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## ERRATA

**Page 6, paragraph 7, line 2:** "regents" should read "reagents".

**Page 37, paragraph 1, line 8:** "hyper-glycoyslation" should read "hyper-glycosylation".

**Page 99, paragraph 1, line 3:** "minimize the any bias" should read "minimize any bias".

**Page 149, paragraph 3, line 3:** "(Fig.1)" should read "(Fig.4)".

**Page 152, paragraph 2, line 5:** "(Fig.2A; $P$=0.02)" should read "(Fig.5A; $P$=0.02)".

## ADDENDUM

**Page 86, paragraph 1, line 8 and page 177 paragraph 2, line 9:** Reference 121 was used as an example of the effectiveness of CTLA4 vaccine targeting, however the findings summarized in this reference were previously described in **"Chaplin, P.J., R. De Rose R, J.S. Boyle, P. McWaters, J. Kelly, J.M. Tennent, A.M. Lew, and J.P. Scheerlinck.** 1999. Targeting improves the efficacy of a DNA vaccine against Corynebacterium pseudotuberculosis in sheep. Infection and Immunity 67: 6434-6438.". Please refer to this reference for a more thorough description of the effectiveness of CTLA4 DNA vaccine targeting using *C. pseudotuberculosis* in the sheep model.

# Evaluation of DNA vaccine targeting strategies and Expression Library Immunisation against lethal erythrocytic stage Malaria

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PhD Thesis

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# DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any University or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Adam Rainczuk

## Abbreviations

| | |
|---|---|
| AMA-1 | Apical membrane antigen-1 |
| APC | Antigen presenting cell |
| CFA | Complete Freund's adjuvant |
| CMV | Cytomegalovirus |
| CSP | Circumsporozoite protein |
| CTL | Cytotoxic T lymphocyte |
| CTLA4 | Cytotoxic T lymphocyte antigen 4 |
| DC | Dendritic cell |
| ECL | Enhanced Chemiluminescence |
| ELI | Expression library immunisation |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| HRP | Horse radish peroxidase |
| ID | Intraepidermal |
| IFA | Incomplete Freund's adjuvant |
| IM | Intramuscular |
| IP | Intraperitoneal |
| IRBC | Infected red blood cell |
| MCP-3 | Monocyte chemotactic protein-3 |
| MSP | Merozoite surface protein |
| $P.$ | Plasmodium |
| p/b | Prime/boost |
| RBC | Red blood cell |
| TNF-$\alpha$ | Tumor necrosis factor alpha |
| TPA | Tissue plasminogen activated |

# Acknowledgments

I would like to acknowledge the following people for their assistance and advice during the course of my PhD studies:

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# Abstract

Malaria is estimated to kill between 1.5 and 2.7 million people every year, with 300 to 500 million people having the disease, and one third of humans living in malaria endemic regions. DNA vaccination allows the delivery of multiple malarial antigens which may eventually form the basis of a vaccine against malaria. This thesis describes new methods of DNA vaccination involving targeting and bicistronic vectors using known malarial candidate antigens, and genomic libraries. This thesis also describes attempts to further characterise immune responses after Expression Library Immunisation (ELI) of mice, and enhance ELI against lethal *P.chabaudi adami* DS malaria challenge.

The enhancement of immunogenicity of malarial DNA vaccines is important if they are to have practical application in protecting against erythrocytic stage malaria. Three different DNA vaccine vector types were used in conjunction with the erythrocytic stage Merozoite Surface Protein 4/5 (MSP4/5), the murine homologue of *P. falciparum* MSP4 and MSP5, in an attempt to enhance survival against lethal *P.c.adami* DS erythrocytic stage challenge. MSP4/5 was inserted into VR1020 (secretory), Monocyte-Chemotactic Protein-3 (MCP-3) (chemoattractant), and Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) (lymph node targeting) vectors. Mice were immunised intraepidermally (ID) via gene-gun, intramuscular injection (IM), or boosted with recombinant MSP4/5 protein. Antibody responses after boosting were predominantly of the IgG1 and IgE isotypes, with low avidity antibodies produced in DNA primed groups. Despite antibody responses comparable to recombinant protein immunisation, boosting mice primed with antigens encoded by MCP-3 and CTLA4 vectors did not enhance survival compared to vector control groups. Gene-gun vaccination using VR1020/MSP4/5 followed by recombinant MSP4/5 boosting, or gene-gun DNA vaccination alone using MCP-3/MSP4/5, resulted in enhanced survival compared to empty vector control mice.

The optimal route of delivery and DNA vector type was established when using a candidate antigen, and this knowledge was now applied to ELI in an effort to enhance the technique. ELI is a method for screening genomes of a pathogen to identify novel combinations of vaccine sequences. The immune responses associated with (and the protective efficacy of) genomic *P.c.adami* DS expression libraries constructed in

VR1020, MCP-3, and CTLA4 DNA vaccine vectors was investigated. Using splenocytes from vaccinated mice, specific T-cell responses, as well as IFN-γ and IL4 production, were observed after stimulation with *P.c.adami* DS infected erythrocytes. This demonstrated the specificity of genomic library vaccination in two of the three libraries constructed. Sera obtained from mice vaccinated with genomic libraries promoted the opsonisation of *P.c.adami* DS infected erythrocytes by murine macrophages *in vitro*, further demonstrating the induction of malaria specific immune responses following ELI. Over three vaccine trials using biolistic delivery of the three libraries, protection after lethal challenge with *P.c.adami* DS ranged from 33-50%. These results show that protective epitopes/antigens are expressed within the libraries, and that ELI induces responses specific to *P.c.adami* DS malaria.

The ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex life-cycle. Bicistronic vector constructs containing an internal ribosome entry site (IRES) and expressing two combinations of malaria antigens: malarial candidate antigen sequences MSP4/5 (fused to MCP-3) with Apical Membrane Antigen-1 (AMA-1); and MSP4/5 with a *P.c.adami* DS genomic DNA library (fused to a Tissue Plasminogen Activated secretion signal) were tested. Transfection of COS 7 cells with bicistronic plasmids resulted in secretion of both malarial candidate antigens AMA-1 and MSP4/5 *in vitro*. Vaccination of BALB/c mice resulted in antibody production via ID gene-gun and IM routes against AMA-1 and MSP4/5. Significant *in vitro* proliferation of splenocytes compared to empty vector controls was also detected after vaccination with bicistronic constructs to both AMA-1 and MSP4/5 malarial candidate antigens. Vaccination with the *P.c.adami* DS bicistronic genomic DNA library also resulted in significant *in vitro* proliferation of splenocytes. Survival of BALB/c mice vaccinated with bicistronic constructs after lethal *P.c.adami* DS erythrocytic stage challenge was variable, although significant reductions in peak parasitemia were observed in two challenge trials. This study demonstrates that the delivery of malarial antigens via bicistronic vectors using a murine model is feasible. Further experimentation with multi-valent delivery systems will be required in the future for the optimisation and refinement of DNA vaccines against malaria.

# Chapter 1

# Introduction

## 1.1) Malaria and vaccine approaches

Malaria is estimated to kill between 1.5 and 2.7 million people every year, with 300 to 500 million people having the disease, and one third of humans living in malaria endemic regions (26, 155). Drug resistant malaria parasites and insecticide resistant *Anopheles* mosquitos affect almost every country where malaria is endemic, further emphasizing the need for new control measures such as a malaria vaccine (62). Four species of the genus *Plasmodium*, a unicellular protozoan parasite, infect humans: *P.malariae*, *P.vivax*, *P.ovale*, and *P. falciparum* (155). *P. falciparum* accounts for 90 percent of all infections (26). Each year, approx 10-30,000 tourists visiting malaria endemic areas are infected, and US military campaigns conducted in malaria endemic regions over the past century have experienced more casualties to malaria than hostile fire (52).

During the blood meal of a *Plasmodium* infected *Anopheles* mosquito, sporozoites contained within the salivary glands are injected into the blood of the host, then migrate to the liver and invade the cytoplasm of hepatocytes (Fig.1)
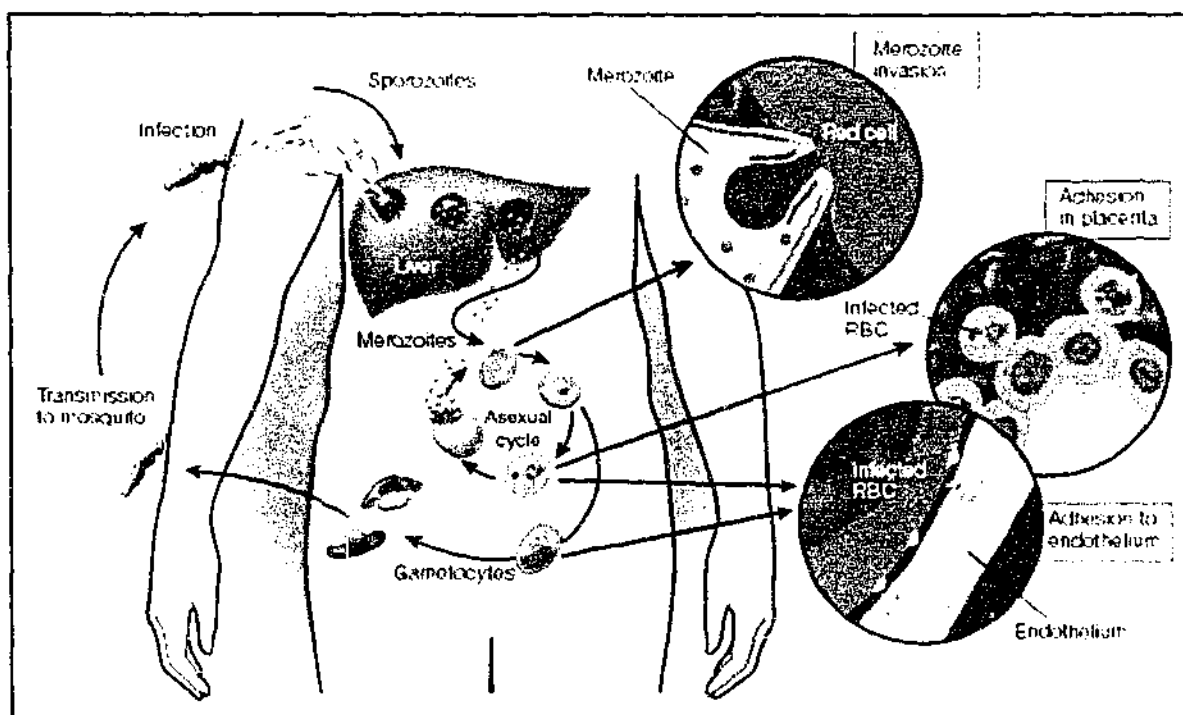
**Figure 1.** Life cycle of *P. falciparum* malaria. Disease occurs only as a result of the asexual blood stage after merozoites leave the liver and begin to invade and grow inside red blood cells (RBCs). RBCs infected with *P. falciparum* must bind to endothelium or placenta for the parasite to avoid spleen-dependent killing mechanisms, but this binding also leads to much of the pathology (taken from 138).

This phase of the life cycle is termed the 'pre-erythrocytic' stage of malaria infection. The hepatocyte is the first stage of parasite proliferation within the host. An effective vaccine directed against the sporozoite stage of the malaria cycle would prevent progression to the next stage of the malaria life cycle, the 'erythrocytic stage' which is associated with clinical illness (138).

The erythrocytic stage of malaria is characterised by the rupture of infected hepatocytes which release merozoites that enter the blood circulation and invade erythrocytes (Fig.1). The merozoite enters the erythrocyte and undergoes a stage of asexual amplification (Fig.2).
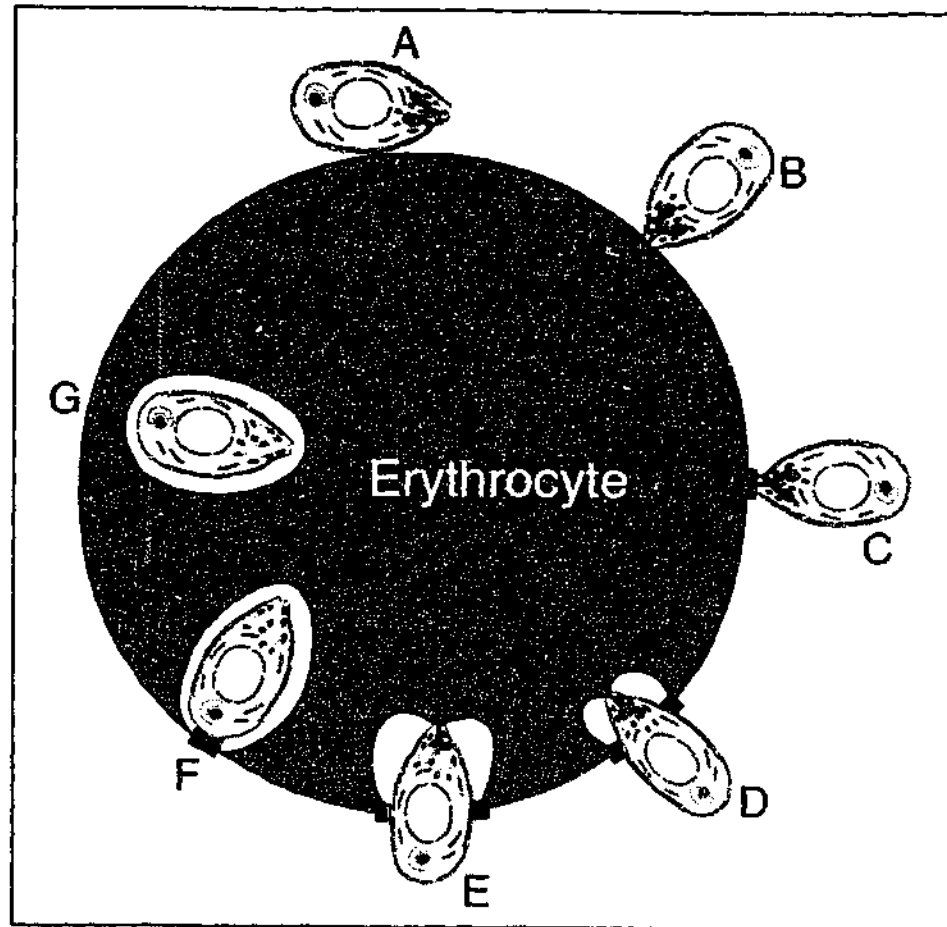
**Figure 2.** Schematic of events associated with *P. falciparum* merozoite invasion of erythrocytes. (A) Attachment; (B) apical reorientation; (C) junction formation and initiation of rhoptry discharge; (D & E) merozoite penetration and parasitophorous vacuole; (F & G) pinching off of junction and re-sealing of erythrocyte membrane. The merozoite surface is stripped as it enters from D to F (taken from 39).

Within 72 hours, the erythrocyte ruptures releasing an average of 16 merozoites which continue the erythrocytic cycle (53). This stage is responsible for clinical illness, and may lead to anaemia, cerebral malaria and possible death. The 'sexual-stage' completes the malaria cycle, occurring when haploid merozoites within erythrocytes differentiate into male and female gametocytes, which then undergo gametogenesis following ingestion by a mosquito (53).

It is believed that the ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex life-cycle, that is, a 'multivalent' malaria vaccine is required (54, 112). An early classical malaria DNA vaccine study first demonstrated that vaccination of mice with two pre-erythrocytic stage antigens can enhance protection over the use of either antigen alone, as well as overcome genetic restriction in different mouse strains (55). Combinations of malarial antigens delivered as malarial DNA vaccines in primates have induced antigen-specific cytotoxic T lymphocytes (CTLs) in pre-erythrocytic stage vaccines (197). In primates it has also been shown that enhanced antibody responses to combinations of malarial antigens can be generated by erythrocytic stage malarial DNA vaccines (100).

The 'First Generation' DNA vaccines (i.e., delivery of only plasmid/antigen DNA) are not optimal for inducing protection against malaria; reasons are discussed in the following Chapter. Immune enhancement strategies for DNA vaccination alone are required for this method of vaccination to be practical (reviewed in 54, 112). Table 1 provides a brief overview of immune responses involved at the pre-erythrocytic and erythrocytic stages of a malaria infection.

**Table 1.** An overview of stages in the malaria cycle whereby vaccines can induce immune responses

| Stage | Immune response |
|---|---|
| Pre-erythrocytic | anti-sporozoite antibodies to reduce hepatocyte invasion |
| | antibodies to opsonise and destroy infected hepatocytes; T cells (cytotoxic and T-helper); cytokines; antigen presenting cells (APCs) |
| Erythrocytic stage | anti-merozoite antibodies to prevent erythrocyte invasion; antibodies to eliminate infected erythrocytes by antibody-dependent T-cell inhibition (ADCI) and antibody-independent T-cell-mediated immunity (AICI); cytokines to kill parasites within erythrocytes; T-helper cells; antigen presenting cells |

Experimental strategies in malaria vaccine design have attempted to incorporate multiple malarial antigens from different stages of the life cycle (54, 73, 109, 113, 164, 189). The most recent multi-stage malarial vaccine effort is the 'MuStDO' (Multi-Stage Malaria DNA-based Vaccine Operation) program (reviewed in 52, 113). The vaccine comprises 15 *P. falciparum* antigens: five from the pre-erythrocytic stage and 10 from the erythrocytic stage. However, as yet, this vaccine formulation has not led to a vaccine for humans and is still in the testing phase (113). Problems after expression of antigens from the encoded DNA, such as antigenic competition between antigens leading to suppression (or complete abrogation) of immune responses have occurred when vaccinating with candidate malarial antigens in mice (49). Generation of antibodies to the pre-erythrocytic stage candidate antigen, the circumsporozoite protein (CSP), could not be detected in the first two malaria DNA vaccine clinical trials in humans, although a cellular response was detected (118, 198). These problems are still yet to be resolved.

The asymptomatic pre-erythrocytic stage has been a major focus in attempting to design a vaccine against malaria (52). These vaccine types aim to prevent the development of all clinical symptoms associated with the erythrocytic stage of malaria

by eradicating sporozoites from the liver. However, the successful growth and replication of a single malaria parasite within an erythrocyte will result in a viable erythrocytic stage infection, which may cause severe disease or death in a malaria naïve individual (reviewed in 112). Erythrocytic stage vaccine design aims to prevent invasion and infection of erythrocytes by merozoites, and to reduce morbidity and mortality by decreasing the parasite load (112, 138). It is this stage of the malaria life-cycle that is under investigation in this thesis.

Sexual-stage 'transmission blocking' vaccines aim to reduce malaria within a community as a whole, by preventing the reinfection of the mosquito vector by malaria parasites contained within infected humans, but confer no benefit for a malaria-infected individual (115).

Theoretically, vaccines can be directed against a single or combination of stages in the malaria life-cycle. However, the way in which a malaria vaccine is designed and processed by the immune system may be the factor determining vaccine effectiveness. It is clear that an effective host defence against the complex nature of the malaria life cycle requires the elicitation of different components of the immune system, and this will require complex strategies in vaccine development (113).

## 1.2) Acquired and vaccine-induced immunity to malaria

### Acquired immunity

In malaria endemic regions the severity of erythrocytic stage malaria infection and its related symptoms decreases with increasing age, indicating that adults develop limited levels of protective immunity (93, 145, 177). Maternally derived antibodies are believed to protect infants against infection. It has been shown that antibodies passively transferred from mothers to infants were positive for major malarial

antigens, with infants able to mount and maintain a strong antibody response against Liver Stage Antigen-1 (LSA-1) compared to other malarial candidate antigens in their first year of life (211).

Each infection survived by a child enhances immunity to malaria (26). Children over the age of five years can survive malaria infection with a substantially larger parasite load and have no adverse symptoms, relative to children that are most at risk from four months of age (reviewed in 2, 62). In humans, natural exposure to malaria induces both $CD8^+$ T cells that have the potential to clear infected hepatocytes, as well as antibodies to erythrocytic stage antigens (177). Passive transfer of antibodies from adults with naturally acquired immunity against malaria, to infected children significantly reduces parasitemia (reviewed in 112). It is has been found that agglutinating antibodies from children can recognise only strains of parasite to which the child was previously exposed; however, sera from adults in the same village were able to agglutinate all strains of parasite found within the community (reviewed in 70). There are approximately 50 variant copies of the gene for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), and the fact that individuals take many years to acquire natural immunity is believed to be dependent on the time taken to acquire antibodies to all PfEMP1 variants (70). Natural immunity observed in humans in malaria endemic regions demonstrates that erythrocytic stage protection is theoretically possible.

*Vaccine induced immunity: Immunisation with irradiated sporozoites*

Sterile immunity against *P. falciparum* malaria can be achieved experimentally in humans upon vaccination with radiation-attenuated sporozoites (91). The sporozoites enter hepatocytes, but are unable to divide and multiply (92). However, it is not yet

known if protection afforded by irradiated sporozoites requires ongoing presence of the parasite inside hepatocytes (91). In rats, it has been demonstrated that protective immunity against sporozoite challenge requires the presence of hepatic stage irradiated parasites, with protection lasting up to six months (170).

It has been shown that *P.berghei* sporozoites invade rat hepatocytes within two minutes after injection (178). As sporozoites rapidly migrate towards the liver, this stage was not believed to be immunogenic and protective immunity directed to this stage was not considered possible (180). Heterologous immunity between different *P. falciparum* strains, lasting up to twelve months, has been shown to be induced in humans after vaccination with a single strain of radiation-attenuated *P. falciparum* sporozoites (59, 91).

Unfortunately, logistical difficulties prevent large scale immunisation using radiation-attenuated sporozoites (83). Sporozoites are irradiated within mosquitos, and are then removed from the salivary glands. Live sporozoites must be delivered into the host and invade hepatocytes to induce a protective immune response (59, 91). Thus, the large-scale delivery of radiation-attenuated sporozoites from infected mosquitos would be impractical. Humans must receive greater than 1000 bites from *P. falciparum* infected mosquitos irradiated with 15,000 rad to be protected against malaria (91). These difficulties involved in generation and delivery of radiation-attenuated sporozoites has prevented large-scale immunisation of individuals using this stage (reviewed in 62). This has driven the development of new strategies for malaria vaccination (reviewed in 54).

## 1.3) Immune pathways induced after malaria infection

The complex effector functions of the host immune system (human or animal) after a malaria infection are not yet fully understood, and this is believed by some to be hampering vaccine development (71). It is known however that T cells play a crucial role in malaria immunity, both by regulating antibody production and by mediating antibody-independent T-cell immunity (AICI) (7, 201). Helper T cells are induced in similar quantities to cytotoxic T lymphocytes (CTLs) in sporozoite-immunised mice, with both exogenous and endogenous immune pathways contributing to parasite clearance (162). This represents the most basic level of understanding for parasite clearance after a malaria infection in humans and animal models, and does not take into account the complex factors of AICI and antibody-dependent T-cell inhibition (ADCI) (reviewed in 70, 71, 113).

The way a malaria vaccine is presented to the immune system determines which T-cell subset is activated. In mice, T-helper cells carrying a $CD4^+$ molecule can be activated by extracellular malarial antigens presented with class II major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs) via the exogenous pathway. T cells bearing $CD4^+$ molecules can be divided into functional subsets, depending on the cytokine they produce. Murine studies have shown that cytokines such as IL-2 and interferon-$\gamma$ (IFN-$\gamma$) are produced by type 1 $CD4^+$ T cells (Th1), which induce cellular immune responses activating $CD8^+$ T cells and the immunoglobulin isotype IgG2a (160). Type 2 $CD4^+$ T cells (Th2) produce cytokines such as IL-4, IL-5, IL-6, and IL-10. Th2 cells primarily induce the IgG1 immunoglobulin in mice, and also promote B cell activation and immunoglobulin class switching (48). The protective antibody types in mice infected with erythrocytic

stage malaria is dependent upon the strain of the infection, and can be altered depending upon the immunising antigen (reviewed in 117, 122).

T-helper cells carrying CD8^{+} molecules are activated by invasive actions such as sporozoite invasion of hepatocytes, with antigens presented on class I MHC molecules via the endogenous pathway (171, 201). In a malaria infection, it was long believed that CD8^{+} CTLs were the major effector cell required for in pre-erythrocytic immunity; however it has been demonstrated that these CTLs alone are not sufficient for the protective immunity generated by irradiated sporozoites (71). Although hepatocytes infected by *P. falciparum* present peptides on both MHC I and MHC II molecules, protection against pre-erythrocytic malaria is primarily mediated by CD8^{+} T cells (reviewed in 71). The cytokines produced by CD8^{+} T cells such as IFN-γ induce infected hepatocytes to produce nitric oxide (NO) and oxygen radicals that kill developing parasites within hepatocytes (135, 136). In contrast, erythrocytes express class I MHC molecules at very low levels; consequently, CD4^{+} T cells, B cells, and their APCs are most important in contributing to vaccine induced erythrocytic stage immunity (14, 42, 71). It has been shown that B-cell deficient mice can control erythrocytic stage infections by limiting parasite growth, emphasizing the importance of T-cell mediated immunity (194, 195). The absence of antibody and the ability of B-cell deficient mice to clear parasitized erythrocytes mostly via the spleen, stresses the importance of complex AICI mechanisms via CD4^{+} MHC II pathways (Fig.3) (70). A major effect of tumor necrosis factor α (TNF-α) during erythrocytic stage malarial infection is to activate cells such as macrophages, causing the release reactive oxygen species (ROS) and NO which are responsible for intra-erythrocytic parasite death (117).
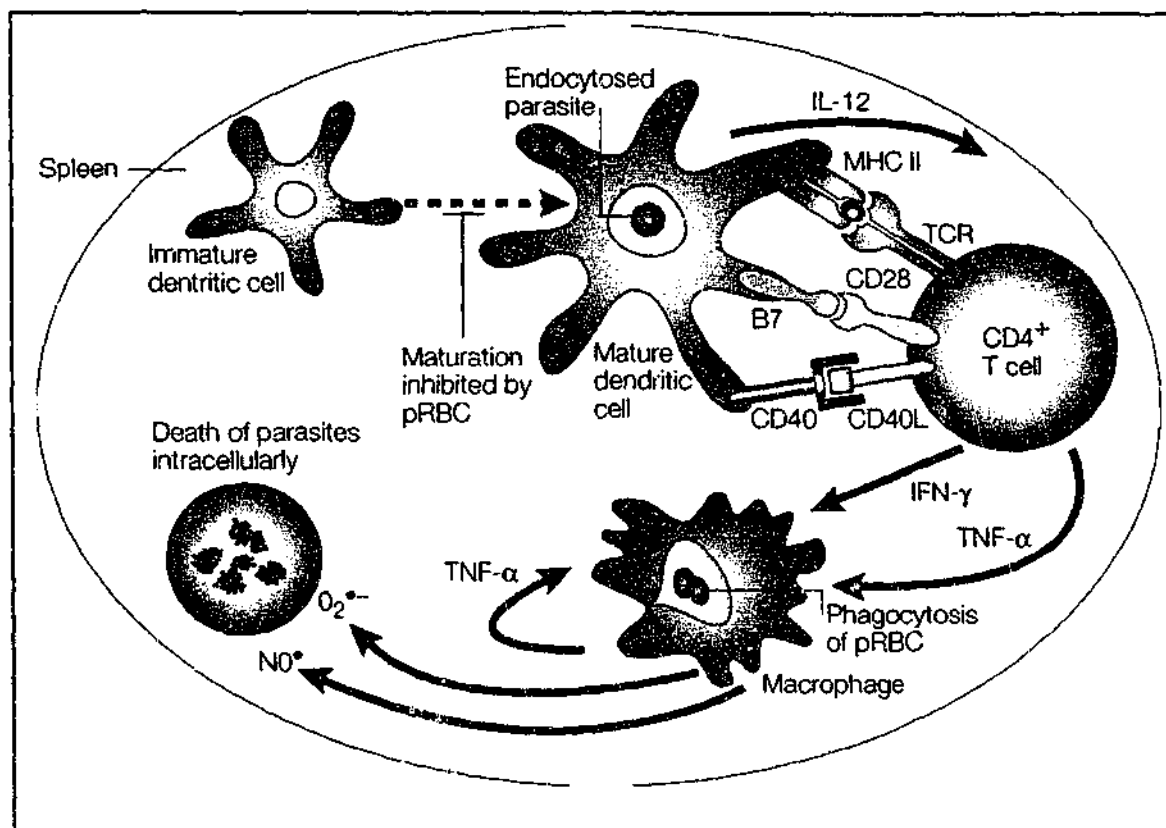
**Figure 3.** A proposed mechanism of antibody-independent T-cell-mediated immunity in the spleen. Activation of CD4^{+} T cells by antigen presenting dendritic cells first occurs via MHCII and T-cell receptor (TCR) interactions (and co-stimulatory B7 and CD40 receptor interactions). This leads to secretion of IFN-γ and TNF-α, macrophage activation, and phagocytosis of infected erythrocytes. Macrophage activation also leads to NO and $O_2$ radical production to kill parasites within erythrocytes (taken from 70).

In humans, IgG1 and IgG3 antibody subclasses have been found to be protective against erythrocytic stage *P. falciparum* infections in passive transfer studies, although this is not the rule in all cases of human malaria infection (reviewed in 66). The equivalent cytophilic isotype is IgG2a in mice and has been associated with protection in *P.yoelii* infections after passive transfer of antibodies from immune mice; however, passive transfer of IgG3 specific to the 19-kDa region of Merozoite Surface Protein-1 (MSP1) can protect mice against *P.yoelii* infection (117). The antibody isotype that is crucial to inducing a protective response can differ depending on the infective species and immunising antigen. Antibody-dependent T-cell-inhibition and phagocytosis however is an important factor in clearance of parasites after erythrocytic stage infection (21, 66). Figure 4 depicts two possible mechanisms of *P. falciparum* infected erythrocyte clearance. This is believed to involve cytophilic antibodies by either monocyte/macrophage phagocytosis, or monocyte/macrophage ADCI of merozoite invasion after the release of an as yet unknown lytic factor or factors (66). The human antibody isotype IgG3 has been found to play a major role in ADCI reactions (187). It has been proposed that TNF-α is involved in the cellular inhibition of ring-stage development of malaria, and is likely to be involved in ADCI reactions (72).
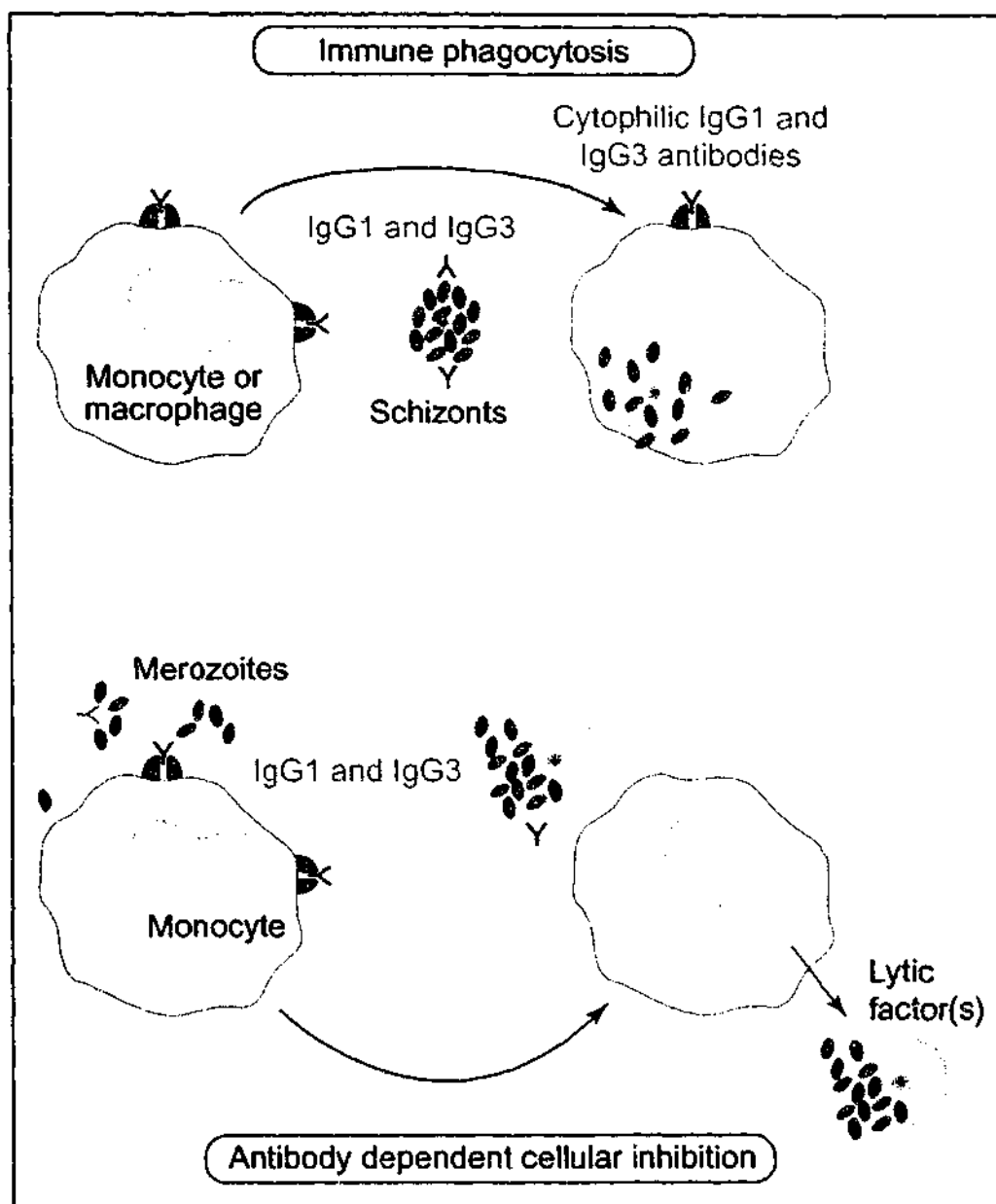
**Figure 4.** Proposed mechanisms of immune phagocytosis and antibody-dependent T-cell-inhibition (ADCI) during erythrocytic stage *P. falciparum* infection. Antibodies specific to the infected erythrocyte bind to phagocytic macrophages and are cleared by phagocytosis. During ADCI, antibodies bridge the infected erythrocyte to an effector cell (which can be a monocyte, macrophage or neutrophil) to release an unknown toxic or lytic factors to inhibit invasion (66).

## 1.4)  Major malarial vaccine candidate antigens

**Table 2.** Antigens from *P. falciparum* included in the MuStDo 5 and 10 vaccines (taken from 113)

| MuStDo antigens | Site of expression | Immune mechanism | References |
|---|---|---|---|
| **Pre-erythrocytic (MuStDo 5)** | | | (31, 51, 157, 175) |
| CSP | Sporozoite surface and micronemes; LSP membrane | | |
| SSP2 | Sporozoite surface and micronemes; LSP | Neutralising Ab to sporozoite surface proteins; CD8$^+$ T cells, IFN-$\gamma$, IL-12 & NO against LSP | |
| LSA1 | LSP vacuole | | |
| LSA3 | LSP and/or Erythrocytic stage merozoite | | |
| Exp1 | LSP vacuole membrane; host cell cytoplasm | | |
| **Erythrocytic stage (MuStDo 10)** | | | |
| MSP1$_{42}$ (two alleles included from *P. falciparum* 3D7 and FVO strains) | | | |
| MSP2 | Surface of merozoite | Invasion blocking Ab | (95, 112) |
| MSP4 | | | |
| MSP5 | | | |
| MSP3 | Surface of merozoite | Invasion blocking Ab & known ADCI | (21, 95) |
| EBA-175 | Micronemes | | |
| AMA-1 | Rhoptries and merozoite surface | Invasion blocking Ab | (95, 112) |
| SERA | Parasitophorous vacuole | | |
| RAP-2 | Rhoptry complex | | |

**Abbreviations:** Ab, antibodies; ADCI, antibody-dependent T-cell inhibition; AMA-1, apical membrane antigen 1; CSP, circumsporozoite protein; EBA-175, erythrocyte binding antigen 175; Exp1, exported protein 1; IFN-$\gamma$, interferon $\gamma$; IL-12, interleukin 12; LSA, liver stage antigen; LSP, liver stage parasite; MSP, merozoite surface protein; NO, nitric oxide; RAP, rhoptry associated protein; SERA, serine repeat antigen; SSP, sporozoite surface protein.

Parasite proteins must contain epitopes that are presented to the immune system  to allow immune recognition (171).  If a vaccine is to be accepted for general use, the antigen under consideration should contain an epitope that is effective across diverse human genetic backgrounds (4, 90).  Inclusion of these epitopes in vaccines may serve to boost low levels of T cells and antibodies in malaria endemic populations (116).

Major malarial candidate antigens have been selected for inclusion in the MuStDo *P. falciparum* DNA vaccine (Table 2). The inclusion of all these antigens may have the potential to sufficiently protect people living in malaria endemic regions, as well as malaria naïve individuals (after optimisation) (113).

This thesis describes vaccination against the erythrocytic stage of malaria. Pre-erythrocytic vaccine candidates described in Table 2 will not be further discussed in detail. The major malarial erythrocytic stage candidate antigens described in Table 2 are positioned at various locations on the merozoite, and are shown in Figure 5. Detailed descriptions of the major erythrocyte stage antigen MSP1, as well as AMA-1 and MSP4/5 (both of which were studied in this thesis) are now discussed further.
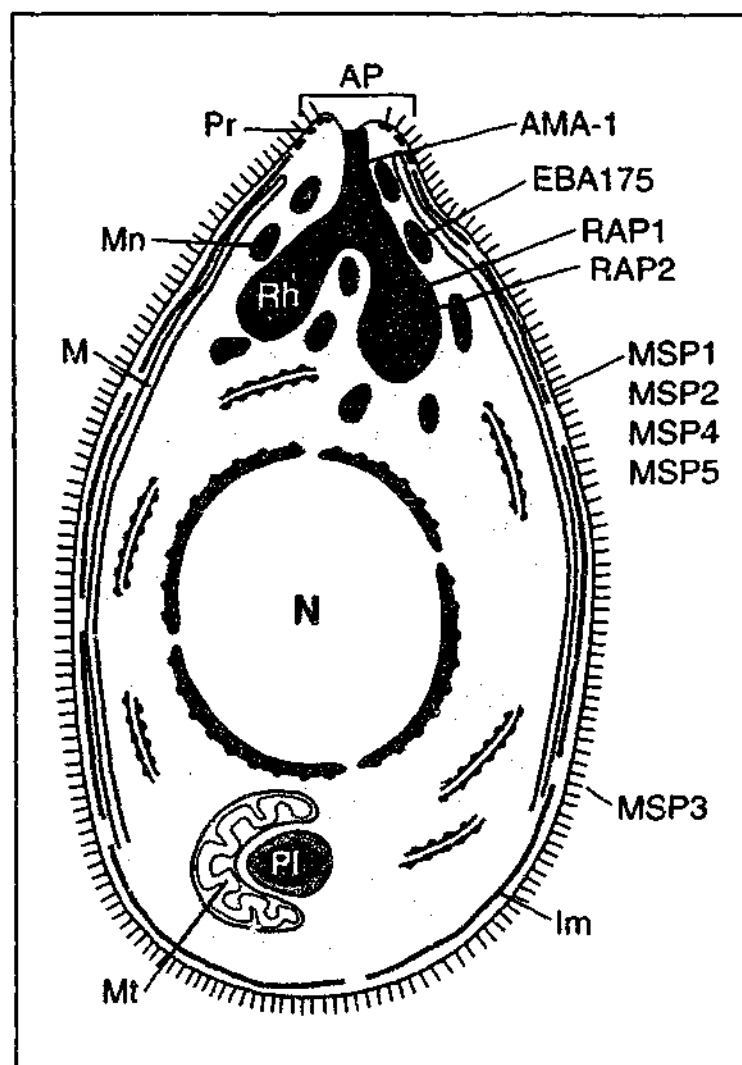
**Figure 5.** Schematic representation of a *P. falciparum* merozoite and locations of major structural features and major erythrocytic stage antigens described in Table 2. N, nucleus; Rh, rhoptries; Im, inner membrane; Mt, mitochondrial; M, microtubules; Mn, micronemes; Pr, polar rings; Ap, apical end; Pl, plastid (taken from 39).

### *Erythrocytic vaccine candidate – Merozoite Surface Protein-1*

Merozoite surface protein-1 (MSP1) was the first antigen to be identified on the surface of erythrocytic stage merozoites (96). MSP1 is synthesised in late schizogony, and contains a polymorphic series of repeats within the amino acid sequence (14). Post-synthetic processing of the 190-230 kDa MSP1 protein results in multiple fragments. At the time of erythrocyte invasion by merozoites, the C-terminal 42 kDa region ($MSP1_{42}$) is further processed to a 33 kDa fragment ($MSP1_{33}$), and a conserved membrane bound 19 kDa fragment ($MSP1_{19}$), containing two epidermal growth factor (EGF)-like motifs believed to assist in erythrocyte invasion (97). The $MSP1_{19}$ fragment remains bound to the merozoite, and is the only merozoite surface protein fragment not shed as it invades an erythrocyte (see Fig.2) (19).

Antibodies to the $MSP1_{19}$ fragment of *P. falciparum* have been shown to be associated with resistance to clinical malaria and the reduction of parasitemia in malaria endemic regions (60). This fragment is also functionally conserved across distantly related plasmodium species (148). Antibodies targeting $MSP1_{19}$ have been shown to inhibit merozoite invasion *in vitro* using the sera from immune individuals (147). The importance of $MSP1_{19}$ in erythrocyte invasion has been demonstrated by allelic replacement of *P. falciparum* parasites with the $MSP1_{19}$ sequence of the rodent parasite *P.chabaudi* (147). $MSP1_{19}$ specific antibodies taken from humans with natural immunity to *P. falciparum* were found to inhibit erythrocyte invasion *in vitro* by the allelic variants of *P. falciparum*.

The unprocessed $MSP1_{42}$ (containing $MSP1_{19}$) and the processed $MSP1_{33}$ fragments of the MSP1 protein have themselves been shown to induce protection in mice. Protection has been demonstrated using recombinant $MSP1_{42}$ in mice against

*P.yoelii* erythrocyte challenge (188). Inhibition of parasite growth in primates has been observed after erythrocytic stage challenge with *P. falciparum* (30) and *P.vivax* (151) using recombinant $MSP1_{42}$. A DNA vaccine using *P. falciparum* $MSP1_{42}$ and the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) also produced enhanced cytotoxic T-cell and antibody responses from monkeys against *P. falciparum* infected erythrocytes (114). Epitopes contained within $MSP1_{33}$ of *P.yoelii* have recently been shown to induce effector T cells producing IFN-$\gamma$, which were capable of delaying erythrocytic stage parasite growth after lethal *P.yoelii* YM infection in mice (in the absence of antibody) (205).

### *Erythrocytic vaccine candidate - Apical Membrane Antigen-1*

Apical Membrane Antigen-1 is believed to be one of the major antigens involved in erythrocyte invasion. AMA-1 is a type I integral membrane protein which appears in the rhoptries and on the merozoite surface during the final four hours of erythrocytic development (40, 154). AMA-1 of *P. falciparum* is synthesised in dividing schizonts as an 80 kDa precursor and is then N-terminally processed to a 62 kDa fragment, which coats the merozoite surface (40).

Unlike most other erythrocytic stage malarial antigens, AMA-1 contains no tandemly arranged immunodominant repeating sequences (149). Therefore AMA-1 may be consistently recognised by an immune system. AMA-1 of *P. falciparum* has been found to contain at least nine epitopes within the extracellular ectodomain that are recognised by human T cells (116). The ectodomain of AMA-1 also contains 16 cysteine residues that are conserved within known sequences of AMA-1 (33, 58, 104, 128, 131, 154, 199). The amino acid sequence similarities between AMA-1 sequences of different *Plasmodium* species are the highest known for any *Plasmodium*

transmembrane protein family (104). Figure 6 shows the amino acid sequence alignment for AMA-1 sequences between different malarial parasites.

The conserved nature of AMA-1 provides additional important practical advantages as a vaccine candidate. Mice can not be infected with *P. falciparum*. Consequently, the immune responses generated from testing a vaccine using *P. falciparum* antigens in mice can not be measured accurately (139). The conserved nature of AMA-1 permits the use of a rodent malarial system such *P. chabaudi* for evaluation of AMA1 as a vaccine model. In *P.chabaudi adami* murine studies, it has been shown that immunisation with the refolded ectodomain of AMA-1 can protect mice against lethal erythrocyte stage challenge, but only after challenge with the homologous parasite strain (8). Immunity to erythrocyte stage challenge was subsequently found to be T-cell dependent in mice (210). However, it has also been demonstrated that antibodies from human immune sera and rabbits immunised with recombinant *P. falciparum* AMA-1, can inhibit merozoite invasion of heterologous and homologous strains of *P. falciparum* (87). Immunisation of primates with *P. fragile* AMA-1 resulted in the clearance of infected erythrocytes and, after subsequent reinfection with heterologous *P. falciparum*, did not develop a detectable parasitemia (37). Vaccination of *Aotus* monkeys using recombinant *P. falciparum* AMA-1 has also resulted in significant protection after lethal erythrocyte stage challenge with *P. falciparum* malaria (183).
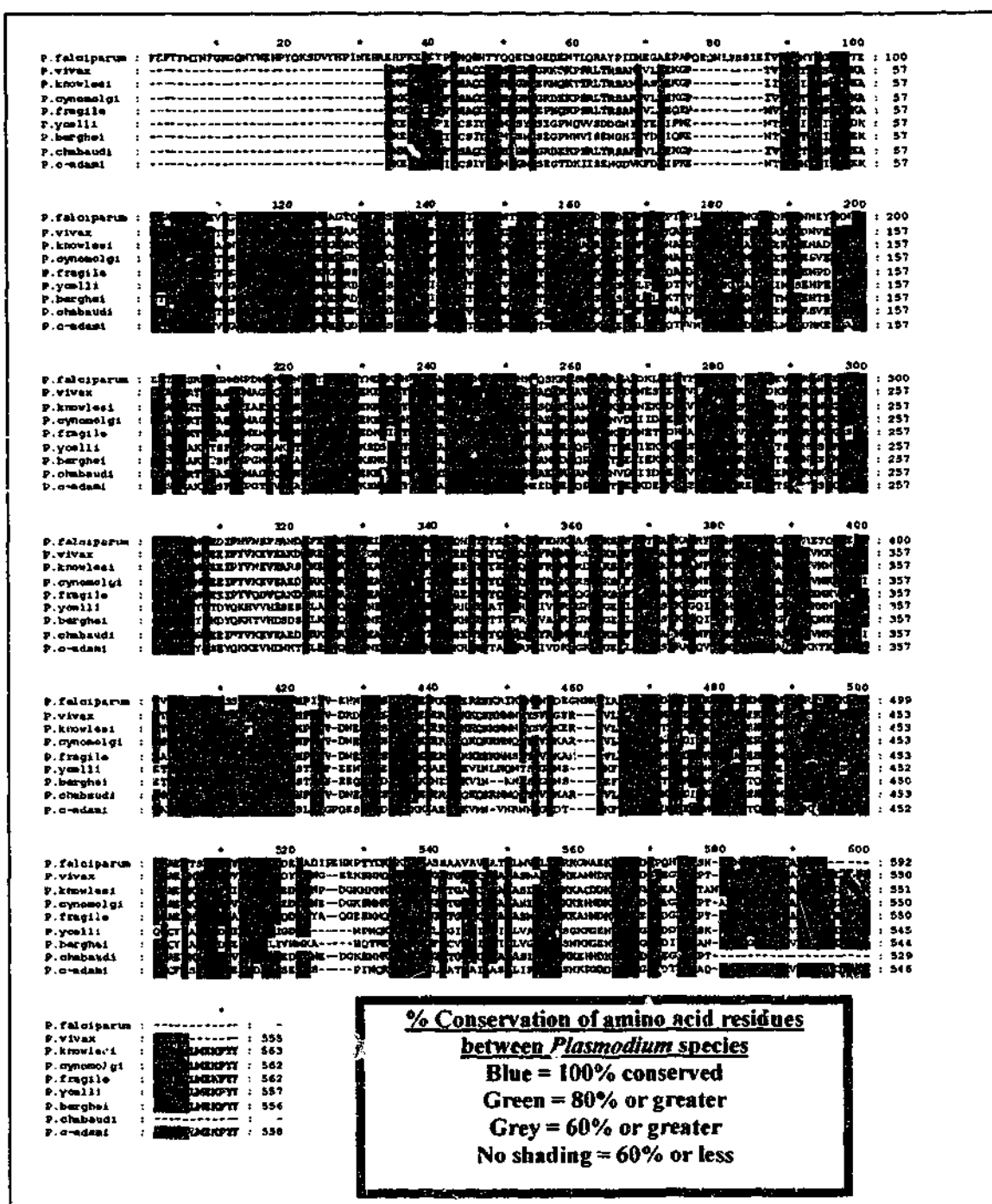
**Figure 6** Multiple amino acid sequence alignment showing percentage conservation of residues between all known *Plasmodium* AMA-1 sequences. The alignment was performed using ClustalX v1.64b, and edited using Genedoc v2.3.000. The sequences were obtained from Genbank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

### Erythrocytic vaccine candidates – Merozoite Surface Proteins 4 & 5

MSP4 and MSP5 are two recently discovered *P. falciparum* and *P.vivax* erythrocytic stage antigens and potential vaccine candidates (17, 129, 130, 209). These proteins are both 272 residues long, contain a single EGF-like domain near the carboxyl terminus, hydrophobic signal sequences, and glycosylphosphatidylinositol anchor signals that attach the proteins to the merozoite surface (17, 130, 209). The similar organisation and location of genes on *P. falciparum* chromosome 2 are believed to have arisen from an ancient gene duplication event (130, 209). Genes encoding *P. falciparum* MSP4 and MSP5 on chromosome 2 are downstream from a highly conserved gene sequence encoding the enzyme adenylosuccinate lyase (ASL) (Fig.7) (17, 129). The syntenic regions of the *P. falciparum* and *P.vivax* genomes in rodent malaria species contain only a single gene with an EGF-like domain, designated MSP4/5 (18).

It has been shown that immunisation using recombinant MSP4/5 can protect mice against lethal *P.yoelii* erythrocytic stage infection (108, 109). MSP4 has been found to be recognised by the immune systems of hum.::. 'iving in malaria endemic regions of Vietnam (196). MSP4 antibodies were detected in 94% of the study population. Antibodies directed to MSP4 were predominantly IgG1 and IgG3 isotypes, suggesting a role for complement and opsonisation of free merozoites during a *P. falciparum* infection. In humans, the IgG1 and IgG3 isotypes are believed to be important in controlling erythrocytic stage infection (reviewed in 66).
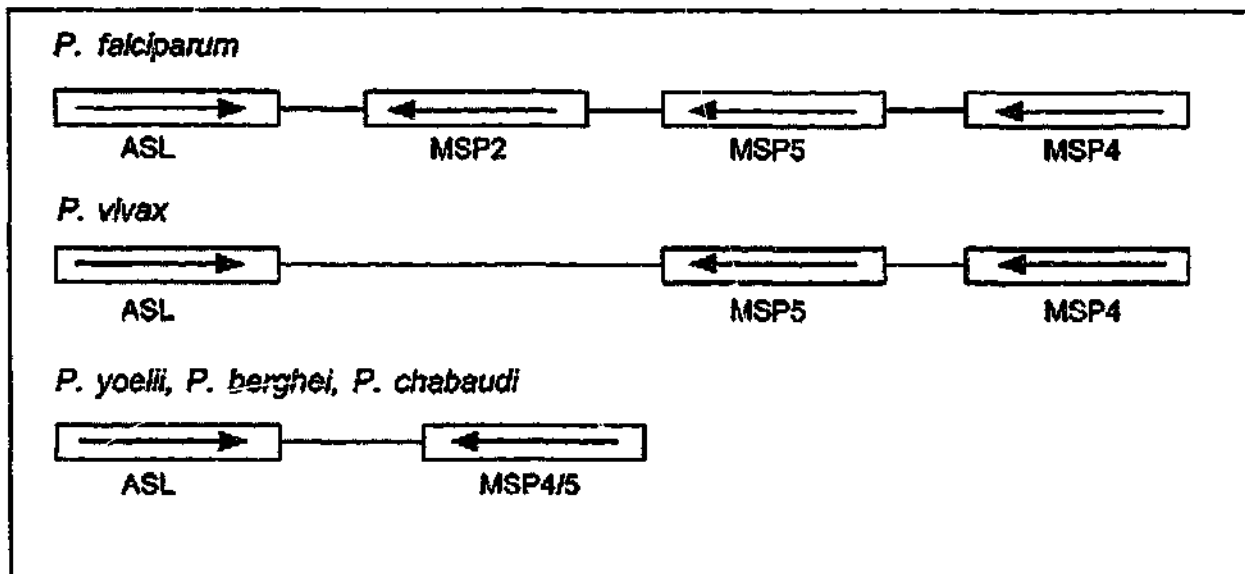
**Figure 7.** Genomic arrangement of the MSP locus between *Plasmodium* species. Arrows indicate the direction of gene transcription; solid black lines represent intergenic regions. Adenylosuccinate lyase (ASL) is upstream from MSP4, MSP5, and MSP4/5 sequences. Merozoite surface protein-2 (MSP2) is located between ASL and MSP5 genes on *P. falciparum* chromosome 2 (from 17).

## 1.5) Conventional malaria vaccine types

### *Subunit vaccines*

The first major vaccine type utilised against malaria was the subunit vaccine. Vaccines of this type stimulate antibody production and T-cell responses due to processing of the injected recombinant protein or synthetic peptides by the exogenous pathway. A major disadvantage of subunit vaccines is the requirement of an adjuvant, as malarial antigens alone are weak immunogens (reviewed in 62, 141). Aluminium hydroxide (alum) -based adjuvants are currently the only type approved for human use (98). The biological activity of this adjuvant consists of at least three major components including: 1) a formation of an antigen 'depot' in tissues to prolong antigen exposure; 2) the production of particulate antigens to facilitate uptake by APCs; 3) the activation of complement, macrophage stimulation, and lymphocyte activation (reviewed in 98). However, in order to be immunogenic, formulations in human malaria subunit vaccine trials must include more potent adjuvants, which have led to side effects and are discussed below. Many recombinant and synthetic subunit vaccines have been developed and tested, however this has not yet led to a subunit vaccine that is effective in humans (reviewed in 141).

Early recombinant subunit vaccines encoding the whole *P. falciparum* CSP have been shown to generate low levels of antibodies in humans, which lasted only two to three weeks (9). High antibody titres have been generated in humans immunised with CSP. Unfortunately, the adjuvant formulations required to generate such responses were so potent that severe side effects including fevers, headaches, malaise, and myalgia were induced (182).

A synthetic polyvalent vaccine containing *P. falciparum* sequences from three erythrocytic stage antigens and repeating sequences from CSP, designated SPf66, was the first human vaccine reported to delay and suppress parasitemia in human volunteers (150). The SPf66 vaccine has undergone the most extensive human clinical testing for any malaria vaccine to date; however the efficacy in reducing clinical *P. falciparum* infections was extremely variable between different populations (with no clear explanation of this variability between trials) (reviewed in 112). A recent human SPf66 Phase I trial attempted to enhance immunogenicity by replacing the aluminium hydroxide adjuvant with the saponin based adjuvant QS-21 (105). This vaccine formulation administered to humans enhanced immunogenicity, compared to use with the alum adjuvant. However, side effects included prolonged pain at the site of injection, erythema, headaches, and fevers. Two subjects developed severe allergy after vaccination and required medical attention. In another recent *P. falciparum* human subunit vaccine trial, the C-terminus of CSP (fused to the hepatitis B virus surface antigen) was found to induce significant protection after homologous challenge in naïve adult volunteers (6). When this vaccine was administered to semi-immune adults, it resulted in low vaccine efficacy against natural infection, but protection was not specific to a single *P. falciparum* strain (6). It remains to be seen if this subunit vaccine will be of benefit to populations in malaria endemic regions.

Administration of recombinant *P. cynomolgi* (a simian analogue of the human *P. vivax* parasite) $MSP1_{19}$ to *Macaca sinica* monkeys results in complete protection upon infected erythrocyte challenge, believed to be due to the contribution of both B and $CD4^+$ T-cell epitopes within $MSP1_{19}$ (151). Subsequent human Phase I trials have demonstrated that administration of recombinant $MSP1_{19}$ and T helper epitopes of tetanus toxoid (in alum) were immunogenic; however, hypersensitivity reactions

occurred in three subjects, including injection site skin reactions in two subjects, and histamine-associated hypotension in one subject (111).

As already described, AMA-1 is a leading recombinant subunit vaccine candidate, with protection against erythrocyte stage challenge against *P.chabaudi* in mice (8) and *P. falciparum* in primates (183). Phase I trails are planned; however problems with the production of recombinant AMA-1 for human use have hampered vaccine testing in humans (1).

## Antigen delivery by live attenuated vaccines

Pre-erythrocytic malarial vaccine strategies using attenuated viral, or bacterial systems, predominantly evoke $CD8^+$ responses through the endogenous pathway. The utilisation of bacterial expression systems such as *Salmonella* in malaria vaccines have not produced promising results in murine (177, 190), or human trials (69). Recombinant vaccinia viruses encoding malarial antigens have also been investigated (139, 173, 189). The use of the modified vaccinia virus Ankara (MVA) encoding the CSP has led to complete protection in mice upon sporozoite challenge (173). In humans, a highly attenuated vaccinia virus containing seven *P. falciparum* genes was tested in volunteers. The gene sequences included CSP, PfSSP2, LSA1, MSP1, SERA, AMA-1, and one from the sexual stage (25 kDa sexual- stage antigen Pfs25). The vaccine was safe, but variably immunogenic, with only one out of thirty-five volunteers protected after sporozoite challenge (146).

The use of attenuated viral vaccines is a strategy whereby $CD4^+$ and $CD8^+$ T cells can be induced in response to sporozoite-infected hepatocytes. The disadvantages of this approach include the difficulty of large-scale production, limited shelf life,

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possible reversion to virulence and cost (62). Another disadvantage is that strong T-cell induction against irrelevant viral proteins may interfere with immunogenicity of the malarial antigen. This may be due to fast clearing of the virus by antibodies, as viruses used as carriers may already be recognised by the immune system (20).

## 1.6) DNA vaccines

In 1990 it was first discovered that injection of plasmid DNA into mouse skeletal muscle could induce expression of encoded marker genes (208). It was subsequently found that expression of the protein from DNA transfected cells lasted up to two years *in vivo* (207). It was then shown that intramuscular injection of DNA encoding a gene from the influenza virus could induce multiple forms of immune responses (192). This resulted in the production of antibodies, $CD4^+$ and $CD8^+$ T cells and immune memory.

The focus of malarial DNA vaccines has been on pre-erythrocytic stage antigens (50). DNA vaccine technology has not yet been optimised for T-cell and B-cell responses required for protective immunity against erythrocytic stage infection (52).

### *Safety considerations for DNA vaccines*

As DNA vaccines are a new technology, a number of safety issues have been raised. One concern is that plasmid DNA may integrate into the host chromosome, resulting in somatic mutations that may inactivate suppressor genes and activate oncogenes (74, 102). Most DNA vaccine plasmids do not possess an origin of replication that is functional in eukaryotic cells; therefore the vaccine does not persist with dividing cells. Another safety issue is the potential for adverse immunological

consequences arising from long-term expression of a foreign gene, such as the induction of anti-DNA antibodies and possible autoimmune disease (74).

In human malaria DNA vaccine trials, it has been established that a *P. falciparum* CSP DNA vaccine was safe and well tolerated after intramuscular (IM) DNA vaccination (118). Human trials using a HIV DNA vaccine have also shown that patients did not develop local or systemic reactions, and no anti-DNA antibodies were detected (126). A quantitative assay for investigating the tissue distribution and integration into chromosomes of plasmid DNA vaccines has been developed (119). Genomic DNA is assayed for integrated plasmid using PCR techniques. After IM vaccination of mice and guinea pigs, it was found that there was no evidence of DNA integration, to a sensitivity of about one copy per microgram of DNA, approximately three orders of magnitude below the spontaneous mutation frequency.

## *Advantages of DNA vaccines*

The major advantage of DNA vaccination is the ability to induce both $CD4^+$ and $CD8^+$ T-cell responses against malarial antigens. The induction of $CD8^+$ T cells and antibodies induced by DNA vaccination has been found to be superior to $CD8^+$ T-cell levels induced by irradiated sporozoites (50, 176). It has been shown that CSP DNA vaccination can protect against sporozoite and erythrocytic stage challenge in animal models in the absence of adjuvants (reviewed in 54). DNA vaccines do not need to be stored at cold temperatures as with conventional vaccines and can therefore be transported to remote areas without refrigeration (48).

One of the most important features of DNA vaccines is that immunisation results in protein expression of the encoded gene within the host. It is possible that the protein expressed may be similar, or even identical to, the native protein. This is important in

terms of vaccine efficacy, as conformational epitopes contained within the antigen may be preserved (52). Proteins made *in vitro* in recombinant expression systems may have different post-translational modifications, or conformations. For example, prokaryotic host T cells do not consistently fold proteins of interest with the correct disulphide-bonding pattern due to the reducing environment in the cytosol (8). Eukaryotic expression systems such as yeast may refold proteins with the correct conformation. However modification of the expressed protein (or gene) may be required, for example, to remove the products of hyper-glycoyslation (82). This may alter the efficacy of a vaccine.

An example whereby the application of DNA vaccines would be beneficial is in the preservation of conformational epitopes contained within the vaccine candidate AMA-1. The eight disulfide bonds in AMA-1 define three possible sub-domains in the ectodomain (Fig.8) (88), which are critical for generating epitopes recognised by antibodies induced by malaria infections in mice (8).
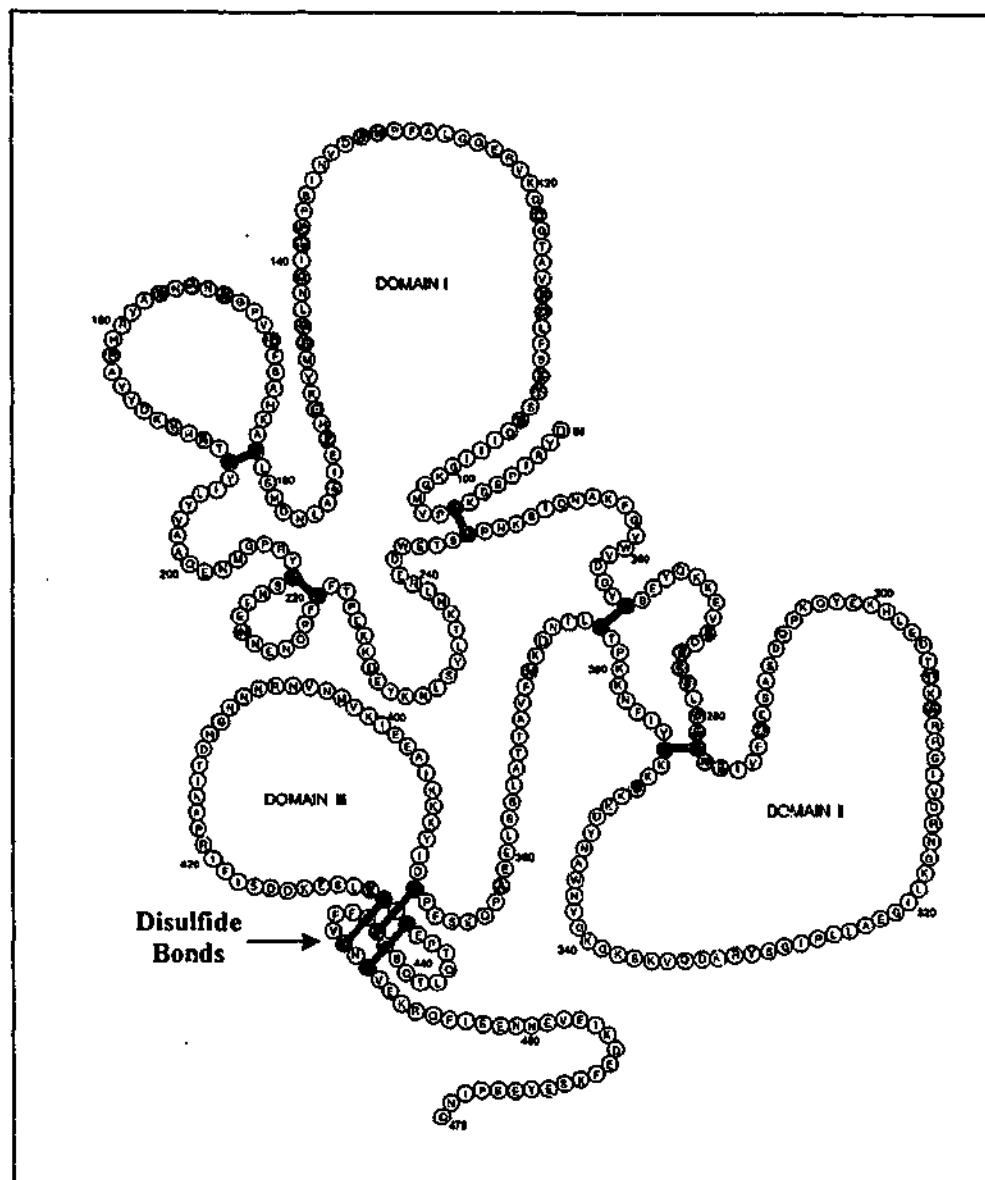
**Figure 8.** The assumed pattern of disulfide bonds forming critical domains in the ectodomain of AMA-1 (from 88).

## Features of a DNA vaccine vector

Most plasmids used for vaccination share the basic attributes of vectors developed for *in vitro* expression of genes in transfected cell lines (48). Figure 9 shows the functional components of a DNA vaccine vector, which includes (A) an origin of replication for producing plasmid in *E. coli*; (B) an antibiotic resistance gene for selective *E. coli* growth; (C) a strong enhancer/promoter (such as the human cytomegalovirus immediate-early promoter); (D) an intron to assist expression of mammalian genes; (E) a gene of interest; and (F) an mRNA transcription termination/polyadenylation sequence for terminating transcription in mammalian cells.
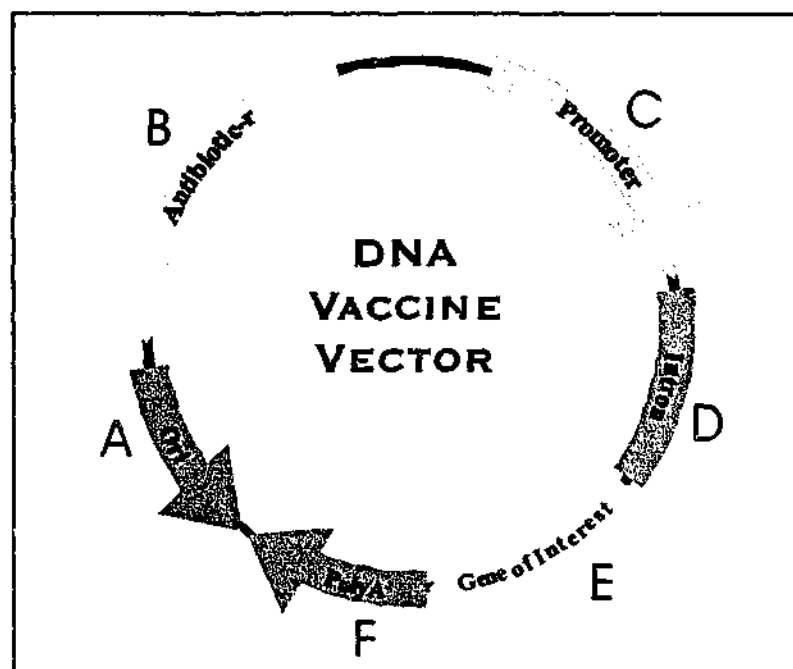


**Figure 9.** Functional components of a DNA vaccine vector.

Signal peptide sequences within the vector may also influence the type of immune response generated. A non-secreted antigen may be optimal for CD8$^+$ mediated protection, as intracellular expression may be preferential for the endogenous pathway (48). Alternatively, a secretion signal sequence may also be added to the vector. This may preferentially stimulate CD4$^+$ cellular and humoral responses by directing antigen presentation through the exogenous pathway (50).

The immune responses generated after DNA vaccination are complex and can change depending on many factors including the route of delivery, type of vaccine vector used, antigen used, and dose of vaccine plasmid.

## *Methods of DNA vaccination: Intramuscular immunisation*

The first DNA vaccine to be used against malaria involved intramuscular (IM) injection of mice with plasmid DNA encoding CSP (176). This induced higher levels of antibodies and CD8$^+$ T cells than immunisation with irradiated sporozoites. Upon sporozoite challenge, protection was found to be CD8$^+$ T-cell dependent. Data from experiments using CSP plasmid DNA also suggest that IM immunisation can be primarily used for the induction of CD8$^+$ T-cell mediated immune responses (50, 52). The method of IM vaccination is not just limited to a syringe/needle to deliver the DNA vaccine. The development of the Biojector$^{TM}$ to deliver vaccines IM without a needle has resulted in enhanced antibody titres to malarial antigens when tested in rabbits (3) and monkeys (52).

IM injection of plasmid DNA is believed to result in the low-level transfection of myocytes (38, 48, 207). Myocytes express MHC class I molecules and do not constitutively express MHC class II molecules at high levels (94). Antibody production may be a result of myocyte cell lysis or cell leakage of the encoded protein

(48). It is also uncertain whether the T-cell response is a direct result of antigen expression by muscle cells. Antigen presenting cells (APCs) in muscle tissue are present in low numbers; however, after injection they are recruited to the injection site (94). Data obtained by Doe *et al* (47) suggest that bone marrow derived APCs (primarily dendritic cells) initiate responses, and that these APCs present proteins synthesised by transfected myocytes. Therefore, APCs resident in (or circulating through) the muscle may take up the injected DNA (or secreted protein) and initiate a $CD8^+$ T-cell response.

## *Methods of DNA vaccination: Intraepidermal immunisation*

Two common methods of intraepidermal (ID) vaccination include either saline injection, or bombardment of the epidermis with DNA coated gold particles via a 'gene-gun'. The advantage of gene-gun vaccination is the amount of DNA required to achieve comparable immune responses is 100-1000 fold less than saline injection. As little as 16ng of DNA has been found to induce an immune response using a gene-gun, while the injection of 5000-fold more DNA (both ID, and IM) was required to achieve comparable results (152).

Intraepidermal vaccination results in the direct transfection of keratinocytes, as well as the direct transfection of MHC class II expressing professional APCs (23, 191). Bone marrow derived dendritic APCs such as Langerhans cells (LCs), a specialised type of dendritic cell, comprise 5% of the cells in the epidermis (10). Keratinocytes, the predominantly transfected skin cell type, secrete cytokines such as granulocyte/macrophage-colony stimulating factor, IL-1 and TNF-α (144). These up-regulate the expression of MHC class II molecules on LC and stimulate migration to lymph nodes. Thus, ID immunisation can result in the transfection of professional

APCs (LC) as well as keratinocytes to enhance the efficiency of vaccination (38, 191).

Malarial DNA immunisation strategies have primarily concentrated on pre-erythrocytic vaccines and stimulating $CD8^+$ T-cell production, while ID immunisation has been shown to induce antibody responses and protection against the erythrocytic stage of malaria infection in monkeys (reviewed in 54).

*The involvement of T helper cell subclasses and mechanisms of DNA immunisation*

It has been shown that the route of DNA immunisation can influence the subclass of antigen-specific T-cell produced, and this in turn may influence the activation of different immune system components. Boyle *et al* (23) have demonstrated that IM immunisation of mice using a model antigen (ovalbumin) produces the IgG2a isotype and IFN-$\gamma$ secretion, which is consistent with a Th1 response. Gene-gun vaccination has been shown to bias the immune system to a Th2 response, generation of antibodies, and activation of $CD4^+$ T cells (50, 63, 64). It was also shown that ID immunisation of mice produced IL-4 and IgG1, which is characteristic of Th2 cells. However, ID injection can also result in raised IFN-$\gamma$ levels produced by dendritic cells (144). This result is not surprising as the resident dendritic cells of the skin, LCs, can express both MHC class I and II molecules to interact with $CD4^+$ and $CD8^+$ molecules in the secondary lymphoid organs (38).

The method of immunisation is a primary determining factor of the type of T-cell class produced, but involvement of T cells in DNA immunisation would not arise unless the naïve T cells are first primed by the DNA vaccine. There are three major mechanisms proposed for T-cell priming, two of which directly involve the presence of dendritic cells. Figure 10 attempts to summarise major mechanisms of T-cell priming after DNA vaccination.

**A. Direct priming by transfected somatic cells**

MHC class I

Somatic cell (myocyte, keratinocyte, fibroblast)

Somatic cells lack co-stimulatory molecules for T-cell expansion

Not supported experimentally

**B. Direct transfection of APC / DC**

MHC class I

Immature DC

MHC class II

Maturation and migration to 2° lymphoid tissue
(Lymph nodes & Spleen)

mature DC

Priming of CD4⁺ (IL-2 secretion & T- and CD8⁺ T cells cell expansion)

**C. Cross priming**

Macropinocytosis

soluble Ag

Apoptotic cell bodies

MHC class II

MHC class I

DC

**Figure 10.** Proposed mechanisms of DNA vaccine priming of naïve T cells. (A) Somatic cells transfected by DNA express MHC class-I molecules on their surface; however, the lack of co-stimulatory molecules (CD80/86) reduce the likelihood that this mechanism is involved in naïve T-cell priming. (B) Direct transfection of dendritic cells (DC) and antigen presenting cells (APC) by injection or gene-gun vaccination (C) Cross priming by IM injection. Presentation of antigen by these routes allows interaction with $CD8^+$ and $CD4^+$ T-cell subsets in secondary lymphoid organs (adapted from 38).

### *Malaria DNA vaccines encoding single antigens: 'First generation' DNA vaccines*

Early malaria DNA vaccine trials in humans using the pre-erythrocytic stage CSP have not been optimal in inducing protection against sporozoite challenge (52). In mice, it has been shown that DNA vaccination using single antigens alone does not result in complete protection, and does not withstand high parasite challenge doses regardless of the mouse model used (reviewed in 52).

The type of T-cell response generated by a DNA vaccine is important for the stage of the malaria life-cycle that is being targeted. It has been shown that IM immunisation of both mice (55), and monkeys (90) using CSP plasmid DNA induces IFN-$\gamma$ and CD8$^+$ CTLs, indicative of a Th1 type response. However CD4$^+$ T-cell responses after CSP immunisation has been found to be poor in mouse, monkey, and human DNA vaccine trials (reviewed in 54). It has since been shown that a CD4$^+$ T-cell response to a conserved epitope on the CSP correlated with protection from *P. falciparum* infection in adults living in malaria endemic regions (161). This emphasizes the importance of including antigens in a vaccine that will activate a broad immune response against malaria infection.

Sedegah *et al* (176) first reported protection after sporozoite challenge in 64% of mice upon IM injection of the *P.yoelii* CSP gene incorporated into plasmid DNA. Immunisation induced CSP specific antibodies and CD8$^+$ T cells against infected hepatocytes. However, this protection was observed using a single mouse strain (BALB/c). It was subsequently found that combining *P.yoelii* CSP and HEP17 pre-erythrocytic stage antigens could enhance protection against sporozoite challenge in three of five strains of mice, while protection after immunisation with CSP alone was restricted to a single mouse strain (55).

A DNA vaccine that includes a single antigen will not be sufficient to protect an outbred human population. Human malaria DNA vaccine trials have mainly concentrated on delivery of a single *P. falciparum* CSP DNA vaccine construct to asses safety, tolerability, and immunogenicity (61, 113, 198). These studies have shown the generation of $CD8^+$ CTL and IFN-$\gamma$ after CSP immunisation, but no antibody responses were detected. For a malaria vaccine to be effective in a heterogeneous human population, a diverse range of class I and II HLA types must be included in the vaccine to overcome the problems of genetic restriction (141).

### DNA vaccines encoding multiple malarial antigens

DNA vaccines encoding multiple malarial antigens from different stages of the life-cycle have been shown to enhance immune responses in animal trials, as well as overcoming genetic restriction between animal strains (52, 54). The early demonstration that synergy between malarial antigens can be achieved by co-immunisation (using *P.yoelii* HEP17 and *P.yoelii* CSP plasmid DNA) (55) was an important observation, and this knowledge has been widely applied to malaria vaccine design.

Antibody levels against a *P. falciparum* $MSP1_{19}$ DNA vaccine tested in mice have been found to be enhanced when injected with multiple plasmids encoding both pre-erythrocytic, and erythrocytic *P. falciparum* antigens (73). In primates, *P. falciparum* pre-erythrocytic DNA vaccines alone have been found to generate low levels of antibody, $CD8^+$ CTL, and IFN-$\gamma$ (197). An erythrocytic stage vaccine comprised of three antigens: AMA-1, $MSP1_{42}$, and erythrocyte binding protein-175 (EBA-175) has also been tested in primates (100). Antibody responses were generated to all three antigens, with the trivalent mixture generating antibody responses 3-12 fold higher

than vaccination with a single plasmid alone. Again, this result demonstrates that the synergy between multiple malarial antigens can be used to advantage when vaccinating with DNA vaccines, which in turn may provide the knowledge for a future human malaria vaccine able to overcome genetic restriction.

It has been shown that using GM-CSF plasmid DNA as an adjuvant with $MSP1_{42}$ plasmid DNA in *Rhesus* monkeys, can induce a rapid induction of antibody after the first dose, when compared to $MSP1_{42}$ plasmid DNA alone (114). In humans the MuStDO 5 pre-erythrocytic DNA vaccine is in the process of being tested with a human granulocyte macrophage-colony stimulating factor (hGM-CSF) plasmid. It was found that the vaccine was safe and well tolerated, however efficacy and immunogenicity results are not yet available (113).

### *Heterologous boosting of DNA vaccines*

Boosting with viral vectors after DNA priming primarily induces responses that generate enhanced $CD8^+$ T-cell responses (often with reduced antibody responses), while DNA priming followed by boosting with recombinant protein in adjuvant helps to generate enhanced antibody responses (but sometimes reduced $CD8^+$ T-cell responses) (52).

Complete protection against *P.berghei* sporozoite challenge has been demonstrated using heterologous prime/boost vaccines in different strains of mice (173). Mice were first primed with a single dose of two plasmids each encoding a single *P.berghei* pre-erythrocytic stage protein. Mice were then administered a single boost with recombinant modified vaccinia virus Ankara (MVA), encoding the same antigens. Challenge with *P.berghei* sporozoites protected 100 percent of mice, with protection associated with $CD8^+$ cells and secretion of IFN-$\gamma$. It has been shown in many studies that boosting with viral vectors encoding pre-erythrocytic stage antigens results in

enhanced $CD8^+$ T-cell responses, and that pre-erythrocytic immunity can be enhanced against sporozoite challenge (44, 67, 78, 163). Natural 'priming' of humans by infected mosquitos results in low levels of naturally acquired $CD8^+$ T cells (177). It may be possible to boost natural $CD8^+$ T-cell levels in humans by using recombinant MVA (173).

Boosting *Aotus* monkeys with pox virus, after priming with plasmids encoding two *P.knowlesi* pre-erythrocytic and two erythrocytic antigens (as well as combinations of GM-CSF and IL4, TNF-α plasmids), resulted in sterile protection of 18% of monkeys, with 78% of monkeys resolving parasitemia spontaneously (165). Control monkeys were treated for high parasitemias after all groups were challenged with a lethal dose of *P.knowlesi* sporozoites. This protocol, with pox virus boosting, is now being planned to be tested in humans (89, 113). Although viral boosting is effective in murine and primate systems, whether boosting humans on a large scale with pox virus will be accepted still remains to be seen.

Attempts to enhance erythrocytic stage protection against malaria have involved DNA priming with antigens expressed at the erythrocytic stage followed by boosting with the homologous protein in adjuvant. Haddad *et al* (76) have reported boosting of antibody titres in three different mouse strains to the *P. falciparum* antigen Pf155/RESA. As the antigen used in this murine experiment was *P. falciparum*, no protection data was obtained.

Boosting monkeys with recombinant malarial protein has been shown to enhance antibody levels, and protect against erythrocytic stage infection. Priming *Rhesus* monkeys with a $MSP1_{42}$ DNA vaccine, followed by boosting with recombinant $MSP1_{19}$ has lead to enhanced antibody responses that were reactive with infected *P. falciparum* erythrocytes *in vitro* (114). Protection against the erythrocytic stage has

also been demonstrated in *Aotus* monkeys using *P. falciparum* EBA-175 DNA priming and recombinant boosting (101). Vaccination with EBA-175 plasmid DNA three times, followed by a single boost with recombinant EBA-175, produced an antibody response equivalent to four doses of recombinant EBA-175. Three out of four monkeys receiving the EBA-175 prime/boost did not require treatment, while all monkeys receiving DNA alone and recombinant protein alone required treatment. This demonstrates the power of a single recombinant protein boost after focussing the immune system with using DNA vaccine priming.

### *Enhancement of DNA vaccines alone*

Several new strategies are now in the process of being investigated to improve the DNA based priming of the immune system. These include optimisation of codons in malarial genes for efficient mammalian expression, DNA vaccine targeting and multi-valent expression systems.

### *Codon optimisation*

Hoffman *et al* (89) have optimised the codon usage of *P. falciparum* genes which have a bias toward adenosine and thymidine nucleotides (compared to mammalian genomes). *P. falciparum* genes were changed to more closely reflect codon usage in mammalian genes, resulting in a 5-40 fold enhancement of *in vitro* expression in mammalian cells, and 5-100 fold higher antibody titres in outbred mice (52, 89).

### *DNA vaccine targeting*

Antigen presenting cells, in particular dendritic cells, have been shown to be potent initiators of immune responses following DNA vaccination, and are important in the uptake of antigen expressed from cells transfected by a DNA vaccine (reviewed in

144). Improvement of DNA vaccine efficacy by targeting antigen to APCs will be required if DNA priming alone is to be sufficient to provide a significant level of protection against erythrocytic stage malaria. The use of the targeting ligand cytotoxic T lymphocyte antigen 4 (CTLA4) in DNA vaccination has been shown to improve the magnitude and speed of the antibody response (22, 56, 121). Chemokines such as monocyte chemotactic protein-3 (MCP-3) have also been shown to enhance protective efficacy after DNA vaccination in a tumor challenge model (16) and HIV mouse model (15). However, both of these targeting methods have not yet been reported in malaria DNA vaccine trials. Figure 11 is a simplified summary of mechanisms involved with DNA vaccine targeting, the details of which are described in the following results Chapters of this thesis.

## Multi-valent expression systems

Many of the studies already described show that the expression of a single gene by a malaria DNA vaccine on its own will not be sufficient for protection against all stages of the life-cycle (reviewed in 52, 54). As combinations of malarial antigens can evoke enhanced immune responses and protect to a greater extent than a single antigen alone (55, 100, 197), it is possible that epitopes contained within multiple malarial antigens are promoting synergistic immune responses. The delivery of multiple antigens by a single vaccine construct would firstly have many practical advantages. The construction of a single plasmid for vaccine delivery would reduce problems associated with production costs, manufacturing time, quality control, and use in a clinical setting (179).

# DNA vaccine targeting

| Lymph node targeting | Activation of naïve T cells in lymph nodes | Chemoattractant |

B7

CTLA4 attracted

CTLA4-ligand/antigen fusion

Chemokine

Dendritic Cell attracted

Chemokine receptor

MCP-3/antigen fusion

DNA Transfected Keratinocytes
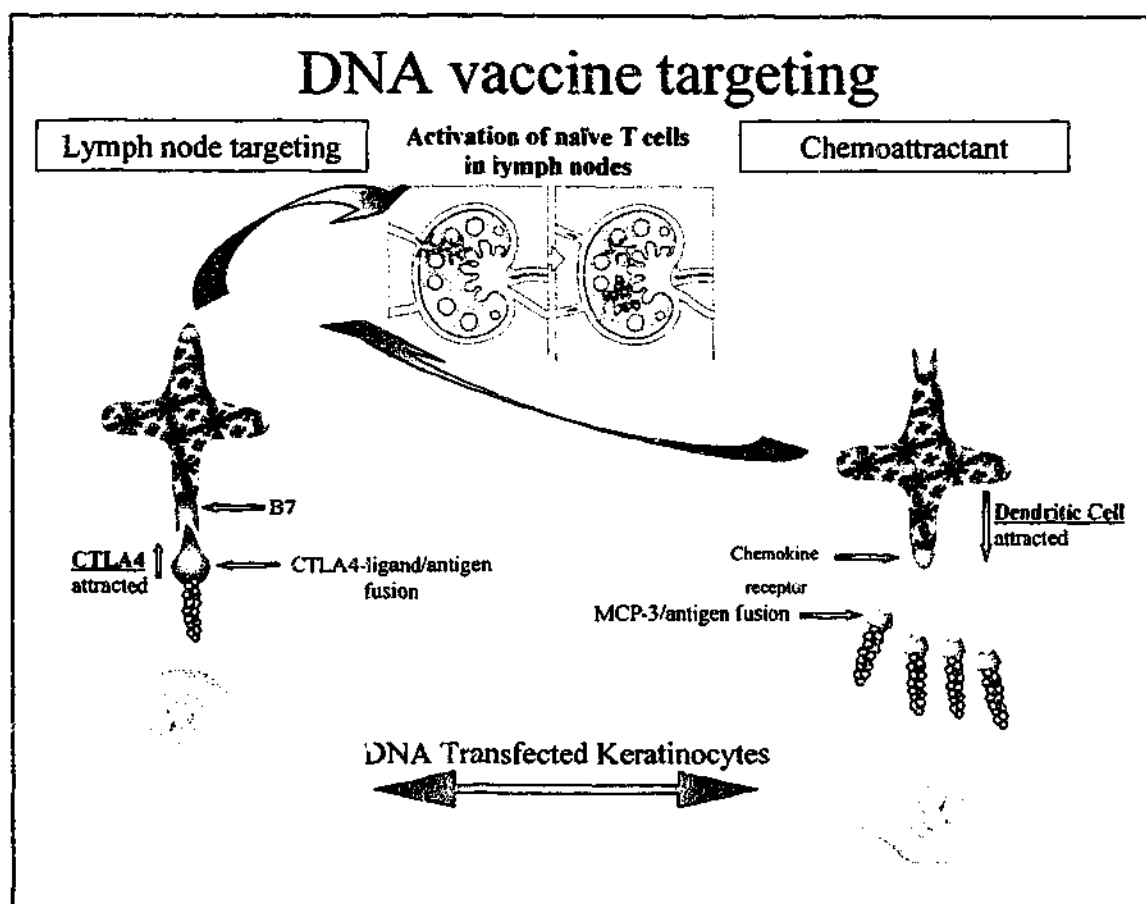
**Figure 11.** DNA vaccine targeting using either CTLA4 (lymph node targeting) (121) or chemoattractants such as MCP-3 (137). ID transfection of cells such as keratinocytes producing CTLA4/ligand fusions can bind to B7 expressing APCs, which then initiate immune responses in the lymph nodes. Expression of MCP-3/antigen fusions from transfected cells attracts APCs to the site of transfection along a chemokine gradient, which then prime naïve T cells in lymph nodes.

Bicistronic plasmids, which utilise an internal ribosome entry site (IRES) placed between two coding regions, allows ribosomes to attach to mRNA and translate the downstream coding sequence, while the upstream sequence is translated by the cap-dependent mechanism (84). The elements of IRES sites have been found in viral and eukaryotic mRNA, each differing in primary sequence, nucleotide length, and secondary structure, although most share a stable hairpin nucleotide structure (containing two conserved motifs: GNRA and RAAA, where N is any nucleotide and R is a purine) that promotes small ribosomal subunit binding (reviewed in 132). A common IRES sequence used in bicistronic vector construction is derived from encephalomyocarditis virus (EMCV), although internal initiation of translation can sometimes be inefficient and is dependent upon the nucleotide composition and arrangement of flanking coding regions (84).

Vaccination with multiple plasmids does not guarantee that all plasmids will be delivered into the nucleus of a single cell, thereby reducing the efficiency of DNA vaccination. It has been shown that using a hepatitis C bicistronic DNA vaccine, the delivery of a bicistronic plasmid containing hepatitis antigens and GM-CSF significantly enhance T-cell proliferative and antibody responses over vaccination with two separate plasmids (34). This has also been shown for hepatitis B DNA vaccination (35). Bicistronic DNA vaccination has also been used for B-cell lymphoma to produce light and heavy chain immunoglobulins from a single cell (179). This was found to be important for their generation, release from transfected cells and enhancement of immune response. In terms of malarial vaccine design, the interactions between malarial antigens that have been found to produce synergistic responses (e.g., as in 55) may be enhanced by their delivery from bicistronic plasmids

(although this is yet to be reported). Polycistronic vectors intended for vaccination are now also in development in other pathogen models (43). The screening of multiple malarial antigen pairs (for example, a known antigen with a yet to be characterised unknown antigen) in bicistronic vectors could also lead to the discovery of new vaccines. Another advantage of bicistronic delivery of malarial antigens is the reduction in redundant control regions such as promoters, untranslated domains, and terminators, reducing the size of the plasmid and increasing the stability of production in the bacterial cell (43). New combinations of antigens may be required to overcome problems with genetic restriction, to allow binding of diverse HLA types to malarial epitopes, when immunising effectively against malaria in outbred human populations.

## DNA Expression Library Immunisation (ELI)

DNA Expression Library Immunisation (ELI) is a true multi-valent expression system, as it utilises the genome of an entire pathogen. The identification of more protective malarial antigens may be possible through the construction of 'DNA libraries' containing DNA sequences from the entire genome. ELI has been applied to bacterial (11, 25) and parasitic infections (5, 134, 156, 181), and recently against simian immunodeficiency virus (184). Genomic ELI libraries encode antigens from all stages of the life-cycle, potentially allowing the discovery of antigens from particular stages of a life-cycle. ELI involves digestion of genomic DNA from an organism, fusing fragments into a DNA vaccine vector and immunising animals (Fig.12) (reviewed in 193). Sequential partitioning of protective libraries into smaller subsets of the genome may result in additional antigen discovery, although the discovery of a single antigen using genomic ELI has not yet been reported.
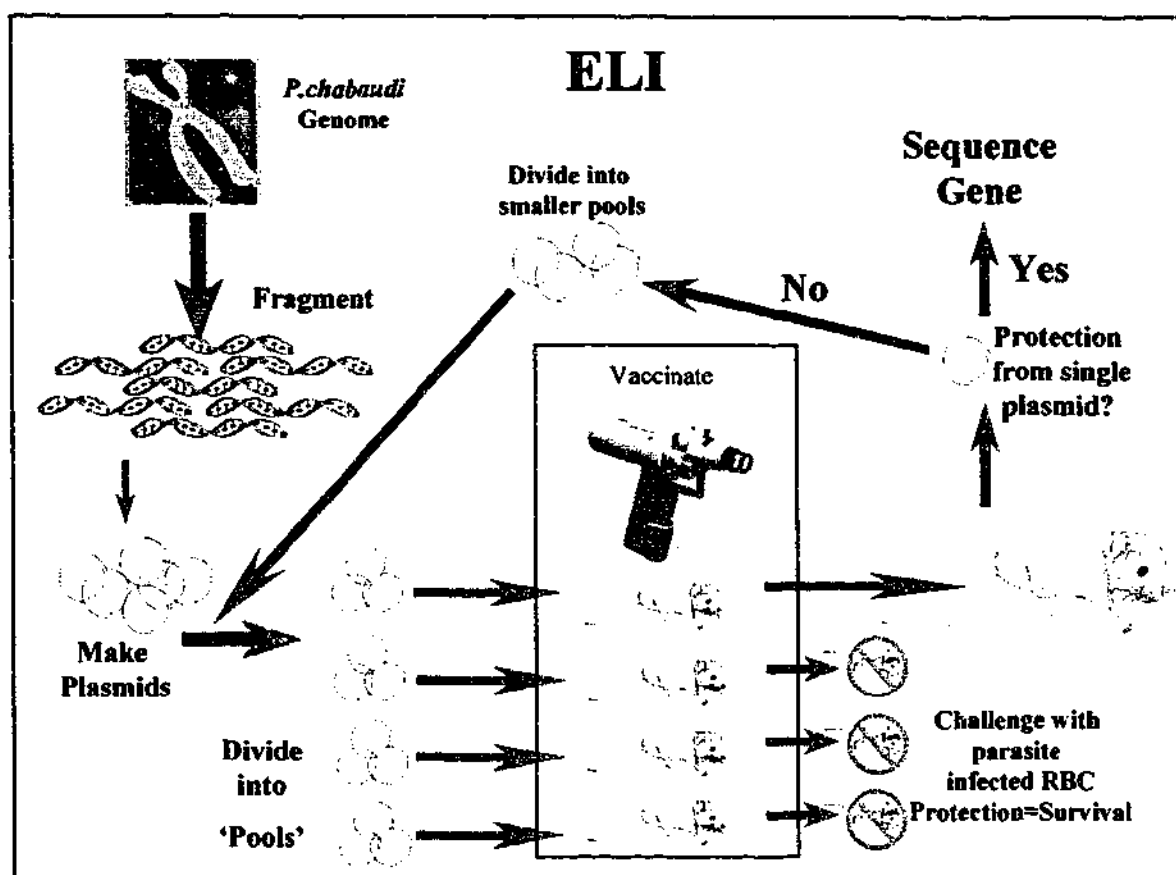
**Figure 12.** The steps involved in ELI toward eventual antigen discovery. The genome is fragmented with a restriction enzyme, followed by insertion into a DNA vaccine vector. The plasmids are then divided into 'pools' of known number, followed by vaccination into groups of animals. Protective groups are selected for, and the whole process is repeated until protection from a single or group of plasmids results.

Existing candidate antigens may be sufficient to protect against malaria in humans with corresponding HLA types, although it is possible that more antigen sequences will be required to protect across diverse genetic backgrounds. The discovery of new full length antigens or epitopes by gradual elimination of non-protective pools is the ultimate goal of ELI. Support for a multivalent vaccine comes from studies of Doolan who showed that it is the combination of malarial genes acting synergistically which have been shown to provide the greatest protection after challenge (e.g., 55, 100, 197). The body of evidence already discussed suggests, at least for malarial vaccination studies, that the removal of antigens acting synergistically is detrimental to protection. It has also been shown that vaccination of mice with combinations of 10 aa peptide epitopes derived from $MSP1_{33}$ can enhance protection of mice over vaccination with individual peptides (205). Therefore, maximising protection by including all combinations of potentially many diverse antigens/epitopes encoded within a genomic DNA vaccine library itself cannot be ignored, since this may lead to improved multivalent vaccines.

## 1.7) Aims and outcomes of this thesis

Work described in this thesis aims to contribute to the optimisation of DNA vaccine delivery against erythrocytic stage malaria, the stage attributed to morbidity and mortality associated with malaria (138). Pre-erythrocytic malarial vaccines are suited to subjects who have never been exposed to malaria, as the elimination of sporozoites would completely prevent malaria (112). However people in malaria endemic regions where drug resistance is spreading and are already exposed to the parasite urgently require effective erythrocytic stage vaccines (155). It is believed that it will not be necessary to induce sterile immunity in clinically immune sufferers of malaria, although if sterile immunity is induced transmission between sufferers would cease (70). DNA vaccination may therefore effectively 'prime' the immune system, before natural boosting by malaria carrying mosquitos occurs (112). A successful erythrocytic stage vaccine would at the least have a therapeutic effect, thereby reducing the morbidity (and potential mortality) associated with malaria.

It can be argued that rodent models of malaria are not relevant to human infection however the principles of immune recognition and activation of effector pathways are likely to be similar in different host and parasite combinations, so mouse models may be used to establish "proof-of-principle" (reviewed in 117, 122). Several studies demonstrate the ability of cross-protection using *P. falciparum* antigens in rodent models of malaria. Antibodies against the *P.falciparum* P0 ribosomal phosphoprotein (PfP0) have been detected in malaria immune individuals, and passive transfer of sera raised in rabbits against PfP0 recombinant protein has been shown to protect mice against lethal *P.yoelii* erythrocytic stage challenge (32). Passive transfer of human antibodies to *P. falciparum* LSA3 (Table 2) protect mice against *P.yoelii* sporozoite challenge (24). DNA immunisation with *P. falciparum* LSA3 also protects against

*P.yoelii* sporozoite challenge in mice (169). Investigation into how different mouse and human models resemble each other in their responses, allows more insight into how this data can be extrapolated to humans (24, 147).

The first Chapter of results (Chapter 2) entitled "The protective efficacy of MSP4/5 against lethal *P.chabaudi adami* DS challenge is dependent on the type of DNA vaccine vector and vaccination protocol" attempts to establish the optimal DNA vaccine vector type, and route of vaccination (IM versus gene-gun) using the recently discovered MSP4/5, the murine homolog of human MSP4 and MSP5 (159). This involved comparing MSP4/5 DNA priming in a non-targeted VR1020 vector, the CTLA4 lymph node targeting vector, and MCP-3 chemoattractant vector. This Chapter also investigates MSP4/5 recombinant protein boosting after DNA priming in an effort to enhance antibody responses to protect against lethal *P.c.adami* DS erythrocytic stage challenge.

Chapter 3 entitled "Induction of specific T-cell responses, opsonising antibodies and protection against *P.chabaudi adami* DS malaria in mice vaccinated with genomic expression libraries expressed from targeted and secretory DNA vect rs" involved the construction and testing of genomic expression libraries in either VR1020, MCP-3, or CTLA4 vectors via the ID route using a gene-gun (158). The major aim of this Chapter was to assess the immune responses induced by ELI in mice, in order to confirm that ELI induced an antigen-specific response. This study was also performed to determine whether the efficacy of ELI could be enhanced using targeting vectors.

The second part of Chapter 3 involved the evaluation of Direct Expression Library Immunisation (DELI). The aim of this was to find large open reading frames contained within the VR1020/30K library which could be potentially used in future

DNA vaccine trials in Professor Spithill's laboratory. This technique has been successfully implemented for vaccination against *Mycoplasma hyopneumoniae* in pigs (140). It was demonstrated that when using DELI, a library of 20,000 clones was reduced to just 96 clones in a single screening using a polyHis fusion detection method. This section of Chapter 3 describes a pilot study conducted to evaluate DELI involving the 1-3 kilobase sequences taken from the VR1020/30K library.

Chapter 4 is entitled "Evaluation of bicistronic DNA vaccines against *P.chabaudi adami* DS malaria". With the knowledge gained from Chapters 2 and 3, the construction of a multi-valent delivery system evaluating bicistronic vectors against lethal erythrocytic stage challenge could commence. Two bicistronic vector types were constructed: BC construct 1 contained a Tissue Plasminogen Activated secretion signal sequence (TPA) and cloning site (designated Position 1) with MCP-3/MSP4/5 (Position 2), and BC construct 2 contained an MCP-3 leader sequence and cloning site (Position 1) with MCP-3/MSP4/5 (Position 2). The candidate antigen AMA-1 was inserted into Position 1 of both types of bicistronic vector constructs. A genomic library was also inserted into Position 1 of BC construct 1, which was designated BC 30K. This was constructed in an attempt to produce synergistic responses between epitopes/antigens contained in the genomic library, and MSP4/5, which was also contained in the vector. As the bicistronic expression of malarial antigens had not been reported, an evaluation of immunogenicity *in vitro* and *in vivo* was also performed. Overall, Chapter 4 attempted to assess whether bicistronic delivery of malarial antigens would be an efficient method for delivering multiple antigens, which may eventually be required to protect against the complex life-cycle of malaria.

# Chapter 2

# The protective efficacy of MSP4/5 against lethal *P.chabaudi adami* DS challenge is dependent on the type of DNA vaccine vector and vaccination protocol.

## *Abstract*

The enhancement of immunogenicity of malarial DNA vaccines is important if they are to have practical application in protecting against erythrocytic stage malaria. This Chapter describes three different DNA vaccine vector types used in conjunction with the erythrocytic stage Merozoite Surface Protein 4/5 (MSP4/5), the murine homologue of *P. falciparum* MSP4 and MSP5. These were used in an attempt to enhance survival against lethal *P.chabaudi adami* DS erythrocytic stage challenge. MSP4/5 was inserted into VR1020 (secretory), Monocyte-Chemotactic Protein-3 (MCP-3) (chemoattractant), and Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) (lymph node targeting) vectors. Mice were immunised intraepidermally via gene-gun, IM injection, or boosting with recombinant MSP4/5 protein. Antibody responses after boosting were predominantly of the IgG1 and IgE isotypes, with low avidity antibodies produced in DNA primed groups. Despite antibody responses comparable to recombinant protein immunisation, boosting mice primed with antigens encoded by MCP-3 and CTLA4 vectors did not enhance survival compared to vector control groups. Gene-gun vaccination using VR1020/MSP4/5 followed by recombinant MSP4/5 boosting, or gene-gun DNA vaccination alone using MCP-3/MSP4/5, resulted in enhanced survival compared to empty vector control mice. The results suggest that the enhancement of survival against lethal erythrocytic stage malaria challenge after utilizing MSP4/5 DNA vaccination is therefore highly dependent on the route and type of vaccine vector employed.

## 2.1) Introduction

This Chapter describes data involving MSP4/5 tested in secretory, lymph node targeting, and chemoattractant vectors against lethal *P.c.adami* DS erythrocytic stage challenge. This Chapter also forms the basis of the manuscript in Appendix I. It was first important to establish the optimal route and DNA vaccine vector type before further studies could commence using ELI. It was also important to establish a positive candidate antigen DNA vaccine as a control for future experiments.

Improvements in the immunogenicity of malarial antigens delivered by DNA vaccines are important if there is to be any possible practical application of this technology to provide protection against erythrocytic stage malaria. It has been demonstrated that immunisation strategies using malarial antigen through priming with DNA plasmids followed by boosting with recombinant protein or viral constructs, can result in enhanced immunogenicity against malaria when compared with DNA vaccination alone (reviewed in 113). Enhancement of antibody titre in malarial prime/boost (p/b) studies has relied heavily on administration of recombinant pox virus expressing malarial antigen, and such studies have also focussed on inducing $CD8^+$ T-cell immunogenicity against pre-erythrocytic stage malaria ( reviewed in 52, 67). In order to help resolve erythrocytic stage malarial infection however, antibody and the activation of $CD4^+$ T cells has been found to be required when vaccinating with recombinant candidate erythrocytic stage antigens such as AMA-1 (87, 210) and $MSP1_{19}$ (42, 85). Enhanced humoral responses to Pf155/RESA after priming with a DNA vaccine, and boosting with recombinant protein in adjuvant has been demonstrated in mice (76). Protection of *Aotus* monkeys against *P. falciparum* after DNA priming using the erythrocytic stage antigen EBA-175,

followed by protein boosting in adjuvant has been observed (101). Priming *Rhesus* monkeys with *P. falciparum* $MSP1_{42}$ DNA vaccine and boosting with recombinant $MSP1_{19}$ has also been shown to enhance antibody titres (114).

It has been suggested that on their own, first-generation DNA vaccines may not be adequate to protect against malaria (reviewed in 89). Antigen presenting cells (APCs), in particular dendritic cells, have been shown to be potent initiators of immune responses following DNA vaccination, and are important in the uptake of antigen expressed from cells transfected by a DNA vaccine (reviewed in 144). Improvement of DNA vaccine efficacy by targeting antigen to APCs may be required if DNA priming alone is to be sufficient to provide a significant level of protection against erythrocytic stage malaria. The use of the targeting ligand cytotoxic T lymphocyte antigen 4 (CTLA4) in DNA vaccination has been shown to improve the magnitude and speed of the antibody response (22, 56, 121). Chemokines such as monocyte chemotactic protein-3 (MCP-3) have also been shown to enhance protective efficacy after DNA vaccination in a tumor challenge model (16).

The mouse homologue of *P. falciparum* MSP4 and MSP5 was first identified in 1999 from *P.chabaudi* (18). Since then, MSP4/5 mouse homologues have also been identified for *P.berghei* and *P. yoelii* (107). Vaccination of mice using recombinant MSP4/5 protein of *P.yoelii* has been shown to protect mice from lethal challenge, with the greatest protection observed in mice producing the highest antibody levels prior to challenge (108). To date, protection studies using MSP4/5 in the *P. chabaudi* model system have not been reported and there is no information about the efficacy of DNA immunisation with this sequence either alone or in combination with protein boosting in any challenge system.

This Chapter investigates the potential of MSP4/5 DNA vaccination to protect mice against erythrocytic stage malarial challenge, and compares the efficacy of MSP4/5 vaccines when delivered by different vectors and protocols. The three different DNA vaccine constructs (VR1020/MSP4/5, CTLA4/MSP4/5, and MCP-3/MSP4/5) were used to prime mice via IM or ID (gene-gun) routes. Antibody responses were assessed in terms of titre, isotype, and avidity. Recombinant MSP4/5 protein was also used to boost antibody responses previously primed via different DNA vectors containing MSP4/5. The protective efficacy of vaccine combinations was evaluated against lethal erythrocytic stage challenge using the *P.c.adami* DS-BALB/c mouse model which is a stringent model for evaluating vaccine efficacy.

## 2.2) Materials and Methods

### *Creation of plasmids.*

The *P.c.adami* DS MSP4/5 sequence (lacking the predicted hydrophobic signal and GPI anchor sequences) was amplified by PCR from the plasmid pTrcHis-A/MSP4/5 which was kindly provided by Dr C. Black and Dr L. Kedzierski (Monash University, Australia) (described in 18) using the oligonucleotides 5'GGAGGCACGCGTATGAAGATCGCAAATTAT (containing an *Mlu*I restriction site) and 5'CTCTAGAGATTAATATAATAATGCTATTAT (containing an *Xba* I restriction site). The CTLA4 vector was provided by Dr A. Lew (Walter and Eliza Hall Institute, Australia) (22) and was prepared by digestion with *Mlu*I and *Xba*I, and the PCR amplified MSP4/5 fragment was inserted to produce CTLA4/MSP4/5. Again, the MSP4/5 sequence was amplified from the pTrcHis-A/MSP4/5 plasmid using the oligonucleotides 5'ATGATGGGATCCATGAAGATCGCAAAT (containing a *Bam*HI site) and 5'GAAGTAGATCTTTATGAATCTGCACTGAG

(containing a *Bgl*II site). The VR1020 vector (VICAL, San Diego, CA, USA) (81) was prepared by digestion with *Bam*HI and *Bgl*II and the amplified MSP4/5 sequence inserted to produce VR1020/MSP4/5.

Murine MCP-3 cDNA was supplied by Dr Harshall Nandurkar (Monash University, Australia). The oligonucleotides 5'TATTATTAGCGGCCGCATGAGGATCTCTGCCACGCTT (containing a *Not*I site) and 5'TATGGATCCTCCACCTCCACCTCCAGGCTTTGGAGTTGGGGT (containing sequence encoding 6xGly aa followed by a *Bam*HI site) were used to amplify MCP-3 by PCR. The MCP-3 PCR fragment was digested with *Not*I and *Bam*HI, and inserted into the VR1012 (VICAL) vector digested with the same enzymes to produce the MCP-3 vector (provided by Dr P.Smooker, RMIT University). MSP4/5 was amplified by PCR from pTrcHis-A/MSP4/5 using the oligonucleotide 5'ATGATGGGATCCATGAAGATCGCAAAT (containing a *Bam*HI site) and 5'CTACTGGATCCTTATGAATCTGCACTGAG (containing a *Bam*HI site), then inserted into the *Bam*HI site of the completed MCP-3 vector.

Ligation reactions were transformed into *E. coli* DH5α and selected on solid media containing 50 µg/ml kanamycin for VR1020/MSP4/5 and MCP-3/MSP4/5 vectors, and 100 µg/ml ampicillin for the CTLA4 vector.

### *Expression and purification of recombinant proteins*

Expression and purification of MSP4/5 ectodomain recombinant protein was performed as described in (18). Protein for initial MSP4/5 protein pilot experiments was provided by Dr C. Black and Dr L. Kedzierski (Monash University, Australia). Recombinant protein for future vaccine trials was prepared according to the following protocol. Briefly, the pTrcHis-A/MSP4/5 vector was transfected into *E. coli* BL21

(DE3) (Novagen, Milwaukee, Wis, USA) for expression of recombinant protein. Large scale purification of the recombinant protein was performed using TALON metal affinity resin (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

### Mammalian cell transfection with MSP4/5 DNA plasmids

Plasmid constructs were tested for expression in COS 7 cells prior to use in animals. Freshly grown COS 7 cells were seeded at $2 \times 10^5$ cells per 35 mm tissue culture dish. Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. COS 7 cells were then incubated in 5% $CO_2$ until 80% confluent. 1μg of plasmid DNA was used to transfect COS 7 cells using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Media was changed 24 hours after transfection. After incubation for a further 2 days at 37°C the cells were washed with PBS, and media was replaced with serum free media to remove FCS that may have interfered protein detection, and cells were grown for a further 24 hours. The supernatant was then collected and subjected to SDS-PAGE and Western Blotting.

### SDS-PAGE and Western Blotting

Protein and COS 7 supernatants were fractionated by SDS-PAGE on 12% (v/v) polyacrylamide gels under reducing conditions and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked in 5% milk powder overnight at 4°C. The membranes were probed using an anti-MSP4/5 rabbit antibody,

followed by an anti-rabbit Ig conjugated to horseradish peroxidase (HRP) (Silenus Laboratories, Melbourne, Australia). The reactive antibodies were then visualized by Enhanced Chemiluminescence (Amersham, Piscataway, NJ, USA).

### *Enzyme-linked immunosorbent assay (ELISA)*

Antibody reactivity with recombinant MSP4/5 protein was measured by ELISA. Microtitre plates were coated with 0.1 ml/well of recombinant MSP4/5 (1 μg/ml) overnight at 4°C using carbonate-bicarbonate buffer pH 9.6. Plates were washed with PBS and 0.05 % TWEEN 20 (Sigma, St Louis, MO, USA)(PBS-T), followed by blocking overnight at 4°C in 5 % skim milk powder and PBS-T. Plates were again washed and diluted sera incubated at 37°C for 2 hours. After washing plates again using PBS-T, total humoral responses were obtained using HRP-conjugated sheep anti-mouse Ig (Silenus) diluted 1:2000 and incubated for 1 hr, followed by washing and addition of substrate. For detection of antibody isotypes, non-conjugated sheep anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgE (The Binding Site, Birmingham, UK) was diluted to 1:1000 and incubated for 1 hr at 37°C. This was followed by washing and addition of HRP-conjugated donkey α-sheep Ig (Silenus) diluted to 1:1000 and incubated for 1 hr at 37°C. After final washing, the ELISA was developed by addition of substrate 3,3',5,5'-tetramethylbenzidine (Sigma). Absorbance was measured at 450 nm, and titres defined as the highest dilution required for an absorbance of 0.2.

### Estimation of antibody avidity

The avidity of the antibody response was performed as described (46), with the following modifications. Briefly, MSP4/5 was used to coat microtitre plates and blocked as described for ELISA. Sera were diluted in the following concentrations of guanidine-HCL: 0M, 1M, 2M, 3M, 3.5M, 4M, 4.25M, 4.5M, and 5M. Sera were then incubated on the plates for 1 hr at 37°C. After extensive washing, total humoral responses were obtained using HRP-conjugated sheep anti-mouse Ig (Silenus) and developed as described for ELISA. The antibody titre was described as the dilution for sera that gave, on the linear portion of the dilution curve, an OD of 50% of the maximum O.D observed. Titres were then normalised for comparison by setting the titre observed in the absence of guanidine at 100%. Sera from individual mice were analysed separately and data represented as the mean titre for each group.

### Isolation of plasmid DNA and construction of vaccination cartridges.

Plasmid preparation and endotoxin removal was performed as described by Boyle *et al* (23). Briefly, DNA was purified from a cleared lysate by PEG 8000 precipitation and endotoxin was removed by three extractions with Triton-X114. Purified DNA was precipitated onto gold microcarriers and these attached to plastic supports according to the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, CA, USA). DNA was combined with carriers at a ratio of 100 μg DNA/50 mg carriers. Each cartridge contains approximately 1 μg DNA.

## Mice and vaccinations

All mice were BALB/c, female, and 5-6 weeks of age at the time of first vaccination. Groups of mice were vaccinated with MSP4/5 protein according to (108), with the following modifications. The MSP4/5 protein group mice were initially vaccinated intraperitoneally (IP) with 25 μg of MSP4/5 protein in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich, USA), followed by two booster immunisations IP of 25 μg of MSP4/5 protein in incomplete adjuvant (IFA) at 2 week intervals. The Freund's adjuvant control group received the same protocol minus MSP4/5 protein. DNA vaccinated mice received three immunisations at two-week intervals. For intraepidermal (ID) DNA vaccination the abdominal region was shaven and particles containing 1 μg of DNA were delivered by the Helios gene-gun (Bio-Rad Laboratories, USA) with a pulse of helium gas at 400 lb/in$^2$. Intramuscular (IM) DNA plasmids were delivered into the *tibialis anterior* muscle (100 μg total) in PBS. All prime/boost mice (including vector controls) initially received three immunisations with plasmid DNA at two week intervals (via ID or IM routes), and were then boosted with a single dose of 10 μg of MSP4/5 IP in incomplete Freund's adjuvant 2 weeks after the final DNA vaccination.

## Infection of mice, blood sampling and parasitemia measurements.

Blood from an infected mouse with a known parasitemia (1-10%) was taken and immediately diluted in PBS to give the required dosage (1 x 10$^5$ infected RBC/dose). Mice were infected by intraperitoneal injection at day 0, and parasitemia assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels

and day to peak parasitemia were compared using the Mann-Whitney non-parametric
$t$-test.

*Analysis of survival curves*

Survival curves for vaccinated and control mice were compared using the Mantel-Haenszel test. Statistical analysis was performed using Prism 3.02 software (GraphPad, San Diego, CA, USA).

## 2.3) Results

*Expression of protein encoded by DNA vaccine plasmids in vitro*

The ability of mammalian cells to secrete MSP4/5 after transfection with DNA vaccine plasmids was tested in COS 7 cells. Proteins encoded by three constructs were secreted into the culture supernatant *in vitro* (Fig.1). CTLA4/MSP4/5 ($\approx$95 kDa) and MCP-3/MSP4/5 ($\approx$50 kDa) fusion proteins, as well as MSP4/5 ($\approx$36 kDa) (secreted by virtue of the TPA signal sequence contained in VR1020), were detected in the cell free supernatant after being probed with anti-MSP4/5 rabbit sera by Western Blot. Supernatants collected from COS 7 cells after transfection with DNA vaccine vectors not containing MSP4/5 inserts did not react with anti-MSP4/5 rabbit sera (data not shown).

**Figure 1.** Western Blot of supernatants taken from COS 7 cells transfected with plasmid vectors containing MSP4/5 inserts. The Western Blot was probed with anti-MSP4/5 rabbit sera. Fusion proteins expressed by each construct are secreted into the culture supernatant (A) CTLA4/MSP4/5, (B) MCP-3/MSP4/5, and (C) VR1020/MSP4/5. Control vectors (CTLA4, MCP-3, and VR1020) not containing MSP4/5 inserts did not react with anti-MSP4/5 rabbit sera (not shown). Molecular mass standards are shown.

*IgG responses in mice vaccinated with MSP4/5 DNA vaccine constructs*

The plasmids VR1020/MSP4/5, CTLA4/MSP4/5, and MCP-3/MSP4/5 were used to vaccinate groups of four mice either IM by injection or ID using the gene-gun. A group of mice vaccinated with *E.coli* derived MSP4/5 *P.c.adami* DS protein formulation in Freund's adjuvant was included as a positive control. The resulting antibody responses were measured by ELISA using recombinant MSP4/5 protein. Figure 2 shows the titre of IgG antibodies produced by each group over a fifteen week period. Immunisation using MSP4/5 protein or the CTLA4/MSP4/5 constructs, regardless of route, induced a high antibody response two weeks after the initial dose which peaked by week 4. The VR1020/MSP4/5 and MCP-3/MSP4/5 ID immunised constructs induced antibodies at a slower rate, with titres peaking at week 9 after the initial dose (Fig.2). However, a slower and poorer induction of IgG was observed when both of these constructs were delivered IM relative to their ID counterparts (Fig.2). Immunisation using the three empty DNA vaccine vectors did not produce a detectable antibody response by ELISA to MSP4/5 protein (data not shown).
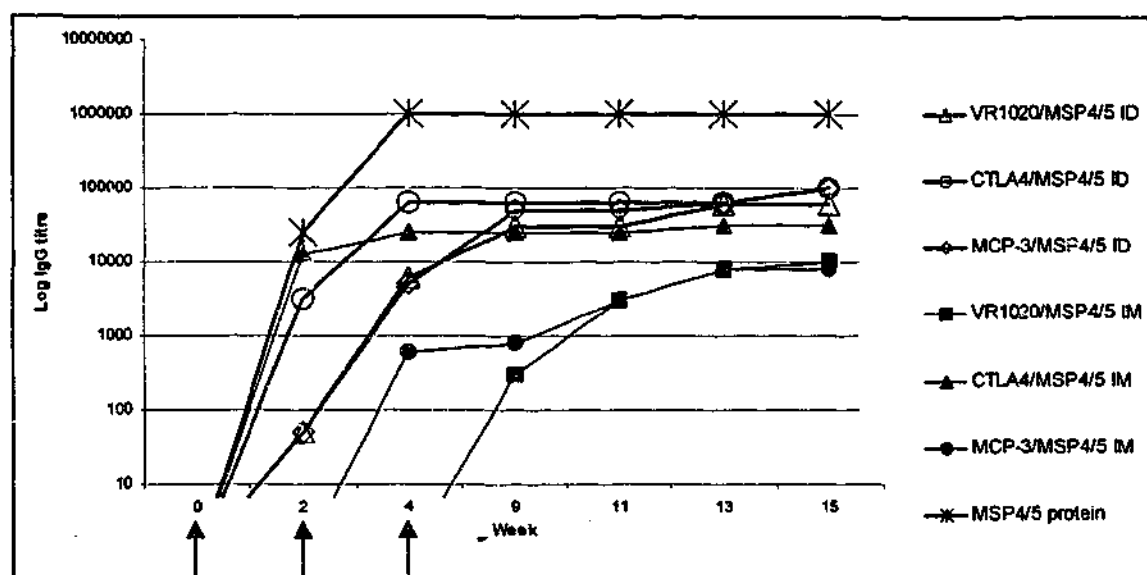
**Figure 2.** IgG responses in mice primed with MSP4/5 DNA or protein vaccines. Pooled mouse IgG antibody responses were measured by ELISA. Vaccines were delivered using either 1μg of DNA ID (gene-gun), or 100 μg of DNA IM using vectors containing an MSP4/5 insert. MSP4/5 protein (25 μg) was administered IP into control animals according to the protocol described in the methods. Arrows indicate a vaccination point. Vaccination with control vectors CTLA4, MCP-3, and MSP4/5 did not induce an antibody response (not shown).

*IgG responses of mice receiving the MSP4/5 prime/boost vaccine protocol*

After receiving three priming DNA vaccinations, four mice in each group received a boosting immunisation with 10 μg of MSP4/5 protein in incomplete Freund's adjuvant IP at week 13. Figure 3 shows a dramatic increase in IgG antibody titre detected at week 15 in each vaccine group, two weeks after MSP4/5 protein boosting. A single dose of 10 μg of MSP4/5 increased IgG titres to 1/64000 in control mice primed with CTLA4, MCP-3, or VR1020 negative control vectors. Boosting the VR1020/MSP4/5 IM or MCP-3/MSP4/5 IM mice achieved a similar titre of IgG. However, boosting with MSP4/5 protein after priming with VR1020/MSP4/5 ID resulted in an antibody response 90 times greater than ID priming alone. Boosting of mice vaccinated with CTLA4/MSP4/5 and MCP-3/MSP4/5 constructs, regardless of vaccination route also resulted in a marked increase of IgG antibodies, which approached the levels observed in the MSP4/5 protein vaccinated control group.
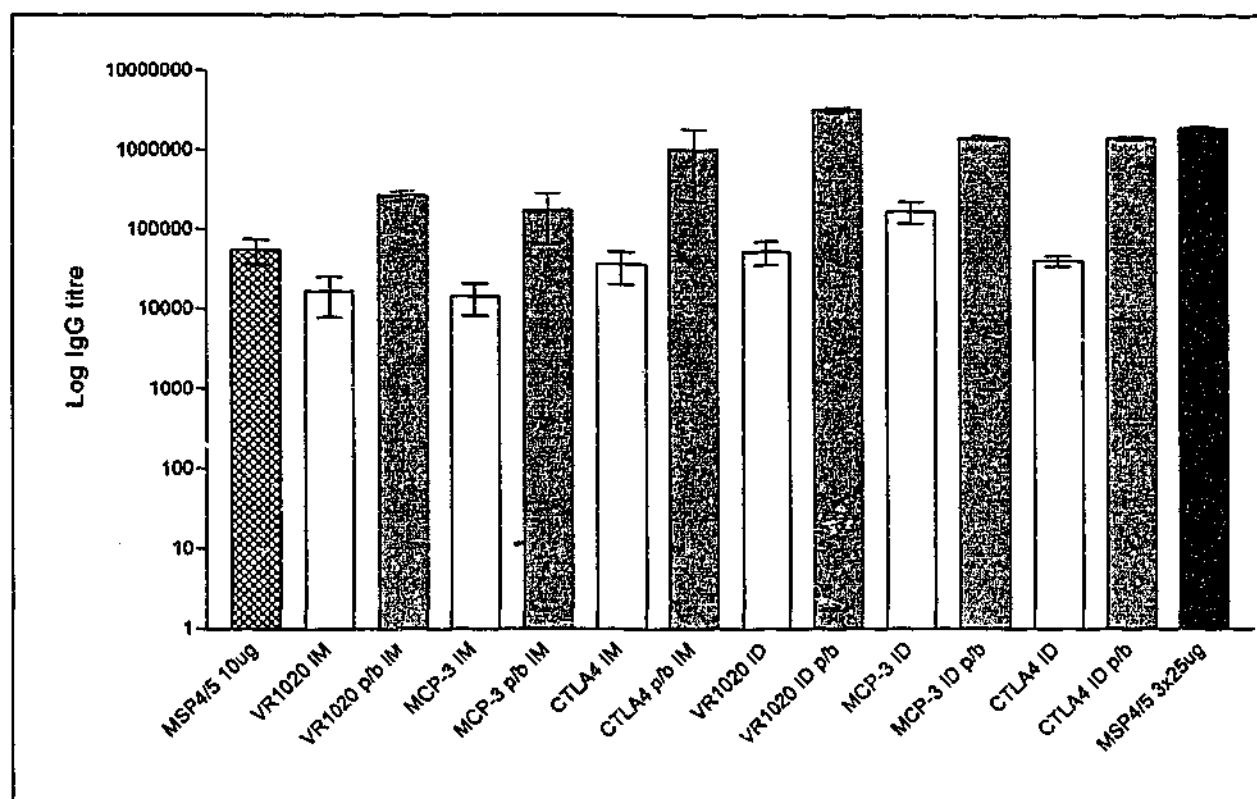
**Figure 3.** IgG responses in mice receiving a prime/boost vaccine protocol. Mouse IgG antibody titres in response to MSP4/5 vaccination in each vector are shown at week 13 after three priming doses of plasmid DNA (empty bars), or at week 15 following boosting with 10 μg of MSP4/5 IP in incomplete Freund's adjuvant (shaded bars). Empty vector primed control groups (hatched bar) received 10 μg of MSP4/5 protein in incomplete Freund's adjuvant. The MSP4/5 protein control group was vaccinated with 3 doses of protein according to the standard MSP4/5 protein protocol described in methods (black bar). Standard error is shown.

### Isotype analysis of pooled Ig responses in boosted mice

Since the isotype of the humoral response may be important in determining the efficacy of malaria vaccines by promoting appropriate ADCI reactions (reviewed in 71), the isotypes of Ig responses of mice vaccinated using the different protocols and boosted with MSP4/5 protein were analysed. Analysis of isotype responses after boosting mice revealed a dominance of IgG1 and IgE isotypes in all vaccine groups. The level of IgG1 and IgE responses are comparable to the total IgG observed (Fig.3). A Th2-type immune response regardless of IM or ID gene-gun vaccination is evidenced by the ratio of IgG1/IgG2a responses, and the level of the IgE responses to the vaccine (Fig.4). Mice primed only with plasmid DNA encoding MSP4/5 revealed a Th2 like response, with levels of IgG1 and IgE antibodies present and very low levels of IgG2a, IgG2b and IgG3 (data not shown). Boosting DNA primed mice with MSP4/5 protein did however enhance IgG2a, IgG2b, and IgG3 antibody subclasses (Fig.4).
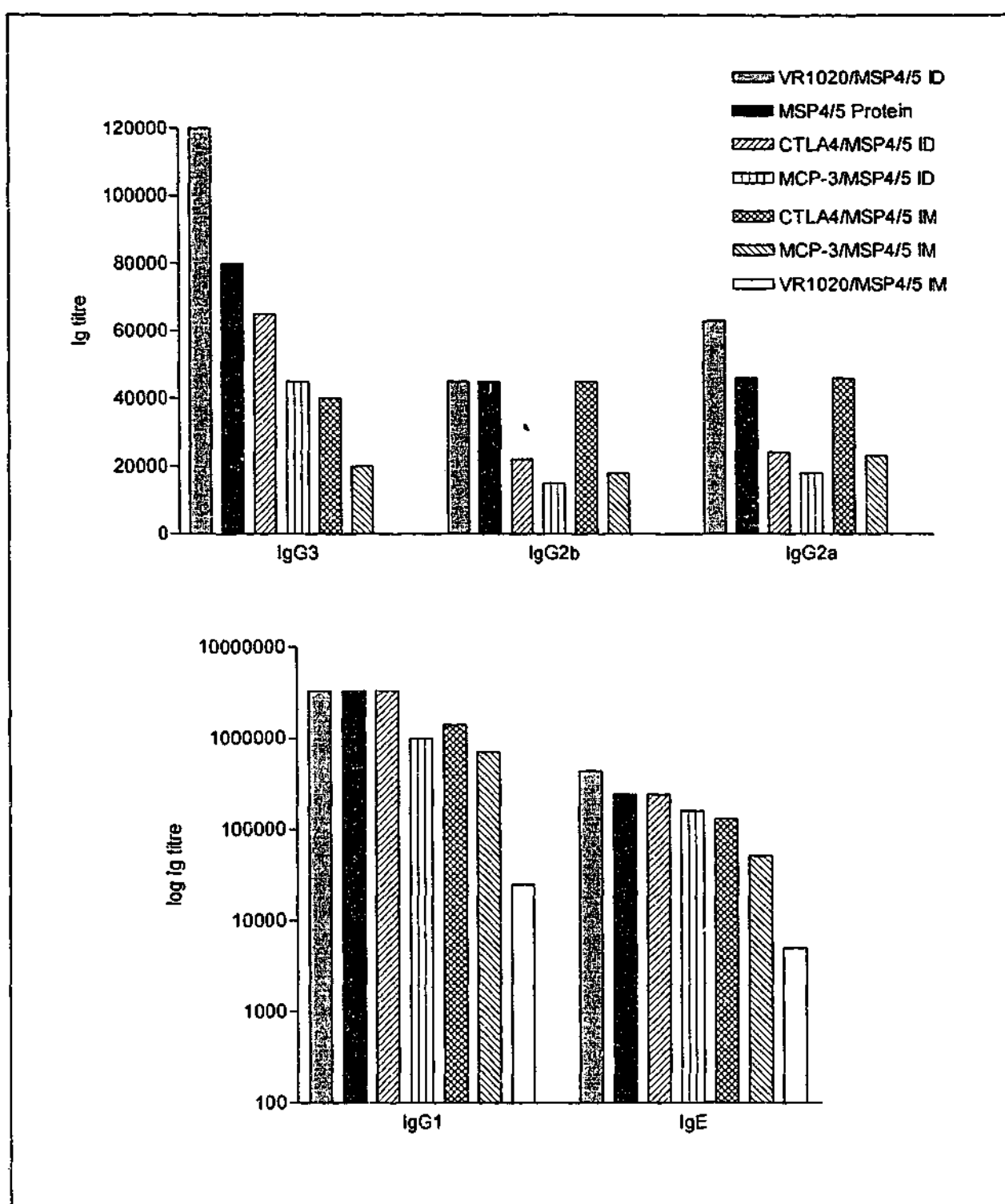
**Figure 4.** ELISA analysis of Ig responses at week 15 in pooled sera from MSP4/5 boosted mice. Mice were vaccinated using protein alone, by gene-gun ID, or by IM injection using 100 μg of plasmid DNA. All DNA primed groups were boosted at week 13 with 10 μg of MSP4/5 in IFA IP. Sera were obtained at week 15 and pooled for analysis.

*Avidity of IgG immune response in MSP4/5 DNA and protein vaccinated mice*

The avidity of the antibody response is an important determinant of the antibody - mediated effector response (71, 103). The average affinity of the antibodies generated by DNA prime/boost (p/b) or protein immunisation was determined by incubation with varying concentrations of the chaotropic agent guanidine-HCL. To determine avidity of the antibody response, the concentration of guanidine-HCL required to disrupt MSP4/5 antibody interactions by 50% was calculated. For each guanidine-HCL concentration the antibody titre at the midpoint of the linear portion of the ELISA curve was estimated (46). The antibody titre observed in the absence of guanidine-HCL was normalised to 100%, and the corresponding groups were compared to this Figure at differing concentrations of guanidine-HCL. Figure 5 shows that a higher guanidine-HCL concentration (>4 M) was required to completely disrupt MSP4/5 IgG interactions in the group vaccinated with MSP4/5 protein, relative to the groups primed ID with MSP4/5 DNA in all 3 vector types and boosted with recombinant protein. In the concentration range of 1-3 M guanidine-HCL, the sera for the CTLA4/MSP4/5 and MCP-3/MSP4/5 vaccines showed a higher avidity than MSP4/5 protein or VR1020/MSP4/5 groups. Above 3 M guanidine-HCL this difference was lost, suggesting that there may be two populations of IgG present in sera for the CTLA4/MSP4/5 and MCP-3/MSP4/5 groups.
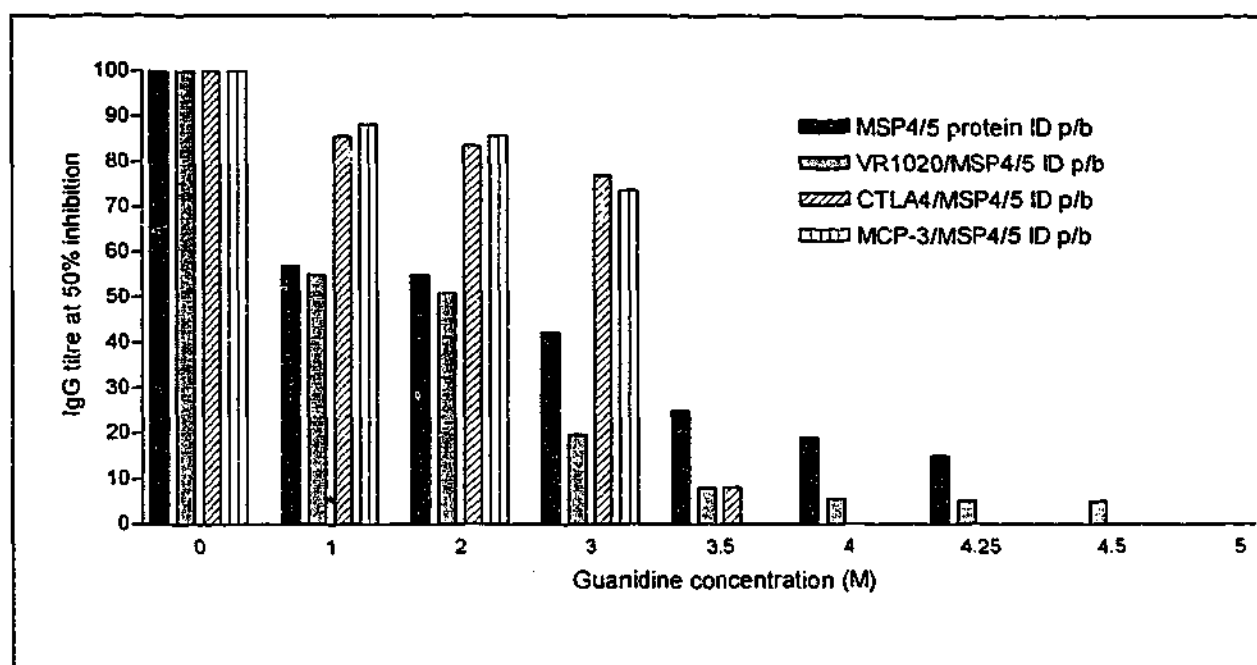
**Figure 5.** Avidity of the antibody response to MSP4/5 in vaccinated mice. ELISAs were performed on sera over a wide range of dilutions ($1/1000$-$1/10^6$) using sera from MSP4/5 protein vaccinated mice or mice given an ID p/b vaccination. Duplicate ELISAs using different concentrations of guanidine-HCL (0-5M) were performed over the same dilution range. A logarithmic trend line was calculated for the dilution curve. The intersection of the trend line which corresponded to a 50% reduction in O.D (in the linear portion of the dilution curve) was used to determine antibody titre. The titre observed in the absence of guanidine-HCL was normalised to 100%. The titres observed in the presence of differing concentrations of guanidine-HCL were compared to the untreated titre and expressed as a percentage of this figure.

### Vaccine efficacy: MSP4/5 P.chabaudi adami DS Pilot study

The *P.chabaudi adami* DS mouse model is a stringent test for vaccine efficacy due to the high virulence of this DS strain (8, 41). In order to determine the protective efficacy of *P.c.adami* DS MSP4/5 against a virulent challenge, a pilot study containing 8 BALB/c mice per group was conducted. The study involved a Freund's adjuvant control group and an MSP4/5 protein group. Mice received an initial IP vaccination of either complete Freund's adjuvant (control) or 25 μg of MSP4/5 in CFA, and two subsequent vaccinations at two week intervals with either incomplete Freund's adjuvant (control) or 25 μg of MSP4/5 protein in incomplete Freund's. Mice were then challenged with 100,000 IRBC two weeks after the final vaccination. A significant difference between Freund's control mice and MSP4/5 vaccinated mice was found, with 75 % survival in the vaccinated mice ($P$=0.015; Fig.6 Panel i), comparable to the protection found with *P.yoelii* MSP4/5 protein vaccination (108).

### Vaccine efficacy: Challenge Trial 1:

The first challenge trial initially contained eight mice per group. Mice were vaccinated IM (100 μg) by injection or ID (1 μg) using the gene-gun. Mice were primed a total of three times at two week intervals for both IM and ID groups (Table 1). The control groups consisted of mice vaccinated with recombinant MSP4/5 in Freund's adjuvant and a group vaccinated with Freund's adjuvant alone (Table 1, groups 13 & 14 respectively). Protein control mice were injected IP with 25 μg of MSP4/5 protein 3 times at two week intervals. At week 13, groups 1-12 (Table 1) were divided into two groups of four mice, with one group receiving 10 μg of MSP4/5 protein in incomplete Freund's adjuvant as a boost. Control mice primed with empty

vector also received 10μg of MSP4/5 in incomplete Freund's adjuvant to control for the effects of a single dose MSP4/5, and any possible synergy with the priming plasmid DNA. The IgG antibody kinetics was followed until week 15 (Fig.3) and then all mice were challenged with 100,000 infected *P.c.adami* DS red blood cells.

Figure 6 shows survival data for both IM and ID vaccination using each vector type. Mice vaccinated ID using VR1020/MSP4/5 p/b were protected against lethal challenge (75 % survival) compared with the VR1020 vector ID p/b control ($P$=0.029; Fig.6 Panel iii). A significant delay in death after administration of VR1020/MSP4/5 IM compared with the VR1020 vector IM control was also detected ($P$=0.038; Fig.6 Panel ii), but not with the VR1020/MSP4/5 IM p/b protocol (Fig.6 Panel ii). Vaccination with the MCP-3/MSP4/5 ID construct also resulted in a significant delay in death compared with the MCP-3 ID vector control ($P$=0.017; Fig.6 Panel v), although vaccination using the MCP-3/MSP4/5 ID p/b protocol did not enhance protection ($P$=0.11; Fig.6 Panel v). No significant differences in survival between groups vaccinated using MCP-3/MSP4/5 IM compared to its MCP-3 IM vector control (Fig.6 Panel iv), or CTLA4/MSP4/5 constructs administered via either route compared to CTLA4 control vectors were detected (Fig.6 Panels vi & vii).

Vaccination using MSP4/5 protein alone resulted in 63 % protection of mice in the MSP4/5 protein control group ($P$=0.011; Fig.6 Panel i). There were no significant differences between survival curves of mice that received MSP4/5 protein alone, when compared against survival curves of other statistically significant groups, namely VR1020/MSP4/5 ID p/b ($P$=0.94), MCP-3/MSP4/5 ID ($P$=0.19), or VR1020/MSP4/5 IM ($P$=0.17).
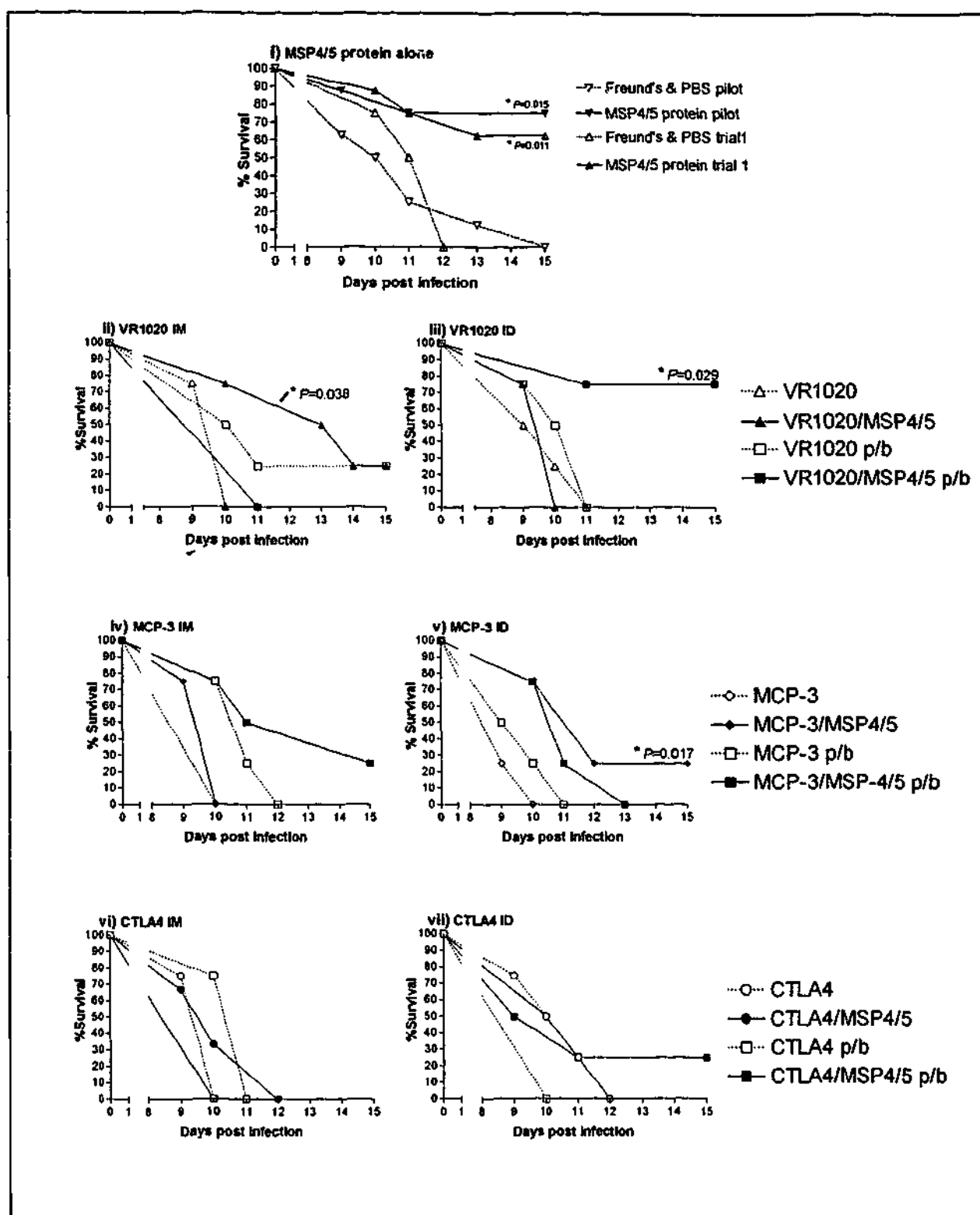
**Figure 6.** Survival curves of mice vaccinated using IM or ID protocols and challenged with *P.c. adami* DS. Panels ii-vii depict Trial 1 groups vaccinated IM by injection with 100 μg of DNA, or ID using the gene-gun. Groups were split in half at week 13, with one group receiving a 10 μg of MSP4/5 protein boost in IFA IP. MSP4/5 protein control groups (presented for clarity in Panel i) initially received 25 μg of MSP4/5 in CFA IP, followed by two extra doses of 25 μg of MSP4/5 in IFA at two week intervals. Control groups in all panels are represented by a dashed survival curve, vaccinates are represented in black. Significant differences between controls and vaccinates are indicated with an *.

*Vaccine efficacy: Challenge Trial 2:*

In order to confirm the protection (primarily in ID vaccinated groups) found in Trial 1, further challenge experiments were performed testing the efficacy of the MSP4/5 vaccine delivered by an ID and p/b protocol, with several modifications made to the protocol. For Trial 2, each group contained six mice, and all mice were primed three times ID at two week intervals. Mice in groups 7-9 (Trial 2, Table 1) were boosted using 10 µg of MSP4/5 protein in incomplete Freund's adjuvant at week 6, two weeks after the final priming vaccination. Half of the vector control groups (1-3, Trial 2, Table 1) were similarly boosted with 10 µg of MSP4/5 protein in incomplete Freund's adjuvant as controls for the protein boost. Significant differences in the efficacy of the vaccines were observed between ID and ID p/b groups, as seen in Trial 1. A significant difference between mice vaccinated with VR1020 vector versus VR1020/MSP4/5 p/b was detected; although mice vaccinated with the p/b formulation did not survive in this experiment, there was a significant delay in death (*P*=0.009; Fig.7 Panel ii). Vaccination using MCP-3/MSP4/5 plasmid DNA alone resulted in a significant delay in death as well as survival of 50 % of animals compared with the MCP-3 vector alone (*P*=0.021; Fig.7 Panel i) confirming the results of Trial 1. Boosting of the MCP-3/MSP4/5 primed animals did not enhance this protection, and no significant difference was found between survival curves when compared to the MCP-3 control vector (*P*=0.37; Fig.7 Panel ii).

As observed in the first trial, there were no significant differences between survival curves of the VR1020 control vector and VR1020/MSP4/5 prime only (Fig.7 Panel i), or any CTLA4 control vector and CTLA4/MSP4/5 vaccinated groups (Fig.7 Panels i & ii). Analysis of parasitemia levels also showed no statistically significant differences between groups vaccinated with vectors containing MSP4/5 or control

mice, except for VR1020/MSP4/5 p/b versus VR1020 vector alone ($P$=0.03, Mann-Whitney non-parametric $t$-test) (Table 1).

### *Vaccine efficacy: Challenge Trial 3:*

The final trial involved confirming observations from groups with survival curves significantly different from their control groups, namely MCP-3/MSP4/5 ID and VR1020/MSP4/5 p/b (Trial 3, Table 1). For Trial 3, each group contained six mice, and all mice were primed three times ID at two week intervals. Mice in groups 1 and 2 (Table 1, Trial 3) were boosted using 10 µg of MSP4/5 protein in incomplete Freund's adjuvant at week 6, two weeks after the final priming vaccination.

Again, a significant difference in kinetics of survival between mice vaccinated with the VR1020 control vector versus VR1020/MSP4/5 p/b was detected ($P$=0.009), with 33 % survival (Fig.7 Panel iii). Vaccination using MCP-3/MSP4/5 plasmid DNA alone resulted in a significant delay in death, as well as 33 % survival of animals compared with the MCP-3 vector alone ($P$=0.03; Fig.7 Panel iii). Analysis of parasitemia showed no statistically significant differences between groups vaccinated with vectors containing MSP4/5 or control mice (Mann-Whitney non-parametric $t$-test).

**Figure 7.** Survival of mice vaccinated by different protocols and challenged with *P.c.adami* DS. Panels i (DNA prime only) and ii (p/b groups) depict survival curves from challenge Trial 2. Panel iii depicts survival data from challenge Trial 3, representing MCP-3/MSP4/5 ID and VR1020/MSP4/5 p/b groups. No significant differences in survival curves were found between control animals receiving ⁀? µg of MSP4/5 protein in incomplete Freund's adjuvant versus control vector primed mice (panels ii & iii). Survival curves for vector and vector/protein boost were combined and represented as one survival curve (panel ii). Control groups in all panels are represented by a dashed survival curve, vaccinates are represented in black. Significant differences between control and vaccinates are indicated with an *.

Table 1. Protocol, parasitemia measurements, and survival curve $P$-values (delay in death) of mice challenged with lethal *P.c.adami* DS over 3 challenge trials. Survival curves between vector control mice and corresponding vaccinates were compared using the Mantel-Haenszel test to determine the significance of a delay in death. MSP4/5 protein group mice received 25 μg of MSP4/5 protein three times at two week intervals as described in the methods. DNA prime and p/b mice all received a total of 3 DNA vaccinations at two week intervals. Prime/boost mice (including vector controls) received 10 μg of MSP4/5 in IFA two weeks after the final DNA priming vaccination.

| Group | Peak % Parasitemia[1] | DNA prime only | | | | MSP4/5 ±DNA prime | | |
|---|---|---|---|---|---|---|---|---|
| | | Route | No. mice | Delay in death P= | % Surviving | No. mice | Delay in death P= | % Surviving |
| MSP4/5 Protein Pilot | | | | | | | | |
| 1. MSP4/5 protein | 27±10 | - | - | - | - | 8 | 0.015 | 75 |
| 2. Freund's adjuvant alone | 32±7 | - | - | - | - | 8 | ns | 13 |
| Challenge Trial 1 | | | | | | | | |
| 1. VR1020 vector (control) | 32±10 | ID | 4 | - | 0 | 4 | - | 0 |
| 2. CTLA4 vector (control) | 34±6 | ID | 4 | - | 0 | 4 | - | 0 |
| 3. MCP-3 vector (control) | 33±6 | ID | 4 | - | 0 | 4 | - | 0 |
| 4. VR1020 vector (control) | 29±4 | IM | 4 | - | 0 | 4 | - | 25 |
| 5. CTLA4 vector (control) | 35±9 | IM | 4 | - | 0 | 4 | - | 0 |
| 6. MCP-3 vector (control) | 32±6 | IM | 4 | - | 0 | 4 | - | 0 |
| 7. VR1020/MSP4/5 | 38±6 | ID | 4 | ns | 0 | 4 | 0.029 | 75 |
| 8. CTLA4/MSP4/5 | 33±6 | ID | 4 | ns | 0 | 4 | ns | 25 |
| 9. MCP-3/MSP4/5 | 37±9 | ID | 4 | 0.017 | 25 | 4 | ns | 0 |
| 10. VR1020/MSP4/5 | 34±10 | IM | 4 | 0.038 | 25 | 4 | ns | 0 |
| 11. CTLA4/MSP4/5 | 33±5 | IM | 4 | ns | 0 | 4 | ns | 0 |
| 12. MCP-3/MSP4/5 | 35±5 | IM | 4 | ns | 0 | 4 | ns | 25 |
| 13. MSP4/5 protein | 36±6 | - | - | - | - | 8 | 0.011 | 63 |
| 14. Freund's adjuvant alone | 39±4 | - | - | - | - | 8 | ns | 0 |
| Challenge Trial 2 | | | | | | | | |
| 1. VR1020 vector (control) | 45±11 | ID | 3 | - | 0 | 3 | - | 0 |
| 2. MCP-3 vector (control) | 36±13 | ID | 3 | - | 0 | 3 | - | 0 |
| 3. CTLA-4 vector (control) | 36±15 | ID | 3 | - | 0 | 3 | - | 0 |
| 4. VR1020/MSP4/5 | 34±4 | ID | 6 | ns | 0 | - | - | - |
| 5. MCP-3/MSP4/5 | 28±7 | ID | 6 | 0.021 | 50 | - | - | - |
| 6. CTLA-4/MSP4/5 | 30±17 | ID | 6 | ns | 0 | - | - | - |
| 7. VR1020/MSP4/5 (p/b) | 32±4* | ID | - | - | - | 6 | 0.009 | 0 |
| 8. MCP-3/MSP4/5 (p/b) | 28±5 | ID | - | - | - | 6 | ns | 33 |
| 9. CTLA-4/MSP4/5 (p/b) | 34±8 | ID | - | - | - | 6 | ns | 0 |
| Challenge Trial 3 | | | | | | | | |
| 1. VR1020 vector (control) | 44±8.7 | ID | - | - | - | 6 | - | 0 |
| 2. VR1020/MSP4/5 (p/b) | 37±9.7 | ID | - | - | - | 6 | 0.009 | 33 |
| 3. MCP-3 vector (control) | 34±6.7 | ID | 6 | - | 0 | - | - | - |
| 4. MCP-3/MSP4/5 | 45±10.1 | ID | 6 | 0.03 | 33 | - | - | - |

*$P$=0.03 Mann-Whitney test compared with VR1020 vector (control)
[1]There were no differences in peak parasitemia between DNA prime and MSP4/5 prime boost mice in Challenge Trial 1, therefore the peak parasitemia was combined for all animals in these groups. ±SD shown. ns – not significant

## 2.4) Discussion

In this study it has been shown that immune and protective responses generated, using the rodent malaria homologue of the human candidate malarial antigens MSP4 and MSP5, is dependent on the route and type of DNA vector used for vaccination. Intraepidermal gene-gun vaccination using a single malarial antigen and a vaccine-targeting approach, namely the MCP-3/MSP4/5 construct, produced a significant difference in protective efficacy compared with vaccination using the MCP-3 vector alone after lethal erythrocytic stage challenge. It was also shown that generation of antibodies after boosting MSP4/5 DNA primed mice with recombinant MSP4/5 does not always correlate with protection against lethal *P.c.adami* DS erythrocytic stage challenge. Boosting with recombinant MSP4/5 protein however did enhance protective efficacy after priming with the untargeted secretory vector construct VR1020/MSP4/5, but not with MCP-3/MSP4/5 and CTLA4/MSP4/5 constructs. Although boosting with MSP4/5 recombinant protein can enhance the generation of antibodies (particularly after gene-gun priming), the avidity of these antibodies varied between the vaccine groups.

Enhanced antibody generation two weeks after the initial vaccination was produced by the CTLA4/MSP4/5 construct, regardless of route. This is presumably due to the CTLA4 ligand binding directly to antigen-presenting cells to increase the likelihood of initiating an immune response (22). This rapid response is in contrast to administration of the VR1020 and MCP-3 MSP4/5 constructs which followed a slower pattern of kinetics after ID administration only. The erythrocytic stage malarial antigen AMA-1 is a leading candidate vaccine antigen (113). Lew *et al* (121) demonstrated that vaccination of mice using AMA-1 of *P.chabaudi* inserted into the

CTLA4 vaccine vector (the same vector type used in the present experiment) could also enhance antibody levels at just two weeks after the initial vaccination. The use of the CTLA4/MSP4/5 construct did not enhance survival regardless of administration route or recombinant protein boosting. Unfortunately, protection data using the CTLA4/AMA-1 construct, or CTLA4 applied to a malarial model has not been previously published. However, high protective efficacy using a CTLA4 construct has been demonstrated against *Corynebacterium pseudotuberculosis* after vaccination in sheep (121). CTLA4 constructs have also been shown to significantly reduce viral titres in mice using a haemagglutinin-based influenza DNA vaccine (45). The lack of protective efficacy observed in the present experiment with CTLA4/MSP4/5 constructs appears to be due to the CTLA4 targeting strategy, given that significant protection was observed when this antigen was delivered using the two other vaccine vectors.

The efficacy of the chemoattractant MCP-3 when applied to malaria DNA vaccines has not been previously described. MCP-3 fusion DNA vaccines have however been successfully applied to induce T-cell dependent anti-tumor immunity in mice (16), and MCP-3 itself is believed to be a potential inhibitor of HIV-1 infection (reviewed in 137). When used ID via gene-gun delivery, the MCP-3/MSP4/5 targeting vector construct provided a significant degree of survival against lethal *P.c.adami* DS erythrocytic stage challenge without the need for protein boosting. The use of the gene-gun to transfect dendritic cells such as Langerhans cells, which are abundant in skin, may provide an environment whereby MCP-3 is most useful. MCP-3 has been shown to bind to chemokine receptors CCR1, CCR2, and CCR3 which are all expressed on immune cells such as monocytes, T-lymphocytes, eosinophils, basophils and dendritic cells (reviewed in 137). Dendritic cells in particular have been shown to

play a major role inducing protection by processing and presenting antigen to naïve T cells after DNA vaccination ( reviewed in 38, 77, 144). It is possible that priming with MCP-3/MSP4/5 ID is enhancing T-cell migration, however further work is required to confirm this.

The use of the untargeted secretory DNA vaccine construct VR1020/MSP4/5 resulted in an enhanced antibody titre, as well as significant protection upon protein boosting when compared to priming with control vector DNA alone and boosting. The use of the VR1020/MSP4/5 DNA vaccine construct alone however did not enhance survival. Secretion of MSP4/5 protein from VR1020/MSP4/5 transfected cells is also likely to represent a form of the protein that more closely resembles the recombinant boosting protein. The fusion of CTLA4 to MSP4/5, when vaccinating with this construct, may affect the tertiary structure of the final secretory protein, and thus affect immunogenicity. The CTLA4/human Ig moiety (approximately 60 kDa) is considerably larger than the MSP4/5 fusion partner (approximately 36 kDa). It is possible that some protective epitopes on MSP4/5 may have been obscured upon folding of the CTLA4 moiety. Further studies evaluating the ability of sera generated by the CTLA4/MSP4/5 and MCP-3/MSP4/5 constructs to react with specific MSP4/5 protein domains (which may or may not have been obscured by the CTLA4 or MCP-3 moieties) are required to confirm this. The production of overlapping *P.c.adami* DS MSP4/5 domains would be useful in determining any differences in the binding specificities of sera generated using each of the three MSP4/5 vector constructs.

In the present study, enhanced antibody levels after boosting were at levels comparable to three doses of *P.c.adami* DS MSP4/5 protein, particularly in the gene-gun primed groups. Gene-gun vaccination itself has been found to bias the immune response to produce IgG1 and IL-4, a Th2-like response, in other DNA vaccine

systems (106, 153, 202). MSP4/5 DNA vaccination in this study biased the immune response towards producing IgG1 and IgE isotypes, regardless of vaccine route or boosting. Vaccinating with DNA ID using the C-terminal region of MSP1 of *P.yoelii* has also been found to produce predominantly IgG1 antibodies (103). Vaccination in the present study with MSP4/5 *P.c.adami* DS protein IP produced a Th2 like IgG1 and IgE response and high levels of protection. Protection after vaccination with *P.yoelii* MSP4/5 protein in female BALB/c mice however has been shown to correlate with high levels of IgG2a and IgG2b isotypes (110). This suggests that there may be major differences in the isotype response induced by the vaccine when using the analogous antigen from two malaria species. This is not surprising given that MSP4/5 for these two species share only 52% amino acid identity. It is therefore difficult to make comparisons of protective efficacy in relation to antibody isotype between these two malarial erythrocytic stage challenge systems. Using *P.chabaudi chabaudi*, it has been shown that IgG1 and IgG2a antibodies from hyperimmune mice coat the surface of schizont infected erythrocytes, and that either of these isotypes can prevent reinvasion of erythrocytes by inhibiting generation of new ring forms (29).

It has been reported that BALB/c mice immunised with ovalbumin via injection with DNA can produce antibodies with a higher avidity, particularly via the ID route, than mice injected by the same route with ovalbumin protein (23). In light of this, results from the present study show that avidity may be antigen specific, and that priming via the ID route does not always enhance the avidity of antibody at high concentrations of guanidine-HCL. A similar pattern of antibody avidity was has been found elsewhere (103). DNA vaccination of three different mouse strains via ID or IM routes using the C-terminal region of *P.yoelii* MSP1 also produced lower avidity antibodies than that observed in protein vaccinated mice. This was attributed to a lack

of affinity maturation of antibodies that were induced by DNA vaccination, with low avidity antibodies still detected at up to 32 weeks after the initial vaccination. Low avidity antibodies were also believed to be a contributing factor in the lack of protection found after lethal erythrocytic stage challenge (103). In the present study however the production of low avidity antibodies after ID vaccination did not result in a lack of protection after lethal erythrocytic stage challenge, as significant survival was observed in prime/boost mice vaccinated using the VR1020/MSP4/5 construct when compared with control mice.

This study demonstrates for the first time that vaccination using MSP4/5 from *P.c. adami* DS can protect against a highly virulent erythrocytic stage challenge when given as a protein only, DNA only (MCP-3 vector), or as a prime/boost vaccine (VR1020 vector). The targeting vector MCP-3/MSP4/5 when administered ID, results in significant protection against death in mice without the need for protein boosting. Priming with DNA, followed by boosting with recombinant protein, however, does have an effect on survival when using the untargeted VR1020/MSP4/5 vector. Although the level of humoral responses and predominant antibody isotypes were similar between DNA primed and boosted groups vaccinated using different vectors, use of the CTLA4/MSP4/5 vector did not enhance survival against erythrocytic stage challenge despite the high levels of antibody obtained. This suggests that the fine specificity of the antibody response is important in determining vaccine efficacy in this model. The results also suggest that the enhancement of survival against erythrocytic stage malaria challenge after utilising MSP4/5 DNA vaccination is therefore highly dependent on the route and type of vaccine vector employed.

# Chapter 3

## Induction of specific T-cell responses, opsonising antibodies and protection against *P.chabaudi adami* DS malaria in mice vaccinated with genomic expression libraries expressed from targeted and secretory DNA vectors

### *Abstract*

It has been proposed that a multivalent malaria vaccine is necessary to mimic the naturally acquired resistance to this disease observed in humans. A major experimental challenge is to identify the optimal components to be used in such a multivalent vaccine. Expression Library Immunisation (ELI) is a potential method for screening genomes of a pathogen to identify novel combinations of vaccine sequences. This Chapter describes immune responses associated with, and the protective efficacy of, genomic *P.chabaudi adami* DS expression libraries constructed in VR1020 (secretory), MCP-3 (chemoattractant), and CTLA4 (lymph node targeting) DNA vaccine vectors. Using splenocytes from vaccinated mice, specific T-cell responses, as well as IFN-γ and IL4 production, were observed after stimulation with *P.c.adami* DS infected erythrocytes, demonstrating the specificity of genomic library vaccination in two of the three libraries constructed. Sera obtained from mice vaccinated with genomic libraries promoted the opsonisation of *P.c.adami* DS infected erythrocytes by murine macrophages *in vitro*, further demonstrating the induction of malaria specific immune responses following ELI. Over three vaccine trials using biolistic delivery of the three libraries, protection after lethal challenge with *P.c.adami* DS ranged from 33-50%. These results show that protective epitopes/antigens are expressed within the libraries, and that ELI induces responses specific to *P.c.adami* DS malaria. This study Chapter demonstrates that ELI has the potential to screening the malaria genome to identify the components of multivalent vaccines.

## 3.1) Introduction

This Chapter describes data involving ELI tested in secretory (VR1020), lymph node targeting (CTLA4), and chemoattractant vectors (MCP-3) against *P.c.adami* DS erythrocytic stage challenge. This Chapter also forms the basis of the manuscript in Appendix II. The optimal route of delivery and DNA vaccine vector for the candidate antigen MSP4/5 was established in Chapter 2. It was now important to determine if ELI could be enhanced using DNA vaccine targeting vectors. The specificity of ELI also needed to be determined, as non-specific stimulation by non-coding genomic DNA included within these libraries remained a possibility.

Although there are several candidate antigens under development to combat malaria, there is no effective single-stage malarial vaccine yet available (reviewed in 204). There is a general consensus proposed that a multivalent vaccine is necessary to mimic naturally acquired resistance in humans. A major challenge is to identify the best antigen components to be used in such a multivalent vaccine (54, 113).

Protection in humans against asexual erythrocytic stages of malaria is believed to include mechanisms such as antibodies that block merozoite entry into erythrocytes, and inhibit parasite development (reviewed in 113). In addition, other studies have shown that malaria specific T cells can adoptively transfer resistance in mice against challenge with *P.yoelii* and *P.chabaudi* indicating that $CD4^+$ T cells alone may also play a role in vaccine induced immunity (206).

ELI enables screening of a pathogenic genome and eventual discovery of potential vaccine candidates from all stages of a pathogen's life-cycle. ELI has been applied to bacterial (11, 25) and parasitic infections (5, 134, 156, 181), and recently against simian immunodeficiency virus (184). Genomic ELI libraries encode antigens from all stages of the life-cycle. A primary focus for malaria vaccine development is the

erythrocytic stage which is responsible for the morbidity and mortality associated with malaria (reviewed in 204).

It has been reported that ELI using a *P.c.adami* DS genomic library significantly protects mice against erythrocytic stage malaria with the lethal *P.c.adami* DS strain (Appendix III) (181), although the protective mechanism(s) induced by this multivalent genomic vaccine remained to be elucidated. This Chapter extends these observations and reports that DNA vaccination of mice using two different libraries of malarial genomic DNA is sufficient in itself to protect against lethal erythrocytic stage malaria. Protection after library vaccination was found to be associated with T-cell responses of splenocytes from vaccinated mice specific to native malarial antigens/epitopes produced in *P.c.adami* DS infected blood. Murine macrophages incubated with sera, obtained after genomic library vaccination, also possessed the ability to opsonise *P.c.adami* DS infected red blood cells (IRBCs) *in vitro*, providing evidence that genomic library vaccination enhances humoral effector responses.

## 3.2)   Materials and Methods

### *Creation of plasmid pools.*

The creation of a genomic expression library is described in (181). Briefly,
*P.c.adami DS* genomic DNA was isolated from Ficoll-purified erythrocytes of
infected BALB/c mice (parasitemia 20-30%). The purified DNA was partially
digested with *Tsp*509I (5 U for 90 seconds at 65°C) and the digested fraction between
1 and 3 kbp isolated by gel elution. VICAL vector VR1020 (VICAL, San Diego, CA,
USA) containing a Tissue Plasminogen Activated secretion signal (TPA) was
prepared by digestion with *Bam*HI and *Bgl*II and the insertion of an *Eco*RI linker
constructed from the oligonucleotides 5'GATCCGGGAATTCAA and
5'GATCTTGAATTCCCG. Once obtained, the new vector was digested with *Eco*RI,
treated with alkaline phosphatase and ligated with *Tsp*509I digested *P.c.adami* DS
genomic DNA. Ligation mixes were transformed into *E. coli* DH5α and selected on
solid media containing 50 μg/ml kanamycin. After overnight growth *E. coli* colonies
were combined into pools. A total of 10 pools (termed 3KA-3KJ) each comprising
approximately 3,000 individual clones were constructed and stored at –80°C in 15%
glycerol.

The MCP-3 vector was prepared as described in Materials and Methods in Chapter 2
and in (159). Briefly, MCP-3 PCR fragment was digested with *Not*I and *Bam*HI, and
inserted into the VR1012 vector (VICAL) digested with the same enzymes to produce
the MCP-3 vector (provided by Dr P.Smooker, RMIT University). The CTLA4
vector (22) was a gift from Dr Andrew Lew (Walter and Eliza Hall Institute,
Australia). Libraries in MCP-3 and CTLA4 vectors were constructed as described for
the VR1020 vector (181) using the same *P.c.adami* DS genomic DNA stock.

Ligation reactions were transformed into *E. coli* DH5α and selected on solid media containing 50 µg/ml kanamycin for the MCP-3 vector, and 100 µg/ml ampicillin for the CTLA4 vector.

### *Isolation of plasmid DNA and construction of vaccination cartridges.*

Library pools stored as glycerol stocks were grown to confluence on solid media prior to inoculation into liquid media. 5-10 confluent plates were used to inoculate one litre of Luria Broth (LB) (1% NaCl, 1% Tryptone, 0.5% Yeast Extract) and grown with shaking at 37°C for 6 hours prior to harvest. Plasmid preparation and endotoxin removal was performed using the QIAGEN Endotoxin-free Plasmid Giga Kit according to the manufacturer's instructions (QIAGEN Inc, Valencia, CA, USA). DNA purified under endotoxin free conditions was precipitated onto gold microcarriers and these attached to plastic supports as per the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, CA, USA). DNA was combined with carriers at a ratio of 100 µg DNA/50 mg carriers. Each projectile contains approximately 1 µg DNA.

### *Mice and vaccinations*

All mice were BALB/c, female, and 4-6 weeks of age at the time of first vaccination. For vaccination, the abdominal region was shaven and particles delivered via the intraepidermal (ID) route using a Helios gene-gun (Bio-Rad Laboratories, USA) with a pulse of helium gas at 400 lb/in$^2$.

### Infection of mice, blood sampling and parasitemia measurements.

Blood from an infected mouse with a known parasitemia (1-10%) was taken and immediately diluted in PBS to give the required dosage ($1 \times 10^5$ infected RBC/dose). Mice were infected by intraperitoneal infection at day 0, and parasitemia assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels and day to peak parasitemia were compared using a Student $t$-test. The Mantel-Haenszel (or Logrank) test was used to measure the parameter of survival. This calculates a P value and tests the null hypothesis that the survival curves are identical between vaccinate and control groups. This was performed using Prism 3 software (GraphPad, San Diego, CA, USA).

### *In vitro spleen cell proliferation*

Ten days after the final DNA vaccination, mice were sacrificed, and single-cell suspensions of splenocytes were obtained by crushing whole spleens with a 5 ml syringe barrel. The splenocytes were then resuspended in complete RPMI 1640 media (Invitrogen Corporation, Grand Island, NY,USA) supplemented with 10% FCS, 2 mM glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St Louis, Mo, USA), and penicillin-streptomycin (100 U/ml; Invitrogen). Suspensions were then passed through a 100 μm pore nylon cell strainer (Beckton Dickinson, Franklin Lakes, NJ, USA), and exposed for three minutes to erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM EDTA, pH 7.4). Splenocytes were again washed and resuspended in RPMI 1640. Cell viability was greater than 80% as determined by trypan blue exclusion (Invitrogen). Splenocytes were stimulated with $2 \times 10^6$ *P.c.adami* DS iRBC, freshly

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extracted from an infected mouse, or $2x10^6$ freshly extracted RBC as a control. As a control for cell viability, splenocytes were stimulated with concanavalin A (Sigma) at a final concentration of 2.5 µg/ml. Splenocytes were cultured for 96 h in flat bottom microtitre plates in triplicate at a final concentration of $5x10^6$ cells/ml ($1x10^6$ cells/well), and pulsed with 1 µCi/well of [$^3$H] thymidine (Amersham Biosciences Corp., Piscataway, NJ, USA) 18 h before harvesting. The splenocytes were harvested onto fibre glass filter mats (Saktron Instruments Inc., Sterling, VA, USA) using an automated cell harvester (Saktron), and incorporated radioactivity measured using a liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, MA, USA).

## *ELISA for IFN-γ and IL4*

Spleen cells from vaccinated and control mice were incubated for 72 h in the presence of $2x10^6$ *P.c.adami* DS infected iRBC, or $2x10^6$ control RBC, and supernatants were harvested after 72 h and stored at -20°C until cytokine levels were measured by ELISA. IFN-γ and IL4 ELISAs were carried out according to the manufacturer's instructions (Endogen, Woburn, MA, USA). Briefly, Nunc Maxisorp (Nunc, Kamstrupvej, Denmark) ELISA plates were coated with anti-mouse IFN-γ monoclonal antibody (Endogen) or anti-mouse IL4 monoclonal antibody (Endogen) and incubated overnight at room temperature. The ELISA plates were then blocked in assay buffer (PBS with 4% BSA, pH 7.4). The supernatants obtained from the splenocyte cultures were tested in duplicate against serial dilutions of recombinant IFN-γ (starting at 5 ng), and IL4 (starting at 1 ng) standards. After incubation at room temperature for 1 h, anti-mouse IL4 biotin labelled monoclonal (Endogen) or anti-mouse IFN-γ biotin labelled monoclonal (Endogen) antibodies were added and incubated for 1 h at room temperature. After plates were washed 3 times, HRP-

conjugated Streptavidin (Amersham) was added at 1:16,000 and incubated for 30 min at room temperature. The plates were again washed 3 times and developed with 3,3',5,5'-tetramethylbenzidine substrate solution (Sigma), stopped with 0.18 M $H_2SO_4$. Absorbance was measured at 450 nm, and titres defined as the highest dilution required for an absorbance of 0.2.

## *Phagocytosis assays*

Phagocytosis assays were performed according to the method described in (143) with the following modifications. Macrophages were obtained from BALB/c mice by peritoneal lavage with 9 ml of ice cold 0.34 M sucrose. The cells were then centrifuged at 1000 g for 10 minutes at 4°C. Peritoneal cell exudates were then resuspended in complete RPMI 1640 (Invitrogen) supplemented with 10% FCS, 2 mM glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma), and penicillin-streptomycin (100 U/ml; Invitrogen) to a final concentration of $2 \times 10^6$ cells/ml. Eight-well chamber slides (Nalge Nunc International Corp, Naperville, IL, USA) were used, with $4 \times 10^5$ macrophages added to each well. The macrophages were allowed to adhere for 2 h at 37°C with 5% $CO_2$. After 2 h, non-adherent T cells were removed by careful washing with 1 ml of 37°C RPMI. Fresh complete RPMI 1640 was added and macrophages left to adhere for a further 2 h. During this time, fresh *P.c.adami* DS IRBC ($10^8$ IRBC/ml, containing trophozoites and schizonts at approximately 40-50% parasitemia) in complete RPMI 1640 were purified with Ficoll (Amersham). The IRBC were washed twice with complete RPMI 1640. After washing, IRBC pellets were placed into 1.5 ml centrifuge tubes in 15 μl aliquots. 30 μl of PBS and 1 μl of sera obtained from groups vaccinated with the genomic libraries, or empty vector controls, was then added to the IRBC pellets and incubated for 1.5 h at 37°C

with shaking. After 1.5 h, IRBC and sera were added to adherent macrophages and incubated for 2 h at 37°C with 5% $CO_2$. The eight-well slides were then washed four times with PBS to remove non-adherent macrophages and non-ingested IRBC. Non-ingested but adherent IRBC, were then lysed by incubation of the slides with cold water for 20 seconds, followed by washing with PBS. The eight-well slides were then fixed and stained using Kwik Diff staining solution (Terno Shandon, Pittsburgh, PA, USA). The number of IRBC taken up by 200 macrophages per individual sample was then quantified by light microscopy.

## *Immunofluorescence*

Fresh *P.c.adami* DS IRBC (containing trophozoites and schizonts at approximately 40-50% parasitemia) were diluted 1:10 in PBS, and smeared onto a microscope slide. The slides containing the IRBC were then immersed in methanol and acetone at a ratio of 1:9 (the ratio of methanol and acetone was varied between experiments to change the permeability of the IRBC to optimize the procedure), and placed at -20°C for 20 minutes. The slides were then air dried and stored in sealed plastic until use. Sera obtained from VR1020/30K, MCP-3/30K, and CTLA4/30K vaccinated mice (and corresponding empty vectors) was used. This sera was diluted 1:100 in PBS before adding to slides containing IRBC. This was then incubated at room temperature for 1 hr before being washed three times with PBS. Next, slides containing IRBC and antibody were allowed to air dry at room temperature. A green fluorophore-labeled goat anti-mouse IgG antibody (Alexa Fluor 488 goat anti-mouse IgG; Molecular Probes, Eugene, OR, USA) was diluted 1:300 in PBS, and placed on the air-dried slide and incubated in the dark at room temperature for 2 hours. The slide was then washed 3 times with PBS and allowed to dry, and visualised under an ultraviolet fluorescence microscope.

## 3.3) Results

### *Characterization of library clones*

All libraries were constructed using the same stock of genomic DNA. Obtaining genomic DNA from one digest batch was critical. This was important in order to minimise the any bias in insert size after enzymatic digestion of the genomic DNA. A partial enzymatic digest (described in the methods section) using *Tsp*509I was required as this enzyme recognises AATT DNA sequences. The *P.c.adami* genome itself comprises more than 80% adenosine and thymidine, therefore differences in digestion times between genomic DNA preparations would result in a bias of insert size.

Table 1 shows the characteristics of random samples of plasmid clones taken from VR1020/30K (181), MCP-3/30K, and CTLA4/30K genomic libraries. These sequences were compared against *P. falciparum* and *P.yoelii* genomic databases (www.ncbi.nlm.nih.gov/PMGifs/Genomes/plasmodium.html), and confirmed the presence of genomic DNA (data not shown). The DNA sequences were also translated and compared against *P.yoelii* and *P. falciparum* protein databases (data not shown), with the size ranges of peptides shown in Table 1. The construction method resulted in three libraries that were almost uniform in terms of size range and the presence of inserts. This was important in order to be able to make comparisons between the three different vectors. The average size of the inserts between the three libraries was 1430 bp. The *P.c.adami* genome is predicted to be approximately 23 Mb (www.ncbi.nlm.nih.gov/projects/Malaria/Rodent/chabaudi.html). Therefore the 30,000 plasmids contained in each of the genomic libraries represent 43 Mbp of DNA, resulting in an overall coverage of approximately 2 times the size of the *P.c.adami* genome for each library.

**Table 1.** Features of *P.c.adami* DS library clones

| Library | VR1020/30K [d] | MCP-3/30K | CTLA4/30K |
|---|---|---|---|
| Plasmid pools created | 10 x 3,000 clones (3KA-3KJ) | 10 x 3,000 clones (3KA-3KJ) | 10 x 3,000 clones (3KA-3KJ) |
| Plasmid clones examined by restriction digestion [a] | 58 | 32 | 45 |
| Number with inserts | 58 (100%) | 32 (100%) | 41 (93%) |
| Plasmid clones examined by sequencing | 24 | 22 | 33 |
| Average insert size | 1.5 Kb | 1.3 Kb | 1.5 Kb |
| Number with reading frame expressing peptide [b] | 18 (75%) | 20 (90%) | 23 (70%) |
| Size range of encoded peptides [c] | 1-115 amino acids | 3-113 amino acids | 2-139 amino acids |
| Number greater than 20 aa | 10 (42%) | 8 (36%) | 11 (33%) |
| Number greater than 50 aa | 3 (13%) | 3 (14%) | 6 (18%) |
| Average size of encoded peptide | 25 amino acids | 32 amino acids | 36 amino acids |

[a]All clones examined were from pool 3KA from each library. [b]In-frame with the TPA signal sequence (VR1020/30K), MCP-3 sequence (MCP-3/30K), and CTLA4 sequence (CTLA4/30K). [c]Not including leader sequences. [d] The VR1020/30K library used in (181).

The proportion of clones that will encode peptides was also estimated. Out of the 30,000 clones, about 50% will encode for a reading frame (since 50% of the genome encodes exons) (28). Of the remaining 15,000 clones, only 50 % will be oriented correctly (since cloning into the vector is not directional) and, of these 7,500 clones, only 1:3 will be in frame with the signal sequence: this leaves approximately 2,500 clones as potentially encoding an in-frame peptide (or about 8% of the clones). From the sequence analysis, it was found that 13-18% of the all the libraries actually encoded in-frame peptides longer than 50 aa (it is assumed anything longer than 50 aa is likely to be a real peptide due to the high AT content of malaria DNA which will create stop codons at high frequency). Based on this, the 30K genomic vaccines should contain approximately 8-18% (2500-5400) of clones that can potentially deliver an encoded peptide.

*Antigen-specific cellular immune responses induced by ELI DNA vaccination in three different vectors*

Groups of mice were vaccinated ID with either the VR1020/30K, MCP-3/30K or CTLA4/30K genomic libraries, or the corresponding empty plasmid DNA control vectors, according to the procedure described in the methods. To evaluate cellular responses to native *P.c.adami* DS antigens induced by each of the genomic libraries (and corresponding empty vector vaccinated control mice), spleens were removed from mice ten days after the final ID vaccination, and cell suspensions from individual mice were stimulated using $2 \times 10^6$ *P.c.adami* DS IRBC, or $2 \times 10^6$ RBC (prepared from naïve BALB/c mice) as a control.

Significant proliferation of spleen cells taken from VR1020/30K ($P<0.01$) and MCP-3/30K ($P<0.05$) vaccinated mice was detected upon stimulation with *P.c.adami*

DS IRBC when compared to VR1020/30K and MCP-3/30K spleen cells stimulated with control RBC (Fig. 1). In contrast, significant levels of proliferation were not detected in spleen cells obtained from CTLA4/30K vaccinated mice upon stimulation with IRBC ($P$=0.17) when compared to CTLA4/30K spleen cells stimulated with control RBC. Spleen cells obtained from control mice vaccinated with the MCP-3 or CTLA4 vector alone and stimulated with IRBC (Fig. 1) or RBC (data not shown) showed no detectable proliferation. However, spleen cells obtained from VR1020 vector vaccinated control mice did proliferate to a very low extent above background levels upon stimulation with IRBC (but not with RBC), but the levels of proliferation observed were significantly lower when compared with VR1020/30K IRBC stimulated spleen cells (Fig.1; $P$=0.013).
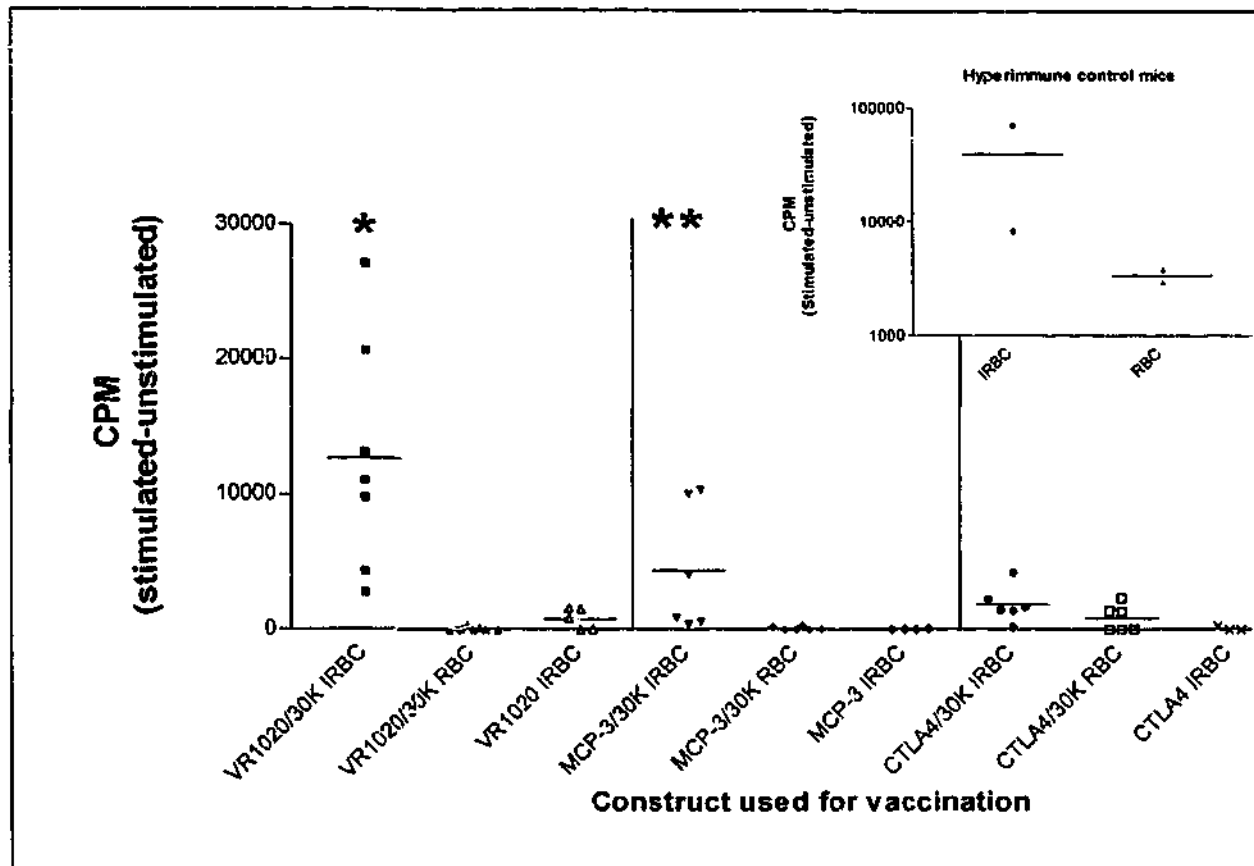
**Figure 1.** *In vitro* proliferation of splenocytes from individual BALB/c mice vaccinated ID with the gene-gun. Mice were vaccinated with VR1020/30K, MCP-3/30K, and CTLA4/30K 3 times at 2 week intervals. Splenocytes were harvested 10 days after the final vaccination. Following 72 h of stimulation with $2\times10^6$ IRBC or $2\times10^6$ RBC, [$^3$H] thymidine was added for 18 h. [$^3$H] incorporated by cells was then measured as CPM. Splenocytes vaccinated with empty vector control DNA (VR1020, MCP-3, CTLA4) were also stimulated with $2\times10^6$ RBC, but no incorporation of [$^3$H] thymidine was observed (data not shown). Splenocytes from all individual splenocyte cultures responded to concanavalin A stimulation (data not shown). The mean CPM (bar) is shown. *, $P<0.01$; **, $P<0.05$. The insert shows the response of splenocytes from hyperimmune control mice (mice that had survived *P.c.adami* DS infection) that were used as a positive control for IRBC.

When compared to splenocytes from control mice vaccinated with vector DNA, VR1020/30K splenocytes stimulated with IRBC produced significantly higher levels of IFN-$\gamma$ (Fig. 2A; $P<0.05$). Spleen cells obtained from MCP-3/30K mice when stimulated with IRBC also produced detectable levels of IFN-$\gamma$ in the culture supernatant (Fig. 2A; $P<0.05$). No IFN-$\gamma$ was detected in culture supernatants of splenocytes taken from mice vaccinated with the CTLA4/30K library and stimulated with IRBC (Fig. 2A), nor in culture supernatants of splenocytes from control mice vaccinated with VR1020, MCP-3, and CTLA4 vector DNA stimulated with IRBC (Fig. 2A) or RBC (data not shown).

IL4 was detected in 3 out of 5 culture supernatants from spleen cells primed with the VR1020/30K and MCP-3/30K genomic libraries and stimulated with IRBC (Fig. 2B). No IL4 was detected with CTLA4/30K primed IRBC-stimulated splenocytes, nor in supernatants of splenocytes from control mice vaccinated with VR1020, MCP-3, and CTLA4 vector DNA that were stimulated with IRBC or RBC (data not shown).
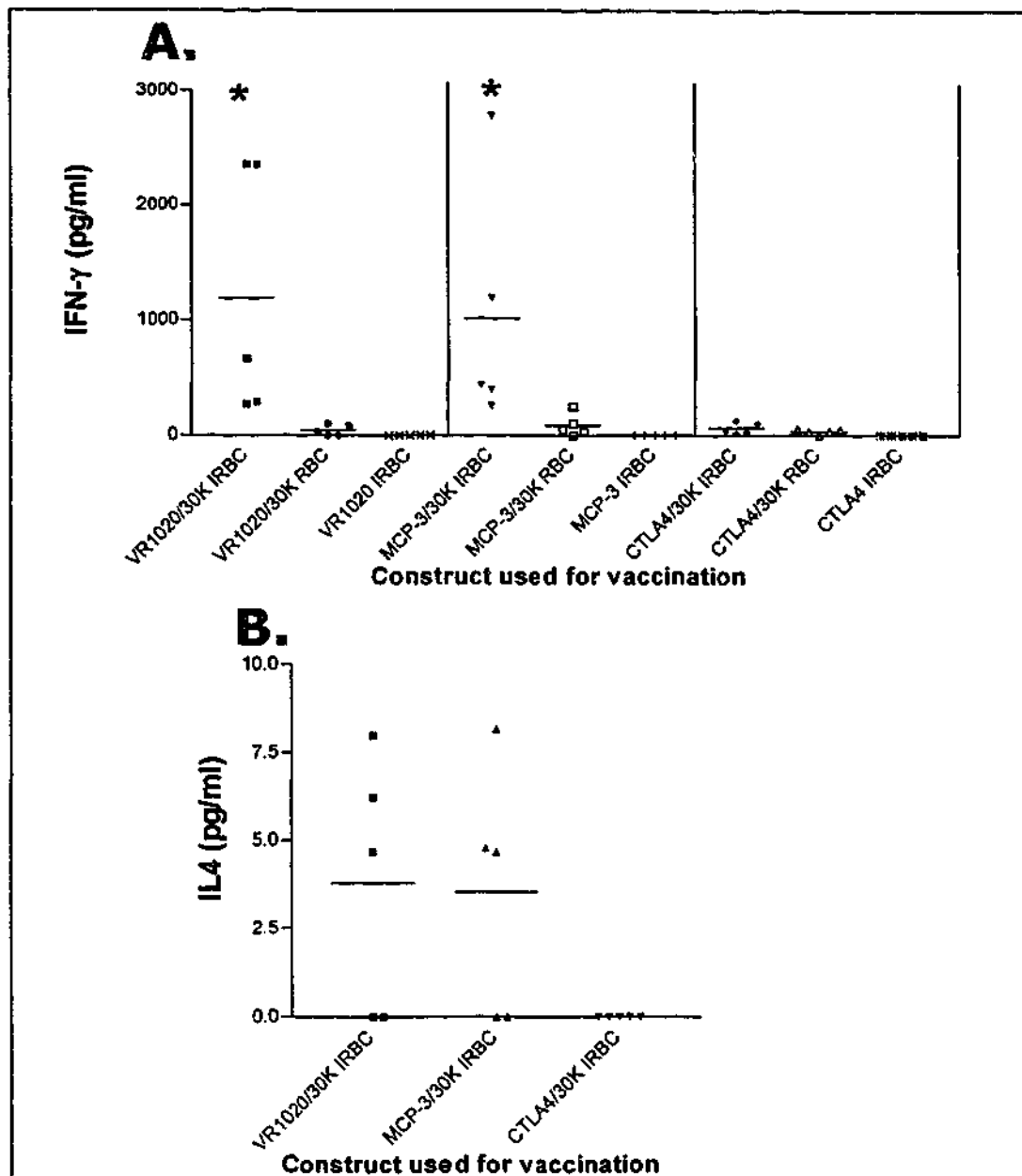
**Figure 2.** IFN-γ production (A) and IL-4 production (B) by splenocytes from BALB/c mice vaccinated with the VR1020/30K, MCP-3/30K, and CTLA4/30K libraries. Splenocytes from individual mice were cultured as for Fig.1. After 72 hours of stimulation with $2\times10^6$ IRBC or $2\times10^6$ RBC, supernatants were harvested and analysed for the presence of IFN-γ and IL4 by ELISA. Splenocytes from mice vaccinated with empty vector control DNA (VR1020, MCP-3, CTLA4) were also stimulated with $2\times10^6$ RBC, but IFN-γ and IL4 responses were not observed (data not shown). Unstimulated splenocytes did not produce detectable IFN-γ or IL-4. The mean IFN-γ or IL4 production is shown (bar). The raw data was $\log_{10}$ transformed and compared using a Student $t$-test; *, $P<0.05$.

*Phagocytosis by macrophages of P.chabaudi adami DS IRBC after incubation with sera from mice vaccinated using each of the genomic libraries*

Intraepidermal DNA vaccination using any of the genomic expression libraries did not produce detectable humoral responses when evaluating sera by ELISA using soluble blood stage *P.c.adami* DS lysate as antigen (data not shown). However, the method of gene-gun vaccination has been shown to produce a strong humoral response after delivery of plasmids containing known antigens in many different vaccine models (reviewed in 144). Antibody-mediated opsonisation of IRBC, and subsequent internalisation and destruction by macrophages, has been shown to be a contributing factor to a reduction in parasitemia during crisis in the *P.chabaudi* mouse model (142, 143).

To determine whether vaccination with the *P.c.adami* DS genomic expression libraries could induce opsonising antibodies, serum from mice vaccinated with each library was tested for the ability to promote opsonisation of *P.c.adami* DS IRBC by BALB/c macrophages. Figure 3 shows that sera obtained from individual mice vaccinated with each of the genomic libraries have the ability to enhance uptake and promote destruction of IRBC by macrophages. The total number of IRBC ingested by macrophages was significantly higher when IRBC were pre-incubated with sera from VR1020/30K ($P<0.05$), MCP-3/30K ($P<0.001$), or CTLA4/30K ($P<0.05$) mice when compared to incubations using sera from control mice vaccinated with vector DNA (Fig.3A). The percentage of macrophages containing IRBC was also significantly higher in incubations using macrophages treated with sera taken from VR1020/30K ($P<0.01$), MCP-3/30K ($P<0.001$), and CTLA4/30K ($P<0.05$) mice when compared

with incubations using sera from control mice vaccinated with vector DNA (Fig.3B). For comparison, Figure 3C shows a pool of the phagocytic activity of sera from six mice vaccinated with the malarial erythrocytic stage antigen MSP4/5 (fused to the MCP-3 vector). This was used as a positive control. It was shown in Chapter 2 that this construct could significantly enhance antibody responses to MSP4/5 (159).

Further experiments to confirm antibody mediated phagocytosis, and not another factor in the serum activating the macrophages, was performed by Dr Tatiana Scorza (McGill University, Quebec, Canada). A commercially-available monoclonal antibody (MAb93, Southern Biotech) was used to block CD16 (Fcγ II) and CD32 (Fcγ III) receptors found on macrophages and prevent Fc binding of IgG antibodies and macrophage activation. Blockage of the Fc receptor with the specific monoclonal antibody MAb93 significantly reduced the uptake of IRBC treated with sera from mice vaccinated with the genomic expression libraries (Fig.3D) (158).

Immunofluorescence studies were also performed using pooled sera from the 3 genomic libraries on IRBCs containing mature parasites (mainly trophozoites and schizonts as used for the opsonisation assays). Figure 4 shows both *infected* and *uninfected* RBC, with white arrows indicating IRBCs. It is clear that all three libraries induced mouse antibodies that could bind to semi-permeablised IRBCs. The sera obtained from animals vaccinated with empty vector control DNA was used as a negative control for the immunofluorescence studies, and did not react strongly to *P.c.adami* DS IRBC (indicated by white arrows).
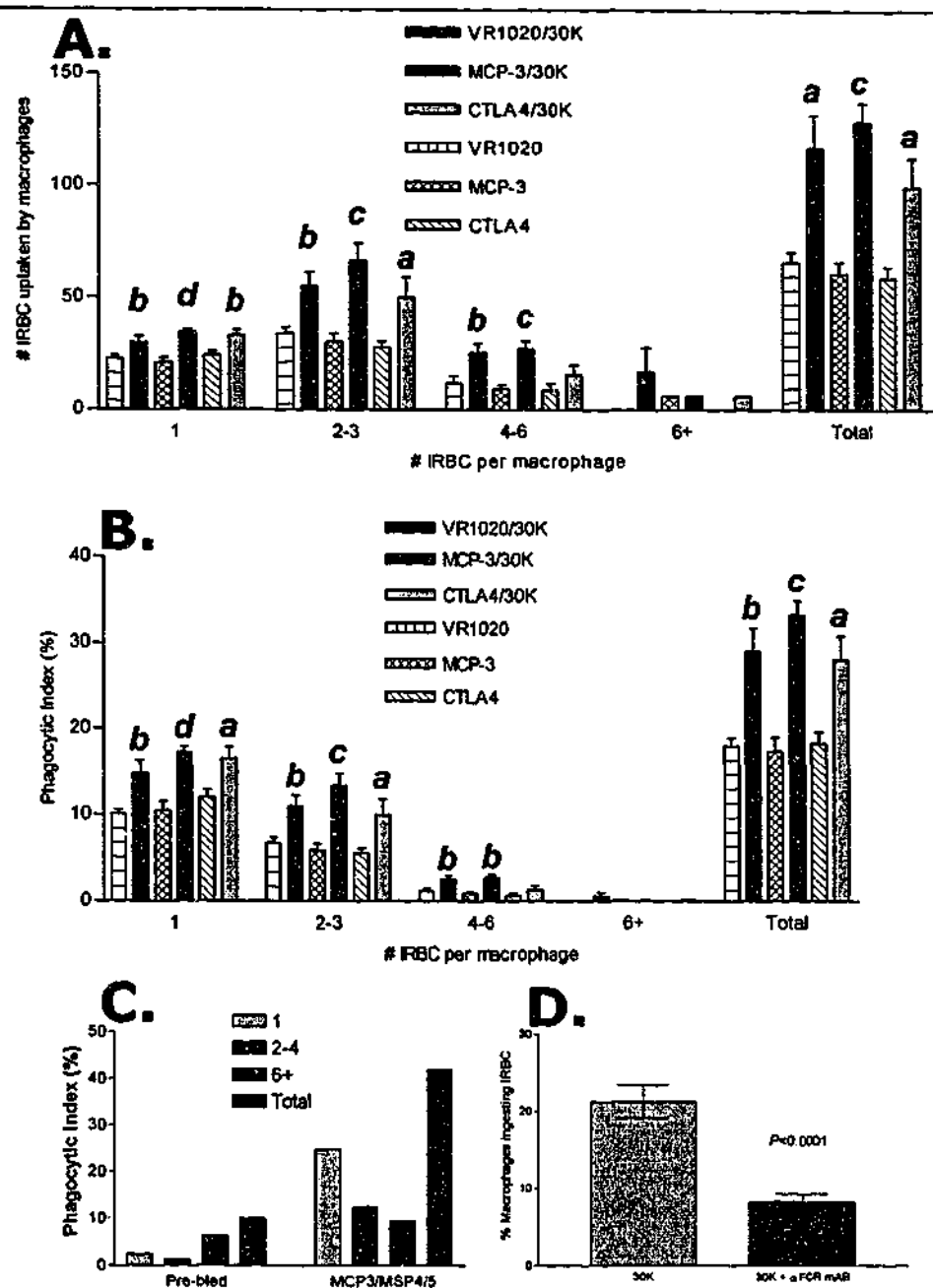
**Figure 3.** Phagocytosis of *P.c.adami* IRBC pre-incubated with sera from mice vaccinated with VR1020/30K, MCP-3/30K and CTLA4/30K (and control vector DNA). All six groups contained 10 BALB/c mice each. (A) The number of IRBC phagocytosed by macrophages after pre-incubation with sera, and the number of IRBC contained within an individual macrophage for each vaccine group is shown. (B) Phagocytic index expressed as a percentage of the number of macrophages containing IRBC out of a total of 200 counted. The number of IRBC contained within an individual macrophage for each vaccine group is also shown. Data are expressed as mean ±SEM. *a*, $P<0.05$; *b*, $P<0.01$; *c*, $P<0.001$; *d*, $P<0.0001$. (C) Phagocytic index of pre-bleed pooled sera (n=6), and pooled sera from mice vaccinated with MCP-3/MSP4/5 DNA (n=6) as a positive control. (D) Incubation of macrophages with a monoclonal antibody specific for Fcγ II and Fcγ III receptors can inhibit the percentage of macrophages ingesting IRBC. Five sera samples were each taken from mice vaccinated with VR1020/30K, MCP-3/30K, and CTLA4/30K libraries, giving a total of 15 individual samples. Macrophages were treated with a α-Fcγ monoclonal antibody, followed by incubation with IRBC previously exposed to genomic library sera (30K + α-Fcγ mAB), or untreated and incubated with IRBC exposed to genomic library sera as a positive control (30K). The data shown is expressed as mean ±SEM of macrophages ingesting IRBC. Treated (30K + α-Fcγ mAB) and untreated (30K) groups were compared using a Student *t*-test.

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VR1020/30K sera          MCP-3/30K sera          CTLA4/30K sera

VR1020 vector control sera    MCP-3 vector control sera    CTLA4 vector control sera
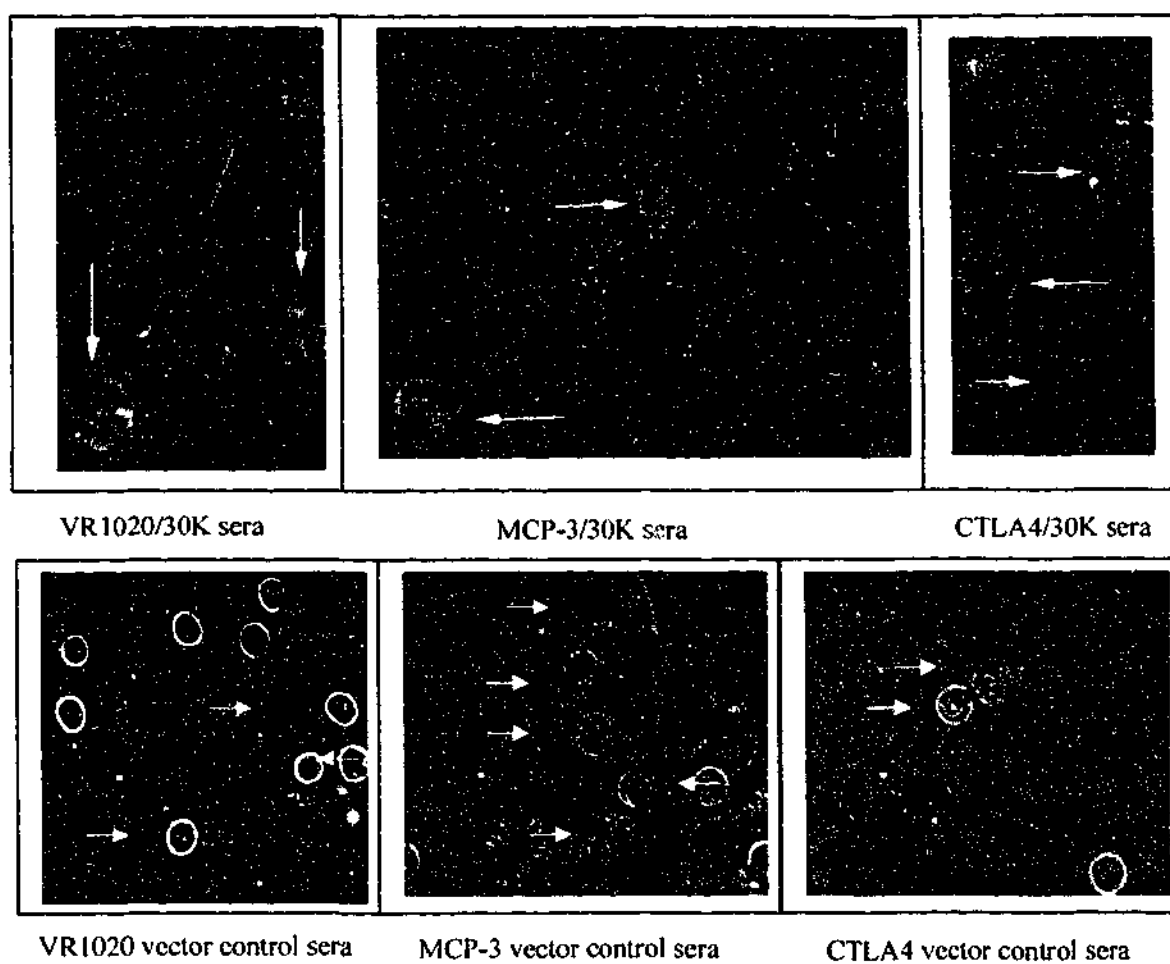
**Figure 4.** Immunofluorescence using VR1020/30K, MCP-3/30K, and CTLA4/30K sera on *P.c.adami* DS semi-permeablised IRBC. White arrows indicate IRBC.

*Protective efficacy induced by DNA vaccination with genomic expression libraries against lethal erythrocytic stage challenge using P.chabaudi adami DS*

The *P.c.adami* DS mouse model is a stringent test for vaccine efficacy due to the high virulence of the DS strain (8, 41). In order to determine the protective efficacy of the VR1020/30K, MCP-3/30K and CTLA4/30K genomic libraries against a virulent challenge three separate mouse trials were conducted (Table 2). It has been previously shown that the VR1020/30K library induces significant (albeit partial) protection against death using the *P.c.adami* DS-BALB/c mouse model (181).

The first challenge trial contained ten mice per group. Mice were vaccinated ID using a gene-gun a total of three times at two week intervals, and challenged two weeks after the final vaccination with $1 \times 10^6$ *P.c.adami* DS IRBC. Trial 1 involved a comparison of the CTLA4/30K and VR1020/30K libraries. The VR1020/30K library was used as a positive control based on our previous experiments where this vaccine has induced 30-60% protection against death (181). There was a significant reduction in peak parasitemia in mice vaccinated with both the VR1020/30K and CTLA4/30K libraries compared to the empty CTLA4 control vector (Table 2). Although there were no significant differences between survival curves of control and vaccinated mice (i.e., a delay in death), increased survival was obtained in the group vaccinated with VR1020/30K library (Fig.5A), which was also repeated in Trial 3 (Fig.5Ciii). In contrast, the CTLA4/30K vaccine did not induce increased survival. Although a significant reduction in peak parasitemia of CTLA4/30K animals was found in Trial 1, this was not reproduced in Trial 3, nor was survival.

The second trial involved the evaluation of the MCP-3/30K genomic library. Mice were vaccinated three times at two week intervals using a gene-gun, and challenged two weeks later with $1 \times 10^6$ *P.c.adami* DS IRBC. Although there was a drop in peak parasitemia between the MCP-3/30K and MCP-3 vector control groups, the differences were not statistically significant due to the large variation in parasitemia between individual mice. A significant delay in death was observed in mice vaccinated with the MCP-3/30K library when compared to the MCP-3 control vector ($P=0.05$; Fig.5B). Half of the mice vaccinated with the MCP-3/30K library survived until day 12 post infection, with two mice surviving to the conclusion of the trial at day 14. No MCP-3 vector control mice survived, as all mice died by day 10 post infection.

The third challenge trial directly compared the efficacy of the VR1020/30K, MCP-3/30K and CTLA4/30K genomic libraries. Although there was a reduction in the mean peak parasitemia for all mice vaccinated with the genomic libraries compared with their respective controls (mice vaccinated with vector only DNA), the reduction was not statistically significant between groups (Table 2). However, there was a significant reduction in peak parasitemia in surviving mice (in the MCP-3/30K and VR1020/30K groups) compared with non-surviving mice (Table 2). There was survival of 50% of mice vaccinated with the VR1020/30K and MCP-3/30K libraries, and a significant delay in death in the VR1020/30K group versus the VR1020 vector control group (Fig.5Ci; $P=0.001$). In contrast, the CTLA4/30K library did not protect mice against lethal *P.c.adami* DS challenge (Fig.5Ciii).
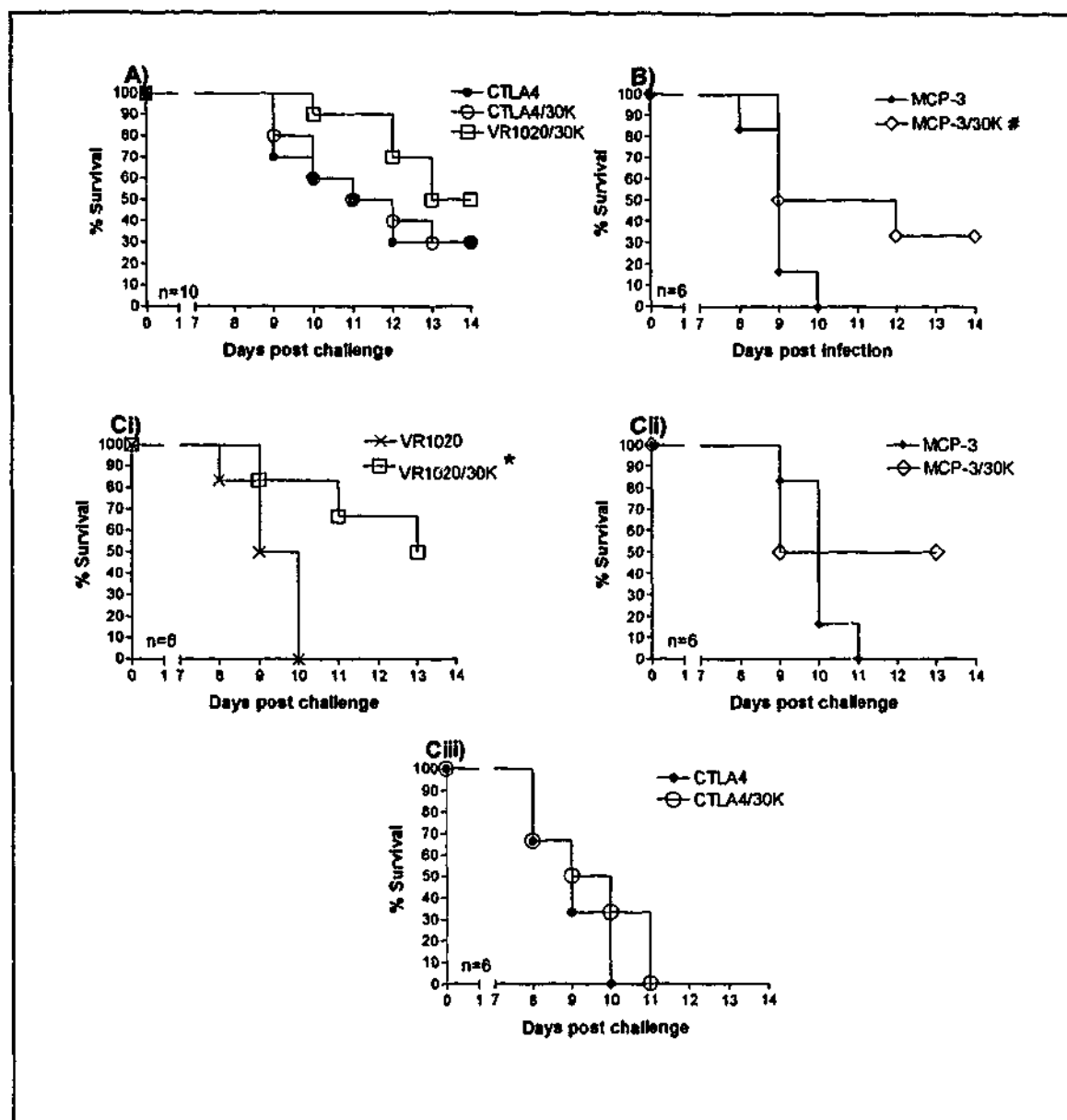
**Figure 5.** Survival curves of mice vaccinated with VR1020/30K, MCP-3/30K and CTLA4/30K genomic libraries, and challenged with virulent *P.c.adami* DS. Mice were vaccinated 3 times at two week intervals via the gene-gun, followed by challenge with 100,000 *P.c.adami* DS IRBC two weeks later. (A) Evaluation of efficacy of the CTLA4/30K and VR1020/30K libraries. (B) Evaluation of the efficacy of the MCP-3/30K library. (C) Comparative evaluation of all three genomic libraries. #, $P=0.05$; *, $P<0.05$ compared to empty control vectors (Mantel-Haenszel test).

**Table 2.** Protocol, parasitemia measurements, and % survival of mice challenged with lethal *P.c.adami* DS over 3 challenge trials.

| Group | No. in group | Peak parasitemia | Survivors (%) |
|---|---|---|---|
| **Trial 1** | | | |
| 1. CTLA4 | 10 | $42\pm6$ | 3 (30%) |
| 2. CTLA4/30K | 10 | $35\pm7^a$ | 3(30%) |
| 3. VR1020/30K | 10 | $35\pm4^a$ | 5 (50%) |
| *Summary* | | | |
| Non-survivors | 19 | $36\pm8$ | |
| Survivors | 11 | $37\pm5$ | |
| **Trial 2** | | | |
| 1. MCP-3 | 6 | $40\pm13$ | 0 (0%) |
| 2. MCP-3/30K | 6 | $31\pm15$ | 2 (33%)$^b$ |
| *Summary* | | | |
| Non-survivors | 10 | $39\pm12$ | |
| Survivors | 2 | $34\pm2$ | |
| **Trial 3** | | | |
| 1. VR1020 | 6 | $45\pm11$ | 0 (0%) |
| 2. VR1020/30K | 6 | $37\pm5$ | 3 (50%)$^c$ |
| 3. MCP-3 | 6 | $43\pm7$ | 0 (0%) |
| 4. MCP-3/30K | 6 | $29\pm18$ | 3 (50%) |
| 5. CTLA4 | 6 | $41\pm7$ | 0 (0%) |
| 6. CTLA4/30K | 6 | $38\pm6$ | 0 (0%) |
| *Summary* | | | |
| Non-survivors | 30 | $39\pm11$ | |
| Survivors | 6 | $25\pm14^a$ | |

Peak parasitemia is expressed as mean ± Standard deviation
a, $P<0.05$; student *t*-test
b, $P=0.05$; Mantel-Haenszel test compared with MCP-3 vector
c, $P<0.01$; Mantel-Haenszel test compared with VR1020
vector

## 3.4) Discussion

Protection of mice against lethal *P.c.adami* DS challenge using ELI has been previously demonstrated in our laboratory (181). The aim of the studies conducted in this Chapter of the thesis was to determine whether the efficacy of ELI could be enhanced using targeting vectors. It was also to assess the immune responses induced by ELI in mice, in order to confirm that ELI induces an antigen-specific response. Both humoral and cellular responses have been found to be required for resolution of infection with *P.chabaudi* malaria in mice ( reviewed in 117, 200). The data presented demonstrate that genomic ELI can induce cellular as well as humoral responses to native *P.c.adami* DS antigens following vaccination. The data also show that protection against lethal *P.c.adami* DS challenge is dependent on the type of DNA expression vector used, with protection correlating with *in vitro* cellular responses that were induced by vaccination.

Vaccination of BALB/c mice with the VR1020/30K or MCP-3/30K libraries induced a specific cellular immune response to native antigens shed by IRBC which was characterised by a significant increase in splenocyte proliferation, as well as detectable levels of IFN-γ and IL-4 secretion.

The ability of the VR1020/30K and MCP-3/30K genomic libraries to promote proliferation of splenocytes in response to live *P.c.adami* DS IRBC demonstrates that the genomic approach to vaccination is specific to epitopes/antigens produced by the parasite, and not an artefact produced by non-specific stimulation of the immune system by foreign DNA. The presence of cellular responses *in vitro* induced by *P.c.adami* DS ELI correlates with the protection observed *in vivo* after lethal erythrocytic stage challenge, as significant splenocyte proliferation after vaccination

with the non-protective CTLA4/30K library was not observed. The production of opsonising antibodies to promote phagocytosis *in vitro* (regardless of the vector used) revealed another possible mechanism by which parasite clearance after ELI may also occur.

The nature of the epitopes/antigens that are recognised by splenocytes from vaccinated mice is clearly of interest, as this may identify sequences in the library encoding protective antigens. Sequencing of a sample of 664 plasmids derived from the VR1020/30K pool, contained open reading fames predicted to encode peptides of various sizes (with many homologous to *P. falciparum* sequences) are indeed present in the library (unpublished data). Recently it has been shown that peptides of 20 amino acids synthesized to span the length of $MSP1_{33}$ could induce T-cell proliferation in mice. Multiple epitopes were recognised in two strains of mice, with two epitopes discovered that were able to induce effector T cells capable of delaying growth of lethal *P.yoelii* YM following adoptive transfer into immunodeficient mice, without inducing detectable antibody responses (205). In addition, peptides were able to protect mice against *P.yoelii*, suggesting the T-cell epitopes may be useful as a vaccine against *P.yoelii* (205). Given that the genomic library DNA plasmids will encode a variety of in-frame peptides, a major contributory factor to protection observed with ELI is possibly due to T-cell help delivered by the library.

Although there was a response to IRBC stimulation in the VR1020/30K and MCP-3/30K vaccinated mice, significant levels of splenocyte proliferation did not occur when mice were vaccinated using the CTLA4/30K library. This result is curious as all three libraries were constructed by the same method, using the same stock of *P.c.adami* DS genomic DNA. One possibility is that the nature of the CTLA4 vector itself is possibly contributing to the lack of proliferation. The CTLA4 vector

expresses proteins as a fusion with the CTLA4/human Ig moiety (approximately 60 kDa) and the fusion partner is thus considerably larger than the fused *P.c.adami* DS sequences, based on a peptide encoding less than 20 amino acids. It is possible that many of the epitopes contained within the CTLA4/30K pool may have been obscured upon folding of the large CTLA4 moiety, and poorly processed or presented to the immune system. CTLA4 *in vivo* is a T-cell receptor involved in cell signalling, and is known to be a negative regulator of T-cell activation (reviewed in 168); however very high doses (50 µg) are required to completely suppress the immune response in mice (123). DNA vaccines producing CTLA4 fusion proteins have previously been reported to enhance antibody titres in mice and sheep (22, 45, 56, 121). However, not all cases of CTLA4 fusion vaccines have resulted in enhanced cellular responses (45), as was found in the present experiment. Overall, although the reason underlying the poor responsiveness of the CTLA4 library is not clear, the result shows that this vector is not useful for delivering malaria ELI vaccines in mice.

Gene-gun vaccination of the genomic libraries did not produce a detectable humoral response upon ELISA analysis of sera, presumably due to the diverse repertoire of *P.c.adami* DS sequences delivered in the library. Therefore another method, opsonisation, was used to establish the presence of antibody after vaccination, and to further explore protective mechanisms mediated by genomic vaccination. Vaccination using all the genomic libraries constructed produced sera that significantly enhanced uptake of IRBC by macrophages *in vitro*, when compared with IRBC incubated with sera from empty vector control animals. In *P.chabaudi* infections, it has been shown that the antibody-mediated immune response is directed primarily to epitopes on the surface of IRBCs, with these antibodies enhancing phagocytosis and subsequent destruction of IRBC *in vitro* (142, 143). In infected

mice, IgG2a and IgG3 antibodies are dominant during ascending primary parasitemia, and help to promote phagocytosis and opsonisation of IRBC (reviewed in 204). Opsonising activity found in sera from immune individuals has also been associated with protection in *P. falciparum* (75). The results show that ELI is able to induce opsonising antibodies that may contribute to the efficacy of the VR1020/30K and MCP-3/30K vaccines.

The use of the three vectors was to compare whether chemotactic attraction or lymph-node targeting would enhance the protection over that observed with the secretory library. This study attempted to enhance immunogenicity of the vaccine and increase the level of protection observed, which would have made subsequent devolution of the libraries an easier task. Comparison of the efficacy of the VR1020/30K (secretory), MCP-3/30K (chemoattractant) and CTLA4/30K (lymph-node targeting) libraries emphasized the need for cellular as well as humoral responses to protect against lethal *P.c.adami* DS challenge. The protective efficacy of the VR1020/30K library against *P.c.adami* DS has been established by our laboratory (181). The MCP-3 and CTLA4 vectors were used in an attempt to try to enhance the efficacy of genomic vaccination above the levels obtained using the secretory VR1020 expression vector; however this was not observed in our studies. It was shown in Chapter 2 that ID DNA vaccination using merozoite surface protein 4/5 (MSP4/5) can significantly enhance survival after *P.c.adami* DS erythrocytic stage challenge when fused to the MCP-3 sequences, but did not significantly reduce the peak parasitemia compared to control animals (159). This construct also produced significant increases in antibody when compared to a VR1020/MSP4/5 construct. However, vaccination with the MCP-3/30K library did not significantly enhance any of the parameters measured (protection, splenocyte proliferation, opsonisation, IFN-γ

and IL-4 production) compared to the VR1020/30K library. The use of MCP-3 as a fusion partner was not detrimental to the library's protective efficacy, but this was not the case when mice were vaccinated with the CTLA4/30K library. Clearly, the choice of fusion partner is important when utilising targeting vectors in DNA vaccine studies. The results of the present study demonstrate however that standard secretory vectors are sufficient to promote protective immune responses using a malarial genomic library, and that the other vector types appear to be more suited to the delivery of candidate antigens, as used in other studies (15, 22, 159).

Priming of mice with the CTLA4/30K library did not promote significant splenocyte proliferation specific to *P.c.adami* DS IRBC, which may have contributed to the lack of protection observed in the present study. Sera obtained after vaccination with CTLA4/30K did however promote phagocytosis of IRBC *in vitro*. This suggests the possibility that CTLA4/30K vaccination primed the immune system with enough T- and B-cell epitopes to produce antibody and promote phagocytosis, but this response was not sufficient to protect mice from death.

The combinations of both specific cellular immune responses, and opsonisation of IRBC by macrophages, are two possible mechanisms which may be responsible for the protection and reduction of parasitemia observed with the VR1020/30K and MCP-3/30K libraries. The detection of IFN-$\gamma$ and IL-4 in splenocyte cultures of mice vaccinated with these two libraries suggests priming of Th1 and Th2 cell subsets within the spleen. Using *P.chabaudi* it has been shown that splenic CD4$^+$ T cells purified from immunologically intact mice during ascending parasitemia produce high levels of IFN-$\gamma$ and IL-2 to limit infection, while IL-4 and IL-10 were produced by splenic CD4$^+$ T cells during descending primary parasitemia (186). B-cell deficient mice however were not able to mount a sufficient Th2 response to completely resolve

the primary parasitemia (186). Acute *P.c.adami* infections in B-cell deficient mice are suppressed at the same rate as normal mice, but complete depletion of $CD4^+$ T cells results in the inability to control parasite growth, emphasizing the importance of $CD4^+$ T-cell activation during erythrocytic stage infection (reviewed in 204).

With these results, a mechanism by which ELI might induce a protective response has been established. The results suggest the possibility that the genomic libraries encode previously undiscovered combinations of malarial epitopes or whole antigens which are protective. Chapter 4 describes a method to potentially exploit the synergistic properties of the VR1020/30K library in combination with known antigens, by co-delivery in bicistronic vectors. This has resulted in a significant reduction in peak parasitemia in challenged mice co-vaccinated with the 30K vaccine and known antigens, which is greater than the reduction observed using the VR1020/30K library alone. Thus, the 30K vaccine may allow us to identify new "unknown" antigens/epitopes that synergise with known antigens. The ELI vaccine may thus represent a method to mimic the multivalent nature of the acquired immune response seen in resistant malaria-exposed humans. Mimicking this response has been proposed to be the best approach to produce an effective malaria vaccine (54). The Spithill laboratory is now in the process of dividing a sub pool of the original 30,000 plasmids contained within the VR1020/30K library into groups based on predicted function (epitope type/homology with known malaria sequences) and size, and these pools are being evaluated in vaccination experiments in an attempt to further define the protective epitopes in the vaccine.

## 3.5)  Direct Expression Library Immunisation (DELI)

### 3.5.1)  Introduction

If ELI is to be used as a tool solely for antigen discovery (disregarding the synergistic effects after vaccination of a genomic library as a whole), the plasmids used to construct the libraries described in Chapter 3 are not optimised for this purpose. A major flaw of ELI described in Chapter 3 was that only a small fraction of the library could potentially express malarial peptides/antigens.

The construction method for libraries in Chapter 3 allows for expression of a malarial protein only if the genomic sequence is in the correct reading frame and in the correct orientation, a 1 in 6 chance of potentially expressing a malarial protein. The complete *P.c.adami* genome sequence is not yet published, however the *P.yoelii* genome shotgun project sequence has been published with 50.6% of the genome predicted to encode proteins (compared with 52.6% of the *P. falciparum* genome) (28). Therefore approximately 50 % of clones will actually begin within a coding region, further reducing the chances of a genuine malarial protein been expressed by the genomic libraries. The calculated fraction of plasmids that express genuine malarial proteins/epitopes is approximately 8% (50 % of one-sixth). Most of the DNA present in the library is therefore unproductive. Our laboratory has sequenced 664 plasmids derived from the VR1020/30K *P.c.adami* DS genomic library (Fig.1). Of the plasmids sequenced, 44% encoded peptides of less than 6 amino acids, with sequences greater than 9 amino acids required to encode epitope motifs for the BALB/c (H2-K$^d$) haplotype used ELI experiments (181). The dilution of potentially protective plasmids amongst unproductive plasmids may also reduce the ability to eventually discover single protective plasmids.
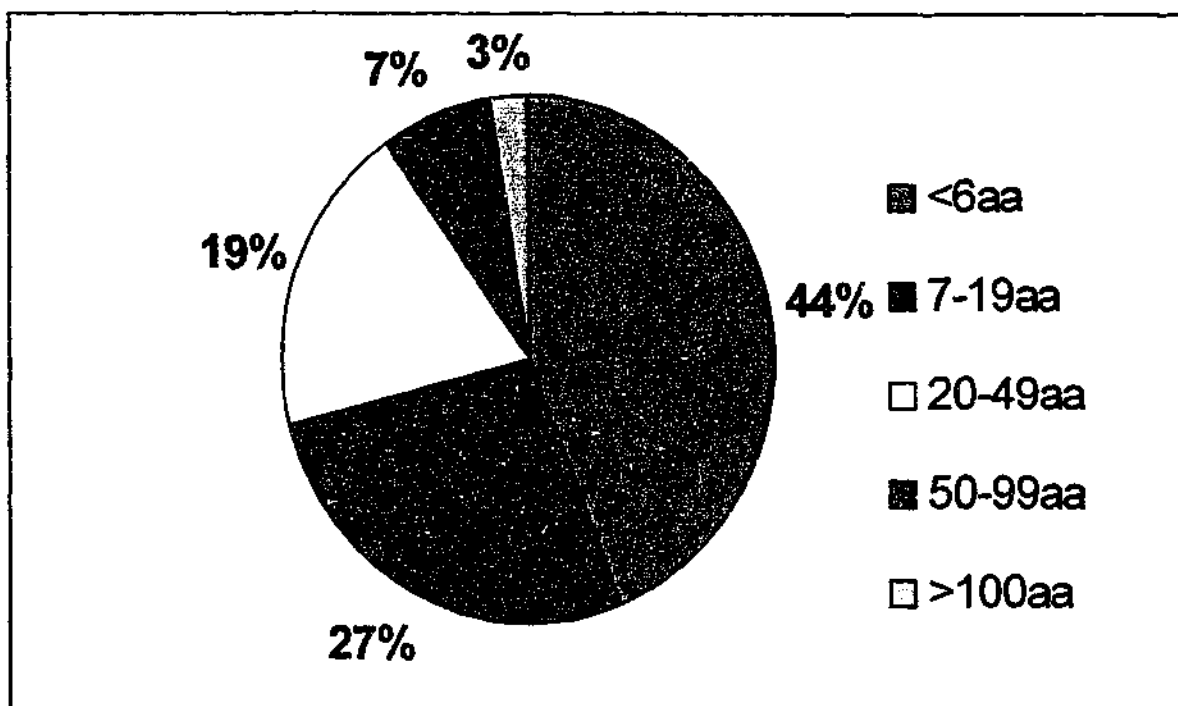
**Figure 1.** Sequencing results of peptide sizes obtained from 664 plasmids derived from the VR1020/30K library (181). Almost half of the peptides sequenced corresponded to sequences encoding less than 6 amino acids.

Improved expression library vectors have been successfully implemented for vaccination against *Mycoplasma hyopneumoniae* in pigs (140). This technique, termed Direct Expression Library Immunisation (DELI) was applied to a relatively small genome (0.9 Mb) (140). It was demonstrated that when using these improved vectors, a library of 20,000 clones was reduced to just 96 clones in a single screening using a poly Histidine (polyHis) fusion detection method. It was found that of the 20,000 clones, 1500 expressed a polyHis fusion protein.

From this positive pool, 96 clones provided enhanced protection after *M. hyopneumoniae* challenge in pigs. Clones not expressing a polyHis fusion protein are not detected (unfused polyHis proteins are degraded in the cytosol), and only polyHis fusion proteins greater than 5-10 kDa produced a positive signal. Screening of libraries for bacterial colonies expressing whole peptides before vaccination, allowed for sorting and subdivision of positive colonies based on function and type after peptide sequences were compared against databases. The following section describes preliminary attempts at applying this technique to the larger *P.c.adami* genome predicted to be approximately 25-30 Mb

(www.ncbi.nlm.nih.gov/projects/Malaria/Rodent/chabaudi.html).

### 3.5.2) Materials and Methods

The following materials and methods for preliminary experiments using *P.c.adami* DS genomic DNA were modified from (140).

*Library construction*

The pCI30 vector was kindly provided by Dr Robert Moore (CSIRO livestock industries, Geelong, Australia) (Fig.2).

The 1-3 Kb *P.c.adami* DS genomic DNA was excised from the VR1020/30K library (181) using *Bam*HI and *Bgl*II restriction enzymes, and purified from an agarose gel. The pCI30 vector was digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Promega, Australia). The purified 1-3 Kb *P.c.adami* DS genomic DNA was then ligated into the *Bam*HI site (with the *Bgl*II restriction site nucleotide overhang destroying a *Bam*HI restriction site) of the pCI30 vector, and transfected into BL21 (DE3) (Novagen, Milwaukee, Wis, USA) *E.coli*.
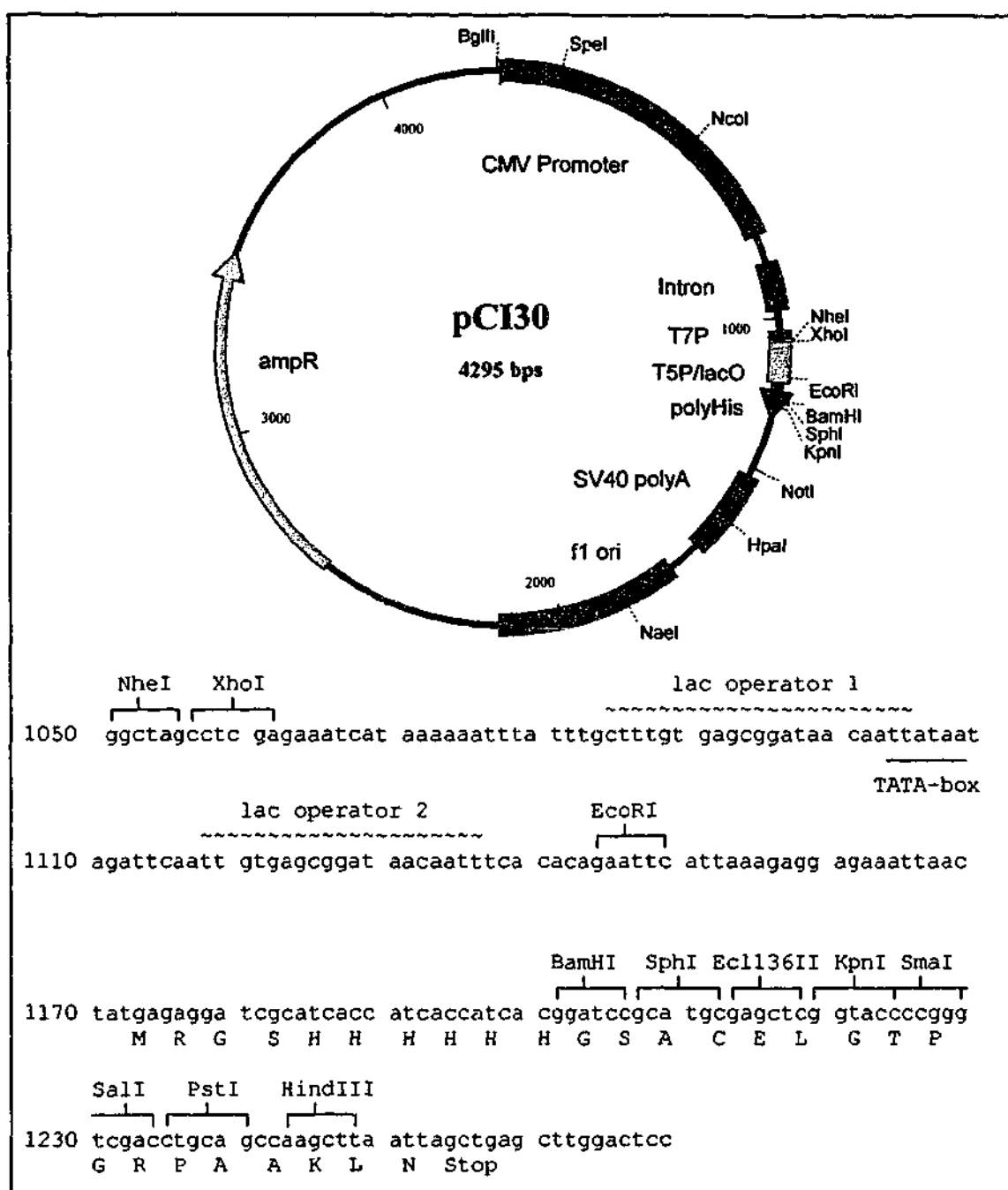
**Figure 2.** Map of pCI30 showing structural features for eukaryotic and prokaryotic expression. The DNA sequence around the multiple cloning site and T5 promoter region with the two lac operator sequences is shown (140).

*Colony screening*

The *P.c.adami* DS genomic library was transfected into BL21 (DE3) cells and grown on Luria Broth (LB) agar plates (1% NaCl, 1% Tryptone, 0.5% Yeast Extract) containing 50 µg/ml ampicillin overnight at 37° C. Thirty-six colonies were then individually picked and again grown on LB agar plates containing 50 µg/ml ampicillin overnight at 37° C. A nitrocellulose membrane was soaked in 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Progen, Australia), allowed to dry, then placed over the colonies and incubated for 4 hours at 37°C. The membrane was then lifted from the LB agar plate (which was then stored at 4°C), and exposed to a saturated chloroform atmosphere for 10 minutes. The membrane was then placed in lysozyme buffer (50 mM Tris-HCL, pH 8; 150 mM NaCl; 5 mM $MgCl_2$; 3% Fetal Calf Serum; Lysozyme, 500 µg/ml; DNase, 1 U/ml) and incubated at room temperature for 1 hour with gentle agitation. After 1 hour, remaining bacterial debris was removed from the nitrocellulose membrane before being placed in fresh lysozyme buffer for a further 2 hours. The membrane was then washed with PBS (1.7 M NaCl; 0.034 M KCl; 0.1 M $Na_2HPO_4$; 0.018 M $KH_2PO_4$) and 0.05% TWEEN 20 (Sigma, St Louis, MO, USA)(PBS-T), followed by incubation overnight at 4° C in 5% skim milk powder and PBS-T. The membrane was washed with PBS-T and then incubated with an anti-polyHis antibody (Sigma) diluted 1/2000 in 1% skim milk and PBS-T for 3 hours at room temperature with agitation. After this time, the membrane was washed with PBS-T and incubated for 1 hour with anti-mouse Ig conjugated to horseradish peroxidase (HRP) (Silenus Laboratories, Melbourne, Australia). The reactive antibodies were then visualised on photographic film using Enhanced Chemiluminescence (ECL) (Amersham, Piscataway, NJ, USA). Positive polyHis-

fusion colonies that exposed the photographic film were then matched to the original LB agar plate and selected. Positive polyHis-fusion encoding plasmids were sent to an outside source for nucleotide sequence determination.

### 3.5.3) Results

A total of 36 randomly selected *E.coli* colonies were screened using the anti-polyHis antibody. After screening with anti-polyHis antibody, positive colonies were selected as described in the methods after developing exposed photographic film using ECL. Two colonies that produced the highest signal strength relative to the other 34 colonies (which did not produce any signal on the photographic film) were selected for sequence determination and analysis.

The plasmid obtained from positive Colony 1 contained a nucleotide sequence predicted to encode a 27 aa peptide (with a predicted molecular weight of 3326 Da) (Fig.3A). The nucleotide sequence was compared against both *P. falciparum* and *P.yoelii* nucleotide databases (www.ncbi.nlm.nih.gov/PMGifs/Genomes/plasmodium.html), however only the *P.yoelii* database produced a single match (Fig. 4). When the translated protein sequence was compared against *P. falciparum* and *P.yoelii* protein databases (www.ncbi.nlm.nih.gov/PMGifs/Genomes/plasmodium.html), no significant similarities were found.

```
(A) Colony 1
ggatccgggaattcgtttttttttttttttttttttgtaaaaacttatctccatattctttа
G  S  G  N  S  F  F  F  F  F  F  C  K  N  L  S  P  Y  S  L
ctcataaccttttccatcaaaaatgtataa
L  I  T  F  S  I  K  N  V  -
```

```
(B) Colony 2
ggatcttcaattgaaaattataaaatacttttaaataaaatttttcaaaaaacgatacaa
G  S  S  I  E  N  Y  K  I  L  L  N  K  I  F  Q  K  T  I  Q
aatattcaaagaaaacaagcatttacaattgaagaaatagaaaaaagacaaaaaaatgaa
N  I  Q  R  K  Q  A  F  T  I  E  E  I  E  K  R  Q  K  N  E
aaaaaaataaaaattaatgatcatttattaagtatatcagatgatataataaataataat
K  K  I  K  I  N  D  H  L  L  S  I  S  D  D  I  I  N  N  N
aacataattaacaatattgaaaatattttaaatccaaattcgattatttcatcgtcagat
N  I  I  N  N  I  E  N  I  L  N  P  N  S  I  I  S  S  S  D
tatgatacatccgatttgggatcagaaatcgatgacaatgaaggacaaaatgatgaagaa
Y  D  T  S  D  L  G  S  E  I  D  D  N  E  G  Q  N  D  E  E
aatgaagacgatgaaaataaaacaatttataatcctttaaatttaccattaggacatgat
N  E  D  D  E  N  K  T  I  Y  N  P  L  N  L  P  L  G  H  D
aataaacctataccttattggttatataaattacatggattatctaaagaatataaatgt
N  K  P  I  P  Y  W  L  Y  K  L  H  G  L  S  K  E  Y  K  C
gaaatatgtggaaattattcttattttggtcgggctgcttttgaaaaacatttttatgaa
E  I  C  G  N  Y  S  Y  F  G  R  A  A  F  E  K  H  F  Y  E
tggcgacactcttttggaatgaagtgtttaaatattccaaatacattacattttaaagag
W  R  H  S  F  G  M  K  C  L  N  I  P  N  T  L  H  F  K  E
attacaaaaatagaagnatgctttaaatctttatga
I  T  K  I  E  X  C  F  K  S  L  -
```

**Figure 3.** Nucleotide sequence and amino acid alignment for plasmids obtained from positive colonies found after using the DELI technique.

```
>gb|AABL01000563.1| Plasmodium yoelii yoelii 17XNL contig MALPY00565,
 whole genome shotgun sequence
                 Length = 13080

 Score = 212 bits (110), Expect = 1e-54
 Identities = 153/173 (88%), Gaps = 6/173 (3%)
 Strand = Plus / Plus

Query: 16     ttttttttttttttttttttgtaaaaacttatctccatattctttactcataaccttttcc 75
              |||||||||||||||||||||||||| |||| |||||||||||||||||||||| ||||||
Sbjct: 11042 ttttttttttttttttttttgtaaaaatttatttccatattctttactcataactttttcc
11101


Query: 76     atcaaaaatgtataaccatctatatcaaaatgtcttaatgctgatttatataattctaaa 135
              || |||||||||||||||||||||||||||||||| |||||||||||||| ||||||||||||
Sbjct: 11102 attaaaaatgtataaccatctatatcaaaataacttaatgctgattcatataattctaaa
11161


Query: 136   aatgtttc-tttagttaagtttcgtttttttt-agtgctcccttc-aaattatt 185
             ||  |||| ||| | ||| || | |||||||| | ||| ||||| ||||||||
Sbjct: 11162 aacatttccttt-gataa-ttgctttttttttaatgc-cccttttaaattatt 11211
```

**Figure 4.** BLAST alignment of the plasmid sequence obtained from Colony 1 when compared against the *P.yoelii* genomic database.

The plasmid obtained from positive Colony 2 contained a nucleotide sequence encoding a peptide 189 aa in length, and a predicted molecular weight of 22399 Da (Fig. 3B). The nucleotide sequence was compared against both *P. falciparum* and *P.yoelii* nucleotide databases, however only the *P.yoelii* database produced a single match (Fig. 5), and indicated that one of the sequences was in the reverse direction. A descriptive annotation of this nucleotide sequence is not yet available in the *P.yoelii* database. The nucleotide sequence obtained from positive Colony 2 was translated in-frame with the polyHis sequence in the DELI vector. The protein sequence was compared against *P. falciparum* and *P.yoelii* protein databases, 107 BLAST matches were found with the highest scores showing similarities to *P.yoelii* and *P. falciparum* nuclear splicing proteins (Fig. 6). This indicated that the nucleotide sequence ligated into the DELI vector was indeed in the positive direction, and part of a malarial polyHis fusion protein was expressed.

```
>gb|AABL01002635.1| Plasmodium yoelii yoelii 17XNL contig MALPY02643,
whole genome shotgun sequence
        Length = 5016

 Score =  546 bits (284), Expect = e-155
 Identities = 427/494 (86%), Gaps = 18/494 (3%)
 Strand = Plus / Minus


Query: 90    tgaagaaatagaaaaaagacaaaaaaatgaaaaaaaaaataaaa-at-taatgatcattta 147
             |||| ||||| ||  || | |||||||||  ||||||||||||| || |||||||||||||
Sbjct: 3765  tgaaaaaata-aatgaa-a-aaaaaaatg--aaaaaaataaaatatctaatgatcattta 3711


Query: 148   ttaagtatatcagatgatataataaataataat-aacataatt-aacaatattgaaaata 205
             ||||||||||| ||| || | ||||| ||| | ||||| || || ||| ||||||||||
Sbjct: 3710  ttaagtatatctgataatctggtaaatgatattcaacat-gttgaa-aatgttgaaaata 3653


Query: 206   ttttaaatccaaattcgattatttcatcgtc-agattatgatacatccgat-ttgggatc 263
             |||| ||||| |||||| || | || || || || |||||||||||| ||| || || ||
Sbjct: 3652  tttttaatccgaattcggttgtatcttcatcaag-ttatgatacatctgatctt-ggctc 3595


Query: 264   agaaatcgatgacaatgaaggacaaaatgatgaagaaatga-agacgatgaaaataaaa 322
             ||||||| ||||| || |||| |||| || |||||||||| ||| | | ||||||||||||
Sbjct: 3594  agaaatagatgaaaacgaagaacaagataatgaagaaactgata-aagatgaaaataaaa 3536


Query: 323   caatttataatcctttaaatttaccattaggacatgataataaacctataccttattggt 382
             ||||||||||||| |||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct: 3535  caatttataatccattaaatttaccattaggacatgataataaacctataccatattggt 3476


Query: 383   tatataaattacatggattatctaaagaatataaatgtgaaatatgtggaaattattctt 442
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||| |
Sbjct: 3475  tatataaattacatggattatccaaagaatataaatgtgaaatatgtgg?aattattcat 3416


Query: 443   attttggtcgggctgcttttgaaaaacatttttatgaatggcgacactcttttggaatga 502
             |||||||||| | |||||||||||||||||||||||||||| ||| |||| || ||||| ||||
Sbjct: 3415  attttggtcgatcagcttttgaaaaacatttttatgagtggagacattcatttggtatga 3356


Query: 503   agtgtttaaatattccaaatacattacattttaaagagattacaaaaatagaagnatgct 562
             | || ||||||||| || |||||||||||||||||||| |||||||||| || | |||||
Sbjct: 3355  aatgcttaaatatacccaatacattacattttaaagaaattacaaaaattgagg-atgct 3297


Query: 563   ttaaatctttatga 576
             ||||||||| |||||
Sbjct: 3296  ttaaatctgtatga 3283
```

**Figure 5.** BLAST alignment of the plasmid sequence obtained from Colony 2 when compared against the *P.yoelii* genomic database. No significant hits were recorded when compared to the *P. falciparum* database.

```
>gb|EAA19688.1| splicing factor 3a subunit 3 [Plasmodium yoelii yoelii]
          Length = 585

 Score =  329 bits (843), Expect = 4e-91
 Identities = 158/190 (83%), Positives = 172/190 (90%), Gaps = 8/190 (4%)

Query: 4    IENYKILLNKIFQKTIQNIQRKQAFTIEEIEKRQKNEK-------KIKI-NDHLLSISDD 55
            IENYKILLNKIFQKTIQN+QRKQAFTIEEIEKRQKNEK       K KI NDHLLSISD+
Sbjct: 335  IENYKILLNKIFQKTIQNMQRKQAFTIEEIEKRQKNEKINEKKNEKNKISNDHLLSISDN 394

Query: 56   IINNNNIINNIENILNPNSIISSSDYDTSDLGSEIDDNEGQNDEENEDDENKTIYNPLNL 115
            ++N+   + N+ENI NPNS++SSS YDTSDLGSEID+NE Q++EE + DENKTIYNPLNL
Sbjct: 395  LVNDIQHVENVENIFNPNSVVSSSSYDTSDLGSEIDENEEQDNEETDKDENKTIYNPLNL 454

Query: 116  PLGHDNKPIPYWLYKLHGLSKEYKCEICGNYSYFGRAAFEKHFYEWRHSFGMKCLNIPNT 175
            PLGHDNKPIPYWLYKLHGLSKEYKCEICGNYSYFGR+AFEKHFYEWRHSFGMKCLNIPNT
Sbjct: 455  PLGHDNKPIPYWLYKLHGLSKEYKCEICGNYSYFGRSAFEKHFYEWRHSFGMKCLNIPNT 514

Query: 176  LHFKEITKIE 185
            LHFKEITKIE
Sbjct: 515  LHFKEITKIE 524

>ref|NP_704786.1| splicesome-associated protein, putative
[Plasmodium falciparum 3D7]
 emb|CAD51929.1| splicesome-associated protein, putative
[Plasmodium falciparum 3D7]
          Length = 589
 Score =  254 bits (649), Expect = 1e-68
 Identities = 134/197 (68%), Positives = 154/197 (78%), Gaps = 19/197 (9%)
```

**Figure 6.** BLAST-P alignment of the translated nucleotide sequence obtained from the plasmid in Colony 2. The translated nucleotide sequence was compared with both *P.yoelii* and *P. falciparum* protein databases. The amino acid sequence when compared against the *P.yoelii* protein database is shown, details of the *P. falciparum* sequence are also shown.

### 3.5.4) Discussion

This study demonstrates that in-frame malarial nucleotide sequences contained within the VR1020 expression library (181) can now be enriched using the DELI technique. This technique has the potential to discover many more in-frame nucleotide sequences from the relatively large *P.c.adami* malarial genome (when compared to 0.9 Mb of *Mycoplasma hyopneumoniae*).

The use of an N-terminal polyHis tag contained within the DELI vector allowed for detection of proteins that were stable (i.e., not degraded) when fused to this tag. Moore *et al* (140) have found that proteins greater than 5-10 kDa are stable when fused to a polyHis tag, and that smaller sequences are degraded in the cytosol and therefore not detected using anti-polyHis antibodies. It is therefore not surprising that only two malarial proteins were detected out of 36 colonies, as approximately half of the peptides contained within the VR1020/30K library are less than 6 amino acids in length (Fig. 1). Even though the sequence obtained from plasmid in Colony 1 was only approximately 3 kDa, the sensitivity of this technique allowed for detection without the selection of any false positive colonies. The detection of the 189 amino acid peptide found in positive Colony 2 was the largest found in the VR1020/30K library, even after nucleotide sequencing of 664 plasmids (Prof. Terry Spithill, personal communication).

Although the nucleotide sequence encoding a 27 amino acid protein was found for Colony 1, there were no similarities with sequences of known function at the nucleotide or protein level. It is possible that this small random genomic sequence is contained within a non-coding region of the *P.c.adami* genome. This sequence did however have similarity with part of a whole genome shotgun sequence from the

*P.yoelii* sequencing project. Analysis of the *P.yoelii* genome has now begun, it is conceivable that this unknown sequence may be characterised, or form part of a much larger nucleotide sequence that will be characterised.

The large 189 amino acid protein encoded by the plasmid in positive Colony 2 shared high similarity with Splicing Factor 3a (subunit 3) from *P.yoelii*. Although the nucleotide sequence was aligned in a reverse direction after comparison using the *P.yoelii* nucleotide database, when the nucleotide sequence was translated in the direction cloned into the DELI vector (in-frame with the polyHis sequence that allowed experimental detection) a large continuous protein sequence was found. A BLAST-P search revealed high similarity to splicing factors from both *P.yoelii* and *P. falciparum*.

Using DELI, it is now possible to extract large *P.c.adami* DS genomic DNA inserts contained within the VR1020/30K library. Although protection found after VR1020/30K vaccination was attributed to epitopes contained within the library (158), DELI will allow the discovery of large open reading frames encoding possible protective sequences. The newly discovered sequences can then be sorted based on predicted function using genomic databases, and eventually be tested in vaccine trials. Further screening of the VR1020/30K library can be accomplished using DELI.

# Chapter 4

## Evaluation of bicistronic DNA vaccines against *P.chabaudi adami* DS malaria

### *Abstract*

The ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex life-cycle. In order to achieve this using DNA vaccine technology, new antigen delivery systems must first be assessed. This study utilised bicistronic vector constructs containing an internal ribosome entry site (IRES) and expressing two combinations of malaria antigens: malarial candidate antigen sequences Merozoite Surface Protein 4/5 (MSP4/5) (fused to a Monocyte Chemotactic Protein-3 chemoattractant sequence) with Apical Membrane Antigen-1 (AMA-1); and MSP4/5 with a *P.chabaudi adami* DS genomic DNA library (fused to a Tissue Plasminogen Activated secretion signal). Transfection of COS 7 cells with bicistronic plasmids resulted in production and secretion of both malarial candidate antigens AMA-1 and MSP4/5 *in vitro*. Vaccination of BALB/c mice resulted in antibody production via ID gene-gun and IM routes against AMA-1 and MSP4/5. Significant *in vitro* proliferation of splenocytes compared to empty vector controls was also detected after vaccination with bicistronic constructs to both AMA-1 and MSP4/5 malarial candidate antigens. Vaccination with the *P.c.adami* DS bicistronic genomic DNA library also resulted in significant *in vitro* proliferation of splenocytes, after incubation with infected erythrocytes. Survival of BALB/c mice vaccinated with bicistronic constructs after lethal *P.c.adami* DS erythrocytic stage challenge was variable, although significant reductions in peak parasitemia were observed in two challenge trials. This study demonstrates that using a murine model the delivery of malarial antigens via bicistronic vectors is feasible. Further experimentation with multi-valent delivery systems may be required for the optimisation and refinement of DNA vaccines for malaria.

## 4.1) Introduction

It is believed that the ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex life-cycle (54, 112). Early malaria DNA vaccine studies have demonstrated that vaccination of mice with two pre-erythroctic stage antigens can enhance protection over the use of either antigen alone, as well as overcome genetic restriction in different mouse strains (55). Combinations of malarial antigens delivered as malarial DNA vaccines in primates have also resulted in enhanced levels of cytotoxic T lymphocytes (CTLs) to pre-erythrocytic stage vaccines (197), and enhanced antibody responses to erythrocytic stage malarial vaccines (100). It is believed that 'First Generation' DNA vaccines (i.e., delivery of only plasmid/antigen DNA) are not optimal to protect against malaria, and that immune enhancement strategies for DNA vaccination alone are required for this method of vaccination to be practical (reviewed in 54).

The use of multi-valent DNA vaccine expression systems such as bicistronic vectors may enable more efficient delivery of antigen in malaria DNA vaccination, as well as enhance synergistic responses between malarial antigens. Testing of viral bicistronic and polycistronic vectors in cancer gene therapy has been widely used to obtain synergistic effects using combinations of anti-tumor genes (reviewed in 43). Examples of non-viral bicistronic vector use as DNA vaccines include hepatitis B (35) and C (34), as well as vaccination against B-cell lymphoma (179). Studies evaluating bicistronic DNA vaccines for use against malaria are yet to be published.

Bicistronic plasmids utilise an internal ribosome entry site (IRES) placed between two coding regions. This allows ribosomes to attach to mRNA and translate the downstream coding sequence, while the upstream sequence is translated by cap-

dependent mechanisms (43). IRES sequences have been found in viral and eukaryotic mRNA, all differing in primary sequence, nucleotide length, and secondary structure, although they do share a hairpin nucleotide structure promoting small ribosomal subunit binding (reviewed in 132). The nucleotide composition of genes flanking the IRES is also an important factor in the expression of the encoded genes contained within bicistronic vectors, both *in vivo* and *in vitro* (43, 84).

Chapter 4 aims to demonstrate that the delivery of bicistronic DNA vaccines using malarial gene sequences is feasible. This study also attempts to determine whether the combination of MCP-3/MSP4/5 and a non-targeted genomic library (as with VR1020/30K) will result in synergy between these two components in a bicistronic vector. The bicistronic delivery of two malarial erythrocytic stage candidate antigens, AMA-1 and MSP4/5, resulted in expression of both antigens *in vitro* and *in vivo*. Bicistronic vaccination produced antibody and splenic T-cell responses to both antigens after immunisation of mice. The parasitemia after lethal erythrocytic stage challenge was also reduced relative to control vectors after bicistronic immunisation of mice. Bicistronic delivery of malarial DNA vaccines may therefore enhance the ability of 'First Generation' DNA vaccines to prime an immune response after exposure to malaria.

## 4.2)    Materials and Methods

### *Creation of bicistronic plasmids*

*Bicistronic vector preparation*

A bicistronic pIRES vector backbone was obtained from Clontech (Clontech, Palo Alto, CA, USA) (Fig.1i).  The pIRES vector was first digested with *Hpa*I and *Bgl*II restriction enzymes to remove the neomycin resistance gene cassette contained within this vector. This resulted in a 1920 bp fragment with two multiple cloning sites and an IRES sequence.  The pIRES-CMV vector was kindly provided by Dr Stephen Hobbs (Institute of Cancer Reasearch, London) (86)(Fig.1ii).  This vector was also digested with *Hpa*I and *Bgl*II to produce a 2791 bp fragment containing an ampicillin resistance gene and a portion of the SV40 polyadenylation sequence. This fragment was ligated to the 1920 bp fragment to produce a 4711 bp bicistronic vector (BC construct) (Fig.1iii).
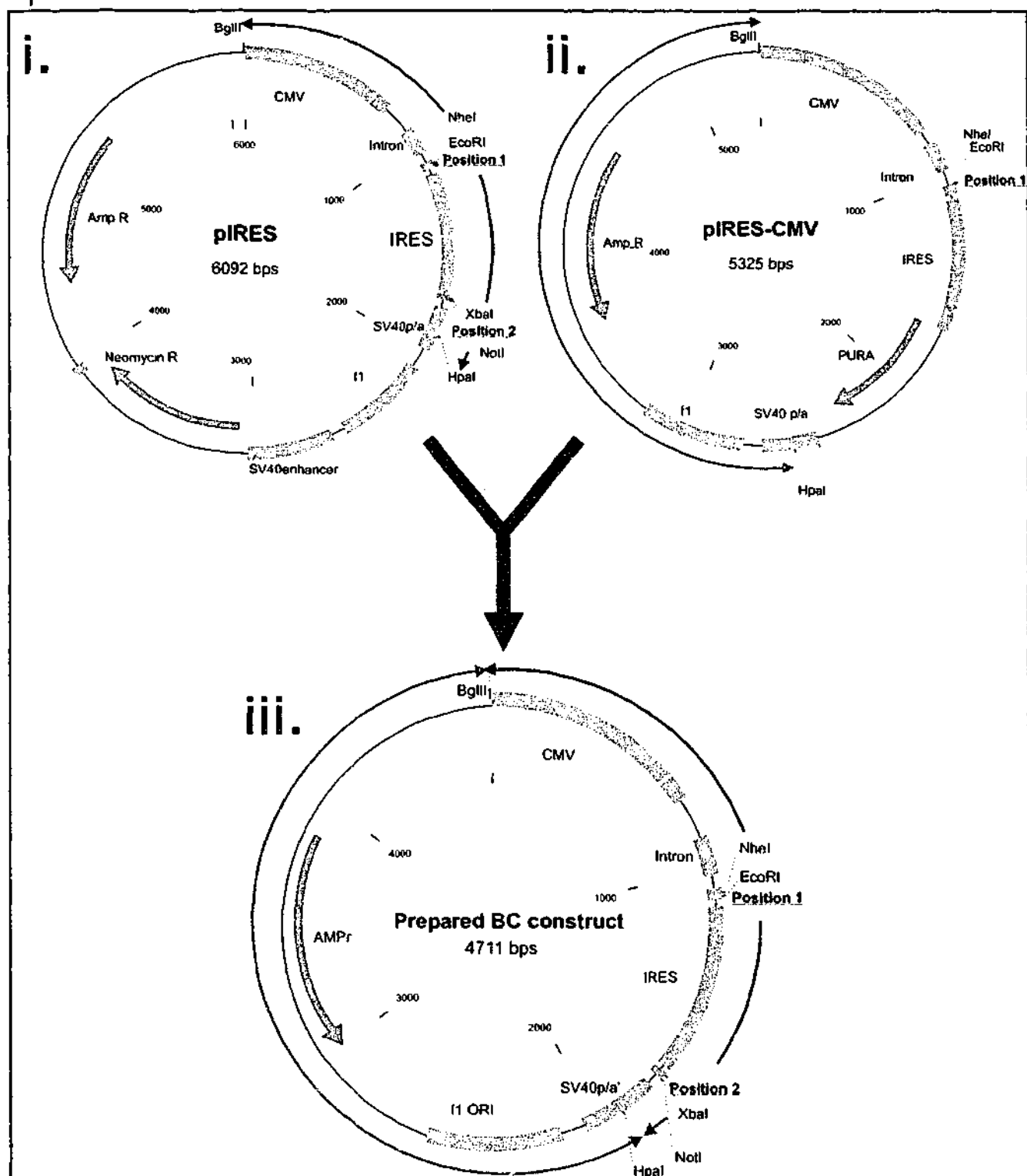
**Figure 1.** The pIRES vector (i) and the pIRES-CMV vector (ii) were combined to complete the bicistronic vector (BC construct) (iii), which was later used for inserting AMA-1, MSP4/5, and the *P.c.adami* DS genomic library. The portions of each vector used for the final Prepared BC construct are indicated by lines on the outer edges of the vector maps.

*Bicistronic vector 1 (BC construct 1) construction*

BC construct 1 contained the AMA-1 *P.c.adami* DS ectodomain sequence fused to a Tissue Plasminogen Activated (TPA) secretion signal in the first position of the vector, and *P.c.adami* DS MSP4/5 fused to the MCP-3 sequence in the second position of the vector (Fig.2i).

The 892 bp MCP-3/MSP4/5 sequence was amplified by PCR from the construct generated in (159), using the oligonucleotides 1 (containing an *Xba*I site) and 2 (containing a *Not*I site) (Table 1). The resulting PCR product was digested with *Xba*I and *Not*I enzymes and ligated into Position 2 of the BC construct.

The AMA-1 ectodomain was amplified from *P.c.adami* DS genomic DNA by PCR using oligonucleotides 3 and 4 (Table 1). The resultant AMA-1 fragment was then digested with *Bgl*II, and inserted into the *Bgl*II site of VR1020 (VICAL, San Diego, CA, USA) in-frame with TPA secretion signal. The TPA/AMA-1 sequence was then amplified by PCR from the VR1020/AMA-1 construct using oligonucleotides 5 and 6 (Table 1) with the resulting product digested with *Nhe*I and *Eco*RI. The BC construct, (containing the MCP-3/MSP4/5 sequence) was digested with *Nhe*I and *Eco*RI, and the TPA/AMA-1 sequence inserted to complete the vector which was designated BC construct 1 (Fig.2i).

*Bicistronic vector 2 (BC construct 2) construction*

BC construct 2 contained the AMA-1 ectodomain sequence fused to the MCP-3 sequence in the first position of the vector, and MSP4/5 fused to the MCP-3 sequence in the second position of the vector. BC construct 1, lacking the TPA/AMA-1 sequence in the first multiple cloning position, but containing MCP-3/MSP4/5 in Position 2, was used as a backbone for BC construct 2 (Fig.2ii).

The AMA-1 ectodomain was amplified by PCR from the VR1020/AMA-1 construct (described in the construction of BC construct 1) using oligonucleotides 7 and 8 (Table 1), and the product digested with *Bam*HI. The PCR product was then inserted into the *Bam*HI site of the VR1012/MCP-3 vector (159) to produce and MCP-3/AMA-1 construct. The MCP-3/AMA-1 (1695bp) sequence was amplified using oligonucleotides 9 and 6 (Table 1), and digested with *Eco*RI. The BC construct 1 backbone was digested with *Eco*RI, and the MCP-3/AMA-1 sequence inserted into the first multiple cloning site to produce BC construct 2 (Fig.2ii)
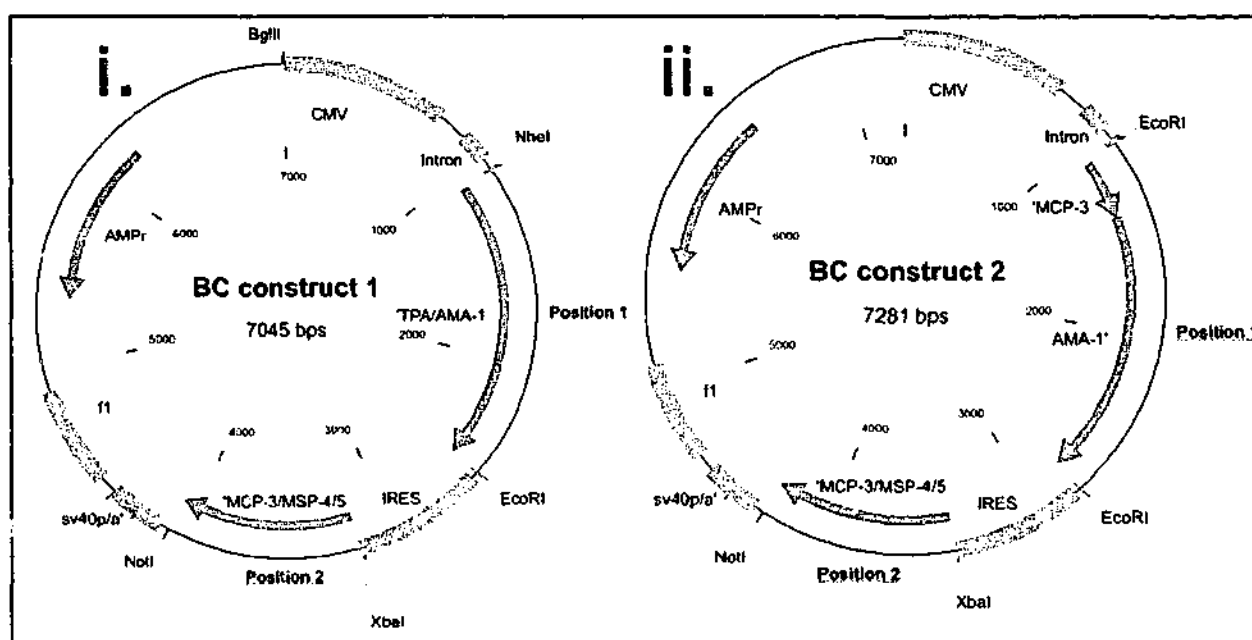
**Figure 2.** (i) BC construct 1 contained a TPA secretion signal fused to AMA-1 in Position 1, followed by an IRES sequence, and MCP-3/MSP4/5 in Position 2 of the vector. (ii) BC construct 2 contained a secretory MCP-3 chemokine sequence fused to AMA-1 in Position 1, an IRES sequence, followed by MCP-3/MSP4/5 in Position 2 of the vector.

*Bicistronic vector 30K (BC 30K) construction*

BC 30K contained *P.c.adami* DS genomic DNA preceded by a TPA secretion signal in the first position of the BC construct 1 backbone (containing MCP-3/MSP4/5 in Position 2). The *P.c.adami* DS genomic DNA was prepared as described in (181) by partially digesting with *Tsp*509I, and then isolating the fraction between 1000 and 3000 bp by gel elution.

The TPA secretion signal contained within the VR1020 vector was amplified by PCR using oligonucleotides 5 and 10 (Table 1). The amplified TPA secretion signal was then inserted into Position 1 of the BC construct 1 backbone after *Nhe*I and *Eco*RI digestion. The completed vector (containing the TPA secretion signal) was then digested with *Eco*RI, and the gel eluted *P.c.adami* DS genomic DNA was ligated into the *Eco*RI site in Position 1 (Fig.3). Ligation mixtures were transfected into DH5α *E. coli*, and grown on agar media containing ampicillin (50 µg/ml) overnight at 37°C before combining into pools of *E.coli* containing library plasmids. A total of ten pools, with each pool containing approximately 3000 individual clones were stored at -80° C in 15% glycerol. Library pools were combined and isolated as described in (181) to produce the BC 30K vaccination library.

Table I. Oligonucleotides used in the construction of bicistronic vectors. Restriction enzyme sites are shaded.

| | Oligonucleotide | Sequence (5'→3') | Description |
|---|---|---|---|
| 1 | MCP-3/XbaI F | GAAGTTCTAGAATGAGGATCTCTGCCACG | PCR amplification of MCP-3/MSP4/5 sequence from (159) |
| 2 | MSP4/5/NotI R | GAAGTGCGGCCGCTTATGAATCTGCACTGAG | |
| 3 | VR1020/AMA1 F | GGGAAGATCTTCCGAAGGTACAGATA | Used for cloning AMA-1 into the VR1020 vector |
| 4 | VR1020/AMA-1 R | GAAGTAGATCTTTACTGATTTA TGGACT | |
| 5 | TPA/NheI F | CGCGGAGCTAGCATGGATGCAATGAAGAGA | PCR amplification of the TPA and AMA-1 sequence from VR1020 |
| 6 | AMA-1/EcoRI R | GAAGTGAATTCTTACTGATTTATTGGACT | |
| 7 | AMA-1/BamHI F | ATGATGGGATCCGAAGGTACAGATAAT | PCR amplification of AMA-1 for insertion into VR1012/MCP-3 vector |
| 8 | AMA-1/BamHI R | GAACTGGATCCTTACTGATTTATTGGACT | |
| 9 | MCP-3/EcoRI F | GAAGTGAATTCATGAGGATCTCTGCCACG | PCR amplification of MCP-3/AMA-1 sequence |
| 10 | TPA/EcoRI R | CTCTCTGAATTCGGTACCGCTGGGCGAAAC | PCR amplification of the TPA secretion signal for BC 30K Position 1 |
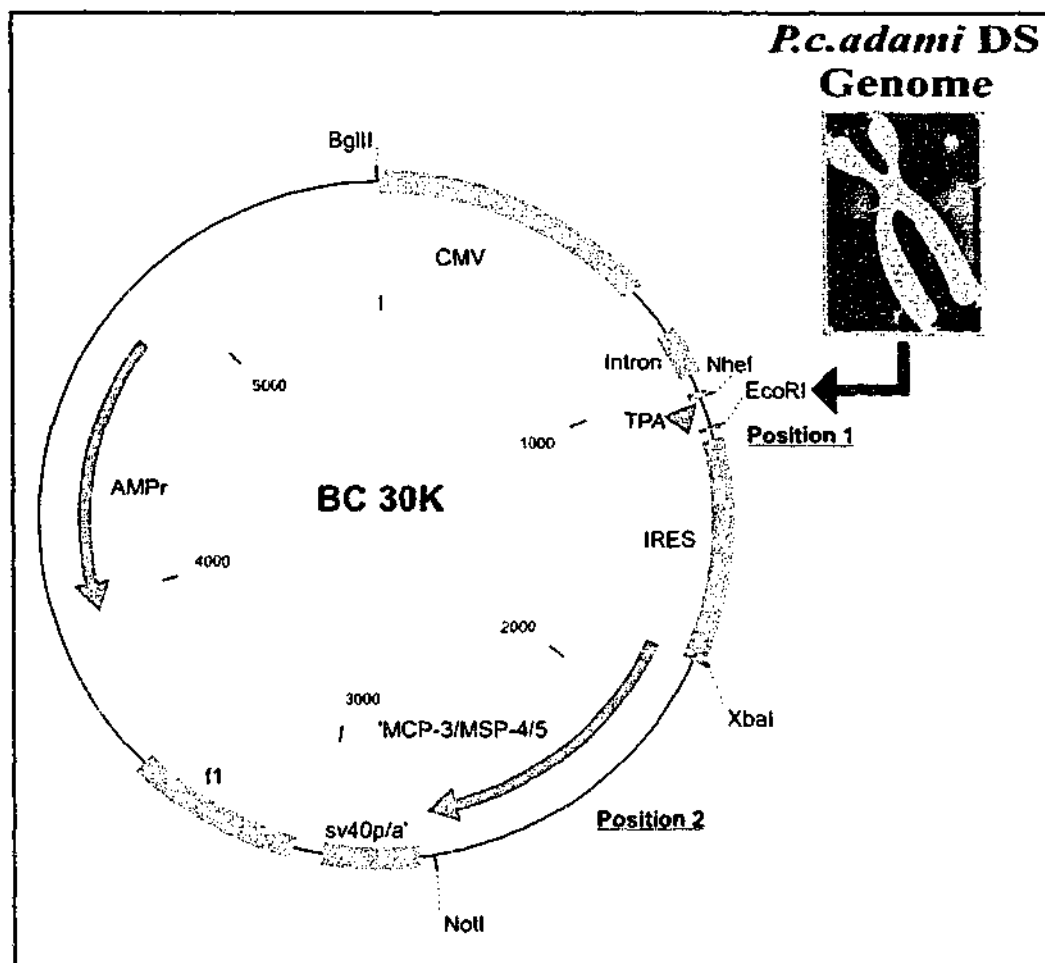
**Figure 3.** The BC 30K construct contained a TPA secretion signal in Position 1, with *P.c.adami* DS genomic DNA inserted into the *Eco*RI site to allow secretion. This was followed by the IRES sequence, and the MCP-3/MSP4/5 sequence in Position 2 of the vector.

## Expression and purification of recombinant proteins

### Purification of MSP4/5

Expression and purification of the recombinant MSP4/5 ectodomain protein was performed as described in (18). Briefly, the pTrcHis-A/MSP4/5 vector was transfected into *E. coli* BL21 (DE3) (Novagen, Milwaukee, Wis, USA) for expression of recombinant MSP4/5 protein. Large scale purification of the recombinant protein was performed using TALON metal affinity resin (Clontech) according to the manufacturer's instructions.

### Purification of AMA-1

The *E.coli* strain JPA101, containing the AMA-1 ectodomain sequence in the expression vector pDS56/RBSii, was kindly provided by Dr Robin Anders (La Trobe University, Australia) (8). A colony of *E.coli* containing the plasmid encoding the AMA-1 ectodomain was used to inoculate 50 ml of Superbroth (3.5% tryptone; 2% yeast extract; 0.5% NaCl) with 50 µg/ml of ampicillin. The 50 ml culture was grown overnight at 37°C, and used to inoculate 500 ml of superbroth (with 100 µg/ml ampicillin) and incubated for a further two hours. Induction of AMA-1 protein expression was performed by addition of 2 mM of isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (Progen, Australia), and incubation for 3 hours at 37°C. The culture pellet was collected by centrifugation for 10 minutes at 3000 g at 4°C.

The purification of the AMA-1 ectodomain was performed under denaturing conditions. The culture pellet was resuspended in 20 ml of Extraction buffer, pH 8 (50 mM $NaH_2PO_4.2H_2O$; 6 M Guanidine-HCL, pH 8; 300 mM NaCl) with 1 mM PMSF. The resuspended pellet was then applied to a French Press for 3 cycles at a pressure of

4 ton. The lysate was then centrifuged at 11000 g for 15 minutes at 4°C, and the pH corrected to pH 8 by addition of 5 M NaOH. The lysate was then applied to 2 ml of TALON metal affinity resin (Clontech) and incubated with rotation for 1 hour at 4°C. The resin was then washed 3 times using 50 ml of Extraction buffer pH 8, before being applied to a column. The final wash step involved the addition of 5 mM imidazole to the Extraction buffer before elution. The AMA-1 was then eluted from the resin using 1x Elution buffer pH 7 (45 mM $NaH_2PO_4.2H_2O$; 5 M guanidine-HCl; 270 mM NaCl; 150 mM imidazole).

To refold the AMA-1 ectodomain, the eluted protein was dialysed at 4°C. The dialysis buffer (20mM Tris-HCL, pH 8) was changed three times over 48 hours to remove any remaining Elution buffer. The dialysis tube containing the AMA-1 was then immersed in Refolding buffer (1 mM reduced glutathione; 0.2 mM oxidised glutathione; 20 mM Tris-HCl, pH 8) in a volume 25 times greater than the AMA- 1 solution in the dialysis tube. This mixture was then degassed by vacuum, sealed under nitrogen, and dialysed overnight at 4°C. The refolded AMA-1 protein was then stored at -80°C in 50% glycerol.

### *Mammalian cell transfection with bicistronic DNA plasmids*

Bicistronic plasmid constructs were tested for expression in COS 7 cells prior to use in mice. Freshly grown COS 7 cells were seeded at $2 \times 10^5$ cells per 35mm tissue culture well. Cells were grown in complete RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. COS 7 cells were then incubated in 5% $CO_2$ until 80% confluent. 3 µg of plasmid DNA was used to transfect COS 7 cells using LipofectAMINE (Invitrogen) in serum free RPMI 1640 according to the manufacturer's instructions.

Serum free RPMI 1640 was changed to complete RPMI 1640 24 hours after transfection. After incubation for a further 2 days at 37° C the cells were washed with PBS, and media was replaced with serum free RPMI 1640 (to remove any FCS that may have masked protein detection by subsequent western blot analysis), and grown for a further 24 hours. The supernatant was then collected and subjected to SDS-PAGE and Western Blotting.

## SDS-PAGE and Western Blotting

Protein and COS 7 supernatants were fractionated by SDS-PAGE on 12% (v/v) polyacrylamide gels under reducing conditions and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked in 5 % milk powder in PBS and 0.05 % TWEEN-20 (Sigma, St Louis, MO, USA) (PBS-T) overnight at 4° C. The membranes were probed using an anti-MSP4/5 rabbit antibody or anti-AMA-1 rabbit antibody, followed by an anti-rabbit Ig conjugated to horseradish peroxidase (HRP) (Silenus Laboratories, Melbourne, Australia). The reactive antibodies were then visualized by Enhanced Chemiluminescence (Amersham, Piscataway, NJ, USA).

## Enzyme-linked immunosorbent assay (ELISA)

Antibody reactivity after vaccination was tested with recombinant MSP4/5 or refolded AMA-1 protein and measured by ELISA. Nunc Maxisorp (Nunc, Kamstrupvej, Denmark) ELISA plates were coated with 0.1 ml/well of recombinant MSP4/5 or AMA-1 (1 μg/ml) overnight at 4°C using carbonate-bicarbonate buffer pH 9.6. Plates were washed with PBS-T, followed by blocking overnight at 4°C in 5% skim milk powder and PBS-T. Plates were again washed and diluted sera incubated

at 37° C for 2 hours. After washing plates again using PBS-T, total humoral responses were obtained using HRP-conjugated sheep anti-mouse Ig (Silenus) diluted 1:2000 and incubated for 1 hr, followed by washing and addition of substrate. After final washing, the ELISA was developed by addition of substrate 3,3',5,5'-tetramethylbenzidine (Sigma). Absorbance was measured at 450 nm, and titres defined as the highest dilution required for an absorbance of 0.2.

## Isolation of plasmid DNA and construction of vaccination cartridges

Library pools stored as glycerol stocks were grown to confluence on solid agar media containing 50 μg/ml of ampicillin prior to inoculation into liquid LB media (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/ml of ampicillin. 5-10 confluent agar plates were used to inoculate one litre of LB media (with 50 μg/ml ampicillin) and grown with shaking at 37°C for 6 hours prior to harvest. Plasmid preparation and endotoxin removal was performed using the QIAGEN endotoxin free Plasmid Giga Kit according to the manufacturer's instructions (QIAGEN Inc, Valencia, CA, USA). Purified DNA was precipitated onto gold microcarriers and these were attached to plastic supports as per manufacturer's recommendations (Bio-Rad Laboratories, Hercules, CA, USA). DNA was combined with gold microcarriers at a ratio of 100 μg DNA/50 mg carriers. Each projectile contains approximately 1 μg DNA.

## Mice and vaccination

All mice were BALB/c, female, and 5-6 weeks of age at the time of first vaccination. DNA vaccinated mice received three immunisations at two-week

intervals. For intraepidermal (ID) DNA vaccination the abdominal region was shaven and particles containing 1 μg of DNA were delivered by the Helios gene-gun (Bio-Rad Laboratories, USA) with a pulse of helium gas at 400 lb/in$^2$. Intramuscular (IM) DNA plasmids were delivered into the *tibialis anterior* muscle (100 μg total) in PBS.

## *In vitro spleen cell proliferation*

Spleen cell proliferation was performed as described in Materials and Methods in Chapter 3 and in (158). Purified AMA-1 and MSP4/5 recombinant proteins were used to stimulate splenocytes at a final concentration of 5 μg/ml. As a control for cell viability, splenocytes were stimulated with concanavalin A (Sigma) at a final concentration of 2.5 μg/ml. Splenocytes were cultured for 96 h in flat bottom microtitre plates in triplicate at a final concentration of $5x10^6$ cells/ml ($1x10^6$ cells/well), and pulsed with 1 μCi/well of [$^3$H] thymidine (Amersham Biosciences Corp., Piscataway, NJ, USA) 18 h before harvesting. The splenocytes were harvested onto fibre glass filter mats (Saktron Instruments Inc., Sterling, VA, USA) using an automated cell harvester (Saktron), and incorporated radioactivity measured using a liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, MA, USA).

## *Infection of mice, blood sampling and parasitemia measurements*

Blood from an infected mouse with a known parasitemia (1-10%) was taken and immediately diluted in PBS to give the required dosage ($1 \times 10^5$ infected RBC/dose). Mice were infected by intraperitoneal injection at day 0, and parasitemia assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection

levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels and day to peak parasitemia were compared using a Student *t*-test.

## *Analysis of survival curves*

Survival curves for vaccinated and control mice were compared using the Mantel-Haenszel test. Statistical analysis was performed using Prism 3.02 software (GraphPad, San Diego, CA, USA).

## 4.3) Results

### *Expression of protein encoded by bicistronic plasmids in vitro*

The ability of mammalian cells to secrete MSP4/5 or AMA-1 after transfection with DNA vaccine plasmids was tested in COS 7 cells. Proteins encoded by the constructs were secreted into the culture supernatant *in vitro* (Fig.1), and detected by Western Blot after probing with either anti-AMA-1 or anti-MSP4/5 rabbit sera.

BC construct 1 contained the TPA/AMA-1 sequence in the first position, followed by MCP-3/MSP4/5 in the second position after the IRES sequence. AMA-1 protein secretion from COS 7 cells transfected with BC construct 1 was detected, as was AMA-1 protein from COS 7 cells transfected with the VR1020/AMA-1 plasmid as a positive control (with AMA-1 secreted by virtue of the TPA signal sequence contained in both vectors). Figure 4 shows that AMA-1 was detected via Western Blot after secretion into the cell supernatant by both COS 7 cells transfected with BC construct 1 (Fig.4C), or the VR1020/AMA-1 control construct (Fig.4A). The MCP-3/AMA-1 fusion protein encoded in the first position of BC construct 2 was also secreted into the cell supernatant after transfection into COS 7 cells and detected via

Western Blot (Fig.4E). The *P.c.adami* DS genomic library was inserted into the first position of BC construct 1 in place of AMA-1 to produce the BC 30K construct which was transfected into COS 7 cells. Due to the low copy number of plasmids expressing peptides in this genomic library, peptide expression levels of encoded sequences were therefore lower and are undetectable via Western Blot.

The secretion of the MCP-3/MSP4/5 fusion protein (from the MSP4/5 sequence contained in the second cloning position after the IRES sequence) was also detected after transfection with both BC construct 1 and 2 plasmid constructs into COS 7 cells. However, the signal strength of the MCP-3/MSP4/5 fusion protein was significantly decreased after transfection of COS 7 cells with BC construct 1 (containing a TPA leader sequence in Position 1; Fig.4G) when compared to BC construct 2 (with an MCP-3 leader sequence in Position 1; Fig.4J).

Supernatants collected from COS 7 cells after transfection with bicistronic DNA vaccine vectors not containing inserts did not react with specific rabbit sera to each recombinant protein (Fig.4B, D, & H).

**A** VR1020/AMA-1 control
**B** VR1020 vector
**C** TPA/AMA-1 in Position 1 of BC construct 1
**D** Empty BC vector
**E** MCP-3/AMA-1 in Position 1 of BC construct 2
**F** MCP-3/AMA-1 control
**G** MCP-3/MSP4/5 in Position 2 of BC construct 1
**H** Empty BC vector
**I** MCP-3/MSP4/5 control
**J** MCP-3/MSP4/5 in Position 2 of BC construct 2
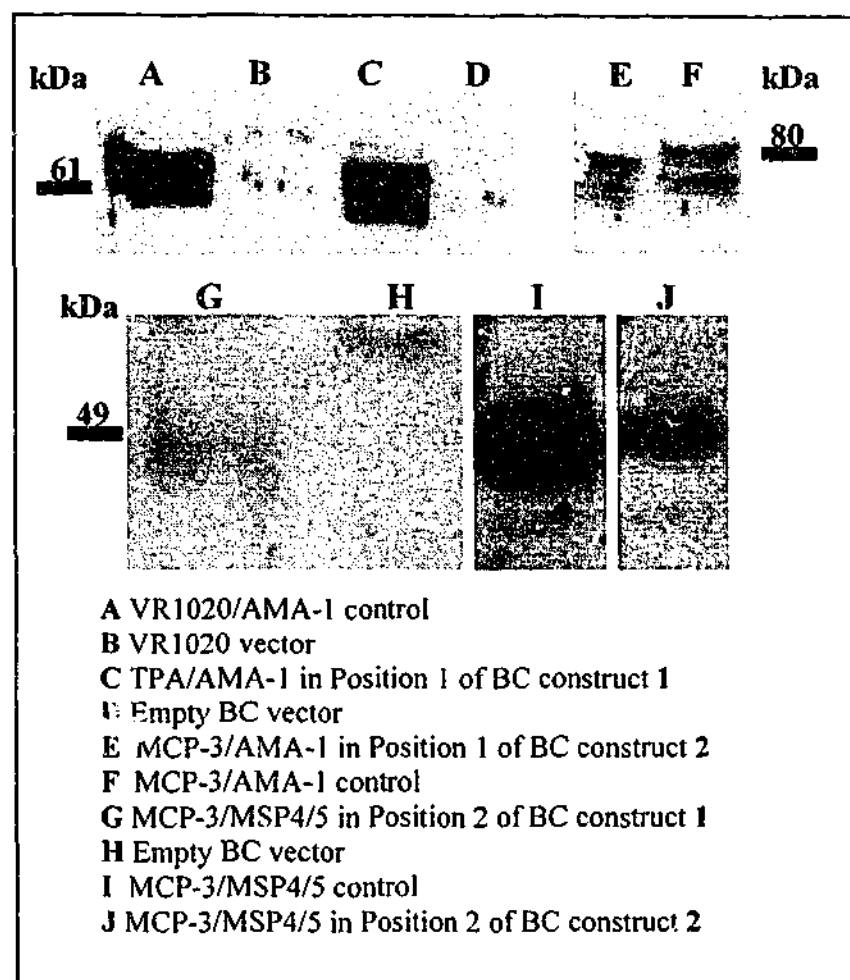
**Figure 4.** Western Blot of supernatants taken from COS 7 cells transfected with bicistronic and monocistronic DNA vectors containing antigen inserts. The Western Blot was probed with anti-AMA-1 rabbit sera (A-F) or anti-MSP4/5 rabbit sera (G-J). Proteins expressed by cells transfected with each construct were secreted into the culture supernatant. Control vectors (B,D, & H) not containing inserts did not react with rabbit sera. Molecular mass standards are shown.

### IgG responses in mice vaccinated with bicistronic DNA vaccine constructs

The bicistronic constructs were used to vaccinate mice either by IM injection or ID using the gene-gun. The resulting antibody responses were measured two weeks after the third vaccination (week 6) by ELISA using recombinant MSP4/5 protein, or recombinant refolded AMA-1 protein. Figure 5 shows the titre (taken as the antisera dilution where the O.D was 0.2) of IgG antibodies of vaccinated mice. Vaccination using the empty DNA vaccine BC control vectors did not produce a detectable IgG antibody response above an O.D of 0.2 by ELISA to MSP4/5 or AMA-1 protein (data not shown).

Mice vaccinated with BC construct 1 via ID (Fig.5A) or IM routes (Fig.5C) produced a detectable IgG response to both AMA-1 and MSP4/5 by ELISA. The antibody response to AMA-1 (untargeted) was significantly higher than MSP4/5 IgG antibody titres after mice were vaccinated via the ID route with BC construct 1 (Fig.2A; $P=0.02$). A higher mean IgG antibody titre to AMA-1 compared to MSP4/5 was also detected after vaccination with BC construct 1 via the IM route, however this was not statistically significant (Fig.5C, $P=0.15$).

The use of MCP-3/AMA-1 in Position 1 of BC construct 2 did not promote an enhanced antibody response to AMA-1; however the mean MSP4/5 antibody response was greater after vaccination of mice via the ID route (Fig.5B). This is in contrast to the use of a TPA/AMA-1 nucleotide sequence in the first position of BC construct 1 which resulted in an enhanced antibody response to AMA-1, as well as detectable levels of MSP4/5 antibodies when delivered via ID and IM routes (Figs.5A & 5C). Therefore, BC construct 2 was excluded from future experiments due to the lack of a detectable antibody response to AMA-1.

As a comparative control experiment, mice (five per group) were vaccinated with the untargeted monocistronic VR1020/AMA-1 construct via ID (gene-gun) or IM routes. Control mice (also five mice per group) were vaccinated with the VR1020 vector via IM and ID routes (from which no antibody response to AMA-1 was detected, therefore data is not shown). For this control experiment, sera were pooled and an ELISA performed in response to refolded recombinant AMA-1 (Fig.5D). Although these data cannot be directly compared statistically to the data generated by the bicistronic constructs containing AMA-1, comparison of BC construct 1 ID (Fig.5A) and BC construct 1 IM in particular (Fig.5C), shows that antibody production to AMA-1 can be enhanced in a bicistronic construct relative to the VR1020/AMA-1 monocistronic construct (Fig.5D). This may be due to the MCP-3 leader sequence in the second position of the vector acting as a chemokine and enhancing APC uptake of AMA1. An experiment with a greater sample size, with sera analysed from individual mice, and the TPA/AMA-1 sequence in the BC vector backbone without the MCP-3/MSP4/5 sequence in Position 2 (to control for CpG motifs between VR1020 and the BC vector) will be required to confirm this finding.

Antibody responses to the variety of antigens expressed in genomic library contained within the BC 30K construct can not be accurately measured by ELISA, due to the low copy number of sequences contained within individual plasmids (181). There was a detectable IgG response to MSP4/5 after vaccination with BC 30K constructs via both ID (Fig.5E) and IM (Fig.5F) routes which were similar to the MSP4/5 titre seen with BC construct 1. T-cell proliferative responses of individual mice to *P.c.adami* DS IRBC (and MSP4/5) are shown below (Fig.6).
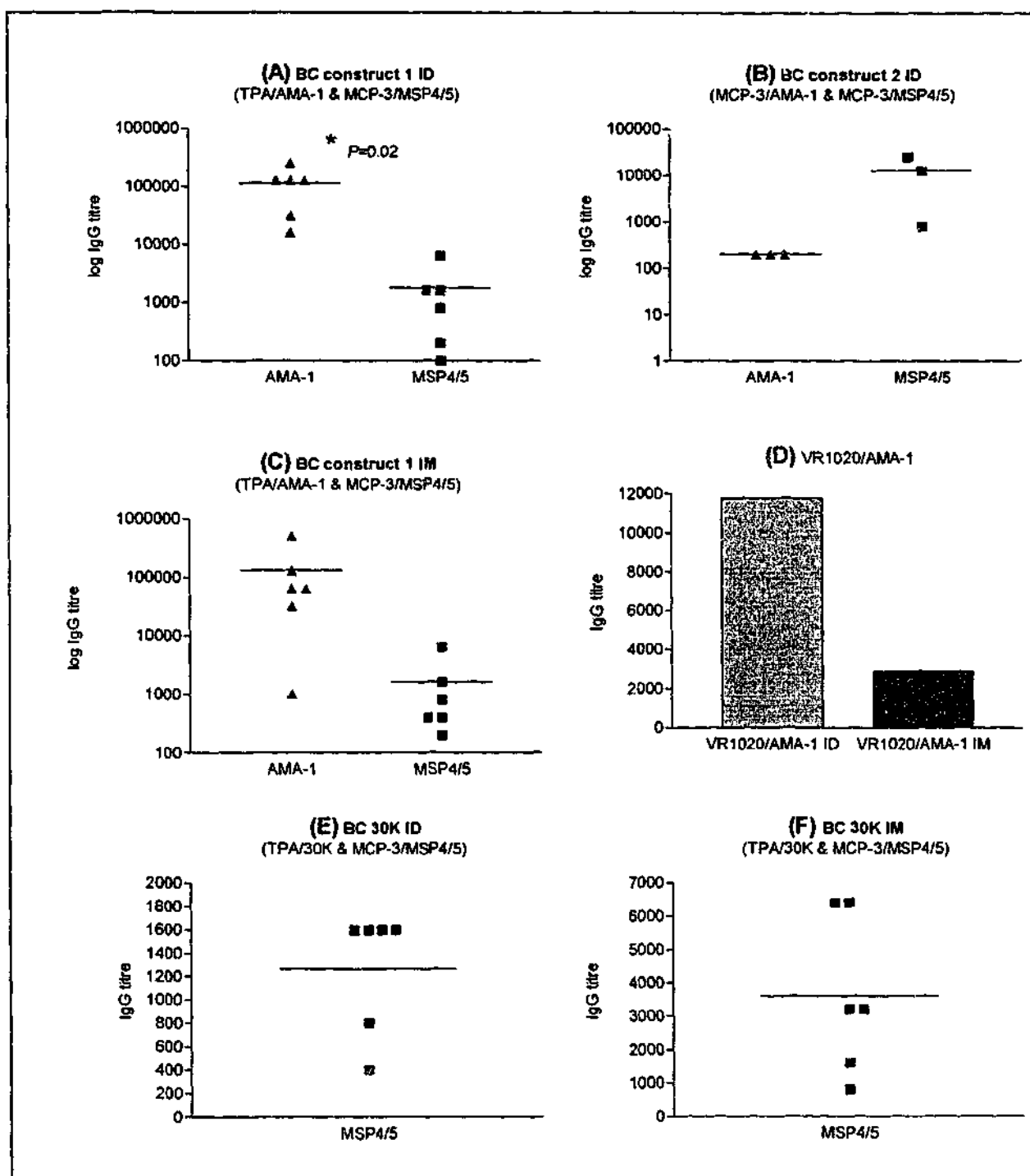
**Figure 5.** IgG responses of mice vaccinated with bicistronic and monocistronic DNA vaccine constructs. IgG antibody responses were measured by ELISA. Vaccines were delivered using either 1 µg of DNA ID (gene-gun), or 100 µg of DNA IM. Graphs **(A)**, **(B)**, and **(C)** show data from sera taken from individual mice reacting to both MSP4/5 and AMA-1 recombinant protein after vaccination with a single bicistronic construct. The mean titre is indicated by a bar. **(D)** Pooled sera from 5 mice vaccinated with VR1020/AMA-1 via both ID and IM routes. MSP4/5 responses after BC 30K administration are shown in **(E)** and **(F)**. Mice vaccinated with vectors not containing AMA-1 or MSP4/5 inserts did not mount an antibody response (not shown). *, *P*<0.05, paired *t*-test.

*Cellular immune responses induced by bicistronic DNA vaccination*

Groups of mice were vaccinated ID or IM with BC construct 1 (containing TPA/AMA-1 and MCP-3/MSP4/5), BC 30K via the ID route, or empty BC plasmid DNA control vectors, according to the procedures described in the Materials and Methods. Cellular proliferation assays were performed on individual mice within these groups based on results of survival and parasitemia data described below. To evaluate cellular responses to *P.c.adami* DS antigens induced by bicistronic vaccination, spleens were removed from mice ten days after the final vaccination. Cell suspensions from individual mice vaccinated with BC construct 1 were stimulated using recombinant AMA-1 and MSP4/5 protein. Cell suspensions obtained from mice vaccinated ID with BC 30K were stimulated with MSP4/5 and equivalent numbers of *P.c.adami* DS IRBC or RBC (prepared from naïve BALB/c mice as a negative control).

Significant proliferation of splenocytes primed using BC construct 1 was observed after vaccination (Fig.6A-D). Significant levels of proliferation were observed after splenocytes were stimulated with MSP4/5, previously primed via the IM (Fig.6A; $P=0.025$) and ID (Fig.6C; $P=0.004$) routes. The resulting proliferation was significantly higher than splenocytes primed with empty vector control DNA. Stimulation of splenocytes using AMA-1 also resulted in a significant increase in proliferation after vaccination by both IM (Fig.6B; $P=0.012$) and ID routes (Fig.6D; $P=0.027$) compared to empty vector DNA. No differences in the level of proliferation were observed between the routes of vaccination. The splenocytes from the empty vector primed mice vaccinated by the ID and IM routes did not proliferate in response

to MSP4/5 or AMA-1. Both antigens from the single BC construct 1 could therefore prime the immune system, resulting in proliferation after stimulation *in vitro*.

Testing the immune priming ability of the genomic library contained within BC 30K required the use of native *P.c.adami* DS antigens in the form of IRBC (with RBC used as a negative control). Splenocytes primed ID with the BC 30K constructs and stimulated with IRBC showed significant proliferation when compared to stimulation with RBC as a negative control (Fig.6F; $P=0.047$). Splenocytes primed ID with BC 30K and stimulated with IRBC showed significantly higher proliferation than splenocytes primed ID using empty vector DNA stimulated with IRBC (Fig.6F; $P=0.027$), or RBC (Fig.6F; $P=0.026$). Proliferation of splenocytes stimulated with MSP4/5 was also enhanced after priming with the BC 30K construct, when compared to splenocytes primed ID with empty vector DNA (Fig.6E; $P=0.013$). The level of stimulation in response to MSP4/5 with the BC 30K ID vaccine was comparable to that seen above using BC construct 1 with MSP4/5 ID (Fig 6C).

IFN-γ and IL4 ELISAs were performed on BC vector 1 splenocytes stimulated with AMA-1 and MSP4/5, as well as BC 30K splenocytes stimulated with IRBC and MSP4/5 (using the methods described in Chapter 3). Although IFN-γ was produced by mice vaccinated with both BC vector 1 (IM & ID) or BC 30K, there were no detectable differences when compared to secretion of IFN-γ by empty vector DNA primed splenocytes, even though empty vector control splenocytes did not proliferate. IL-4 was not detected in supernatant taken from splenocytes primed with any BC construct (data not shown).
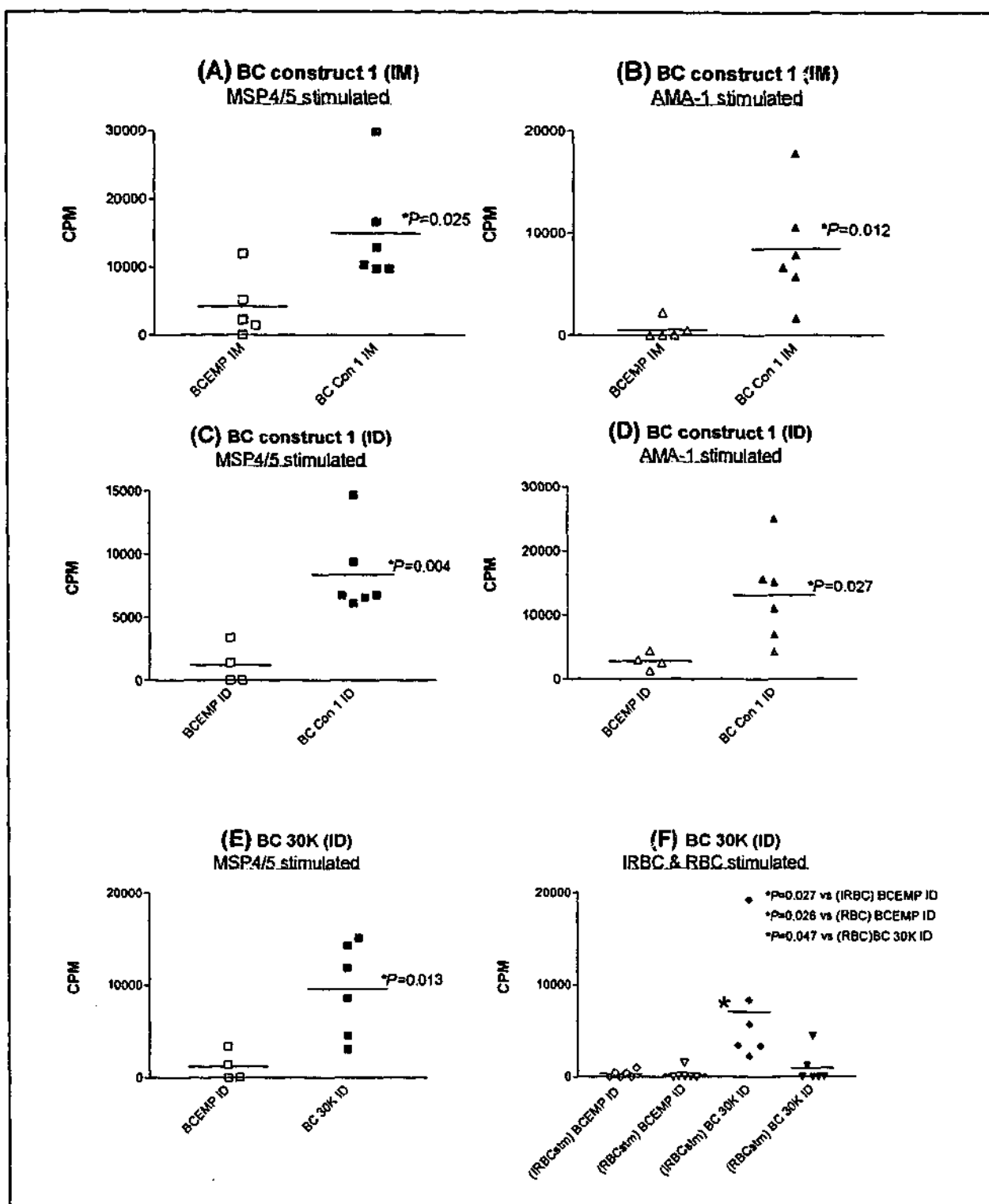
**Figure 6.** Proliferation of splenocytes primed with bicistronic vectors. *In vitro* proliferation of splenocytes from individual BALB/c mice vaccinated ID with the gene-gun or IM by injection. Mice were vaccinated with BC construct 1 or BC 30K 3 times at 2 week intervals. Splenocytes were harvested 10 days after the final vaccination. BC construct 1 primed splenocytes were stimulated with AMA-1 or MSP4/5 (A-D) and harvested at 72 hours, after [³H] thymidine was added 18 hours previously. For BC 30K ID (E-F), after 72 h of stimulation with $2 \times 10^6$ IRBC or $2 \times 10^6$ RBC, [³H] thymidine was added for 18 h. [³H] incorporated by cells was then measured. Splenocytes primed with empty vector control DNA were also stimulated with $2 \times 10^6$ IRBC or RBC. Splenocytes from all individual cultures responded to concanavalin A stimulation (data not shown). The mean CPM (bar) is shown. Statistical analysis was performed using the unpaired *t*-test.

### Vaccine efficacy: Bicistronic DNA vaccine challenge Trial 1.

Bicistronic Trial 1 contained 6 female BALB/c mice per group vaccinated with BC construct 1 and BC 30K. Mice were vaccinated IM (100 μg) by injection or ID (1 μg) using the gene-gun three times at two week intervals, and challenged with 100,000 *P.c.adami* DS IRBC two weeks after the final vaccination. This trial represents an extremely virulent challenge using the *P.c.adami* DS system.

Figure 7 shows survival curves of mice challenged with lethal *P.c.adami*. There were no significant differences in survival found between vaccinated and empty vector control mice (Fig.7A-C). All mice died by day 13.

The parasