candidate vaccine, ICC-1132 formulated in Seppic ISA 720. **Vaccine 2005a**; Vol.23 (7), pp857-64.

Walther M, Tongren JE, Andrews L, Korbel D, King E, Fletcher H, Andersen RF, Bejon P, Thompson F, Dunachie SJ, Edele F, de Souza JB, Sinden RE, Gilbert SC, Riley EM, Hill AV. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. **Immunity 2005b**; Vol.23 (3), pp287-96.

Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, Hedstrom RC, Rahardjo N, Gay T, Hobart P, Stout R, Jones TR, Richie TL, Parker SE, Doolan DL, Norman J, Hoffman SL. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. **Proc Natl Acad Sci U S A 2001**; Vol.98 (19), pp10817-22.

Webster DP, Dunachie S, Vuola JM, Berthoud T, Keating S, Laidlaw SM, McConkey SJ, Poulton I, Andrews L, Andersen RF, Bejon P, Butcher G, Sinden R, Skinner MA, Gilbert

SC, Hill AV. Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. **Proc Natl Acad Sci U S A 2005**; Vol.102 (13), pp4836-41.

Weiss WR, Mellouk S, Houghten RA, Sedegah M, Kumar S, Good MF, Berzofsky JA, Miller LH, Hoffman SL. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. **J Exp Med 1990**; Vol.171 (3), pp763-73.

White N, Nosten F, Bjorkman A, Marsh K, Snow RW. WHO, the Global Fund, and medical malpractice in malaria treatment. **Lancet 2004**; Vol.363 (9415), pp1160.

White NJ, Olliaro P. Artemisinin and derivatives in the treatment of uncomplicated malaria. **Med Trop (Mars) 1998**; Vol.58 (3 Suppl), pp54-6.

Whittle HC, Brown J, Marsh K, Greenwood BM, Seidelin P, Tighe H, Wedderburn L. T-cell control of Epstein-Barr virus-infected B cells is lost during P. falciparum malaria. **Nature 1984**; Vol.312 (5993), pp449-50.

Williamson WA, Greenwood BM. Impairment of the immune response to vaccination after acute malaria. **Lancet 1978**; Vol.1 (8078), pp1328-9.

Wilson NS, Behrens GM, Lundie RJ, Smith CM, Waithman J, Young L, Forehan SP, Mount A, Steptoe RJ, Shortman KD, de Koning-Ward TF, Belz GT, Carbone FR, Crabb BS, Heath WR, Villadangos JA. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. **Nat Immunol 2006**; Vol.7 (2), pp165-72.

Witney AA, Doolan DL, Anthony RM, Weiss WR, Hoffman SL, Carucci DJ. Determining liver stage parasite burden by real time quantitative PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. **Mol Biochem Parasitol 2001**; Vol.118 (2), pp233-45.

Xin KQ, Jounai N, Someya K, Honma K, Mizuguchi H, Naganawa S, Kitamura K, Hayakawa T, Saha S, Takeshita F, Okuda K, Honda M, Klinman DM. Prime-boost

vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. **Gene Ther 2005**; Vol.12 (24), pp1769-77.

Xu H, Wipasa J, Yan H, Zeng M, Makobongo MO, Finkelman FD, Kelso A, Good MF. The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. **J Exp Med 2002**; Vol.195 (7), pp881-92.

Zinkernagel RM, Hengartner H. Protective 'immunity' by pre-existent neutralizing antibody titers and preactivated T cells but not by so-called 'immunological memory'. **Immunol Rev 2006**; Vol.211, pp310-9.

9 Appendices

9.1 TRAP Peptides used in ELISpot assays

9.1.1 TRAP peptides: T996 (vaccine strain)

T1	MNHLGNVKYLVIVFLIFFDL
T2	VIVFLIFFDLFLVNGRDVQN
T3	FLVNGRDVQNNIVDEIKYSE
T4	NIVDEIKYSEEVCNDQVDLY
T5	EVCNDQVDLYLLMDCSGSIR
T6	LLMDCSGSIRRHNWVNHAVP
T7	RHNWVNHAVPLAMKLIQQLN
T8	LAMKLIQQLNLNDNAIHLYV
T9	LNDNAIHLYVNVFSNNAKEI
T10	LNDNAIHLYVNVFSNNAKEI
T11	IRLHSDASKNKEKALIIRS
T12	KEKALIIRSLLSTNLPYGR
T13	LLSTNLPYGRTNLTDALLQV
T14	TNLTDALLQVRKHLNDRINR
T15	RKHLNDRINRENANQLVVIL
T16	ENANQLVVILTDGIPDSIQD
T17	TDGIPDSIQDSLKESRKLSD
T18	SLKESRKLSDRGVKIAVFGI
T19	RGVKIAVFGIGQGINVAFNR
T20	GQGINVAFNRFLVGCHPSDG
T21	FLVGCHPSDGKCNLYADSAW

T22	KCNLYADSAWENVKNVIGPF
T23	ENVKNVIGPFMKAVCVEVEK
T24	MKAVCVEVEKTASCGVWDEW
T25	TASCGVWDEWSPCSVTCGKG
T26	SPCSVTCGKGTRSRKREILH
T27	TRSRKREILHEGCTSEIQEQ
T28	EGCTSEIQEQCEEERCPPKW
T29	CEEERCPPKWEPLDVPDEPE
T30	EPLDVPDEPEDDQPRPRGDN
T31	DDQPRPRGDNSSVQKPEENI
T32	SSVQKPEENIIDNNPQEPSP
T33	IDNNPQEPSPNPEEGKDENP
T34	NPEEGKDENPNGFDLDENPE
T35	NGFDLDENPENPPNPDIPEQ
T36	NPPNPDIPEQKPNIPEDSEK
T37	
T38	DIPEQKP*NIPEDSEKEVPSD*
T39	EDSEKEVPSDVPKNPEDDRE
T40	VPKNPEDDREENFDIPKKPE
T41	ENFDIPKKPENKHDNQNNLP
T42	NKHDNQNNLPNDKSDRNIPY
T43	NDKSDRNIPYSPLPPKVLDN
T44	SPLPPKVLDNERKQSDPQSQ
T45	ERKQSDPQSQDNNGNRHVPN
T46	DNNGNRHVPNSEDRETRPHG
T47	SEDRETRPHGRNNENRSYNR
T48	RNNENRSYNRKYNDTPKHPE
T49	KYNDTPKHPEREEHEKPDNN
T50	REEHEKPDNNKKKGESDNKY
T51	KKKGESDNKYKIAGGIAGGL
T52	KIAGGIAGGLALLACAGLAY
T53	ALLACAGLAYKFVVPGAATP
T54	KFVVPGAATPYAGEPAPFDE
T55	YAGEPAPFDETLGEEDKDLD
T56	TLGEEDKDLDEPEQFRLPEE
T57	EPEQFRLPEENEWN

9.1.2 TRAP peptides: 3D7 strain

(only the 20aa sequences that are variant to T996 are shown)

D3	FLVNGRDVQNNIVDEIKYRE
D4	NIVDEIKYREEVCNDEVDLY
D5	EVCNDEVDLYLLMDCSGSIR
D8	LAMKLIQQLNLNDNAIHLYA
D9	LNDNAIHLYASVFSNNAREI
D10	SVFSNNAREIIRLHSDASKN
D11	IRLHSDASKNKEKALIIIKS
D12	KEKALIIIKSLLSTNLPYGK

D13	LLSTNLPYGKTNLTDALLQV
D27	TRSRKREILHEGCTSELQEQ
D28	EGCTSELQEQCEEERCLPKR
D29	CEEERCLPKREPLDVPDEPE
D31	DDQPRPRGDNFAVEKPNENI
D32	FAVEKPNENIIDNNPQEPSP
D33	IDNNPQEPSPNPEEGKGENP
D34	NPEEGKGENPNGFDLDENPE
D35	NGFDLDENPENPPNPPNPPN
D36	NPPNPPNPPNPPNPPNPPNP
D37	PPNPPNPPNPDIPEQKPNIP
D38	DIPEQKPNIPEDSEKEVPSD
D42	NKHDNQNNLPNDKSDRYIPY
D43	NDKSDRYIPYSPLAPKVLDN
D44	SPLAPKVLDNERKQSDPQSQ
D48	RNNENRSYNRKHNNTPKHPE
D49	KHNNTPKHPEREEHEKPDNN
D50	REEHEKPDNNKKKAGSDNKY
D51	KKKAGSDNKYKIAGGIAGGL
D52	KIAGGIAGGLALLACAGLAY
D53	ALLACAGLAYKFVVPGAATP
D54	KFVVPGAATPYAGEPAPFDE
D55	YAGEPAPFDETLGEEDKDLD
D56	TLGEEDKDLDEPEQFRLPEE
D57	EPEQFRLPEENEWN

9.1.3 Multiple Epitope peptides

Sequence	Epitope	Antigen	Type	HLA restriction
MINAYLDKL	st8	PfSTARP	CD8	A2.2
ISKYEDEI	ls50	PfLSA-1	CD8	B17
SYI^{P}SAEKI	pb9	PbCSP	CD8	mouse H2-Kd
KPNDKSLY	ls8	PfLSA-1	CD8	B35
KPKDELDY	cp26	PfCSP	CD8	B35
KPIVQYDNF	ls6	PfLSA-1	CD8	B53
ASKNKEKALII	tr42/43	PfTRAP	CD8	B8
GIAGGLALL	tr39	PfTRAP	CD8	A2.1
MNPNDPNRNV	cp6	PfCSP	CD8	B7
HLGNVKYLV	tr26	PfTRAP	CD8	A2.1
KSLYDEHI	ls54	PfLSA-1	CD8	B58
LLMDCSGSI	tr29	PfTRAP	CD8	A2.2
DPNANPNVDPNANPNV	CSP	PfCSP	CD4	promiscuously binding
QVHFQPLPPAVVKL	BCG	BCG	CD4	promiscuously binding
QFIKANSKFIGITE	TT	TT	CD4	promiscuously binding
YLNKIQNSL	cp39	PfCSP	CD8	A2.1

MEKLKELEK	la72	PfLSA-1	CD8	B8
ATSVLAGL	ex23	PfEXP-1	CD8	B58
NANPNANPNANPNANP	NANP	PfCSP	B cell	
DEWSPCSVTCGKGTRSRKRE	TRAP-AM	PfTRAP	Binding motif	

The multiple epitopes were pooled together in a single well. Synonymous TRAP peptides

(i.e.1, 2, 6, 7, 14-26, 30, 39, 40, 41, 45, 46, 47, and those available only for T996 (50, 51,

52, 53, 54, 55, 56, 57) were pooled across 3 wells. Variant peptides (i.e. 3, 4, 5, 8-13, 27-

29, 31- 38, 42- 44, 48, 49 were pooled according to strain, in 2 pools of T996 peptides

and 2 pools of 3D7 peptides. The vaccine strain was T996.

9.2   Circumsporozoite Antigen: Peptides used in ELISpot assays

Synonymous regions	CSNT1-D	MRKLAILSVSSFLFV
	CSNT2-D	ILSVSSFLFVEALFQ
	CSNT3-D	SFLFVEALFQEYQCY
	CSNT4-D	EALFQEYQCYGSSSN
	CSNT5-D	EYQCYGSSSNTRVLN
	CSNT6-D	GSSSNTRVLNELNYD
	CSNT7-D	TRVLNELNYDNAGTN
	CSNT8-D	ELNYDNAGTNLYNEL
	CSNT9-D	NAGTNLYNELEMNYY
	CSNT10-D	LYNELEMNYYGKQEN
	CSNT11-D	EMNYYGKQENWYSLK
	CSNT12-D	GKQENWYSLKKNSRS
	CSNT13-D	WYSLKKNSRSLGEND
	CSNT14-D	KNSRSLGENDDGNNE
	CSNT15-D	LGENDDGNNEDNEKL
	CSNT16-D	DGNNEDNEKLRKPKH
	CSNT17-D	DNEKLRKPKHKKLKQ
	CSNT18-D	RKPKHKKLKQPADGN
	CSNT19-D	KKLKQPADGNPDPNA
	CSNT20-D	PADGNPDPNANPNVD
	CSNT21-D	PDPNANPNVDPNANP
	CSNT22-D	NPNVDPNANPNVDPN
	CS1-D	NANPNANPNANPNAN
	CS2-D	ANPNANPNANPNKNNQ
	CS3-D	NKNNQGNGQGHNMPN
	CS4-D	NKNNQGNGQGHNMPN
	CS5-D	GNGQGHNMPNDPNRN
	CS6-D	HNMPNDPNRNVDENA

	CS7-D	DPNRNVDENANANSA
	CS8-D	VDENANANSAVKNNN
	CS9-D	NANSAVKNNNNEEPS
TH2R region, 3D7 strain	CS10-D	VKNNNNEEPSDKHIK
	CS11-D	NEEPSDKHIKEYLNK
	CS12-D	DKHIKEYLNKIQNSL
	CS13-D	EYLNKIQNSLSTEWS
	CS14-D	IQNSLSTEWSPCSVT
	CS15-D	STEWSPCSVTCGNGI
TH3R region, 3D7 strain	CS16-D	PCSVTCGNGIQVRIK
	CS17-D	CGNGIQVRIKPGSAN
	CS18-D	QVRIKPGSANKPKDE
	CS19-D	PGSANKPKDELDYAN
	CS20-D	KPKDELDYANDIEKK
	CS21-D	LDYANDIEKKICKME
Synonymous region	CS22-D	DIEKKICKMEKCSSV
	CS23-D	ICKMEKCSSVFNVVN
	CS24-D	KCSSVFNVVNSSIGL
	CS25-D	FNVVNSSIGLIMVLS
	CS26-D	SSIGLIMVLSFLFLN
TH2R region, 7G8 strain	CS10-G	VKNNNNEEPSDKHIE
	CS11-G	NEEPSDKHIEQYLKK
	CS12-G	DKHIEQYLKKIKNSI
	CS13-G	QYLKKIKNSISTEWS
	CS14-G	IKNSISTEWSPCSVT
	CS15-G	STEWSPCSVTCGNGI
TH3R region, 7G8 strain	CS16-G	PCSVTCGNGIQVRIK
	CS17-G	CGNGIQVRIKPGSAN
	CS18-G	QVRIKPGSANKPKDE
	CS19-G	PGSANKPKDELDYEN
	CS20-G	KPKDELDYENDIEKK
	CS21-G	LDYENDIEKKICKME

The synonymous regions were pooled across 3 peptide pools, and the TH2R and

TH3R regions pooled according to strain, 2 pools of 3D7 peptides and 2 pools of

7G8 peptides. The vaccine strain was 3D7.

9.3 Standard Operating Procedures for ELISpot assays.

9.3.1 Ex vivo ELISPOT

- Make the coating solution by adding 10 µl of catcher antibody (1-D1K for gamma, or capture Il2 antibody for BD) per ml of ELISPOT coating buffer resulting in a concentration of 10 µg/ml. Record the lot number of the catcher antibody on the Standard record sheet. IFN gamma and Il2 concentrations are the same.

- Add 50 µl per well of the coating solution to the ELISPOT plate. Keep at RT for 3-8 h or at +4°C for 8-48 hours.

- Flick off the coating solution, and wash the plates 6 times with 250 µl sterile PBS using a multichannel pipette to dispense the PBS. Flick off the washing solution and blot onto absorbent paper.

- Block the wells with 100 µl R10. Keep the plate at RT for 1-8 h or at +4°C for 8-48 h.

- Flick off the blocking solution, and add peptides and other stimulants to the wells by transferring 50 µl of solution from the stimulant plate (see 3.10) to the corresponding wells on the ELISPOT plate. Add 50 µl of cell suspension (8x10^6 cells/ml in RN10) to the appropriate wells on the plate. Prepare duplicate wells per each sample. Record the time of addition of cells to the plate on the Standard record sheet.

- Incubate the plate for 18-20 h at +37°C 5%CO$_2$ in the TC incubator.

- Flick off the cell suspension, and, using the plate washer, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper.

- Dilute the detector antibody (7-B6-1-Biotin for gamma, or Il2 detector antibody - BD) 1:1000 in PBS resulting in a concentration of 1 µg/ml, and add 50 µl to each well. Record the time of addition of the detector antibody and the batch number of the antibody on the Standard record sheet. Concentrations are the same for Il2 and IFNgamma.

- Incubate the plate for 2-4 h at RT or overnight at +4°C.

- Flick off the detector antibody and, using the plate washer, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper.

- Dilute the SA-ALP 1:1000 in PBS, and add 50 µl to each well. Record the time of addition of the SA-ALP and the batch number of the SA-ALP to the Standard record sheet.

- Incubate for 1-2 h at RT.

- Flick off the SA-ALP and, using the plate washer, wash 6 times with PBS-Tween. Flick off the washing solution and blot onto absorbent paper.

- Make up the development buffer from the AP Conjugate Substrate Kit:

Number of plates	1	2	3	4	5	6	7	8	9
Vol Water /ml	4.8	9.6	14.4	19.2	24	28.8	33.6	38.4	43.2
Vol 25x Dev Buff /ml	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8
Vol Reagent A /µl	50	100	150	200	250	300	350	400	450
Vol Reagent B /µl	50	100	150	200	250	300	350	400	450

- Add 50 µl to each well. Record the time of addition of the buffer and the lot number of the kit to the Standard record sheet.

- Develop for approximately 3-7 min at room temperature.

- Wash thoroughly with tap water and let soak over night. Record the time of washing to the Standard record sheet.

- Dry in air for 1-2 days before counting with ELISPOT reader.

9.3.2   Cultured ELISPOT

- Spin 1 million PBMC at 1400 rpm for 7 minutes.

Day 0

- Add 250 µl RN10 to the cell suspension, transfer to a well (= 2 x10^6 cells/well). Add 250ul of peptide pool.

DAY 3

- Carefully take off 250 µl of media. Add 250ul of RN10 containing 50ul of rIl2 stock solution (50 units).

DAY 7

- Carefully take off 250 µl of media. Add 250ul of RN10 containing 50ul of rIl2 stock solution (50 units)

DAY 9

- Resuspend cells carefully and transfer them to a 15-ml falcon tube. Wash 3 times with 1 ml of RPMI. Resuspend in 0.5 ml of RN10. Incubate at +37°C overnight.

DAY 10

- Prepare the ELISPOT plate as in ex vivo protocol. Add 25 µl of cultured cells (corresponds to 2.5 x 10^4 of the initially plated cells) and 25 µl of RN10 to duplicate wells of an ELISPOT plate containing 50 µl of stimulants transferred from the stimulant plate. Pipette RN10 cultured and stimulated cells as two separate samples. Use the remaining cells to calculate the cell number after at day 10 and possibly for ICS. Incubate at +37°C for 18-20 h.

DAY 11

- Develop the ELISPOT plate as described in ex vivo protocol.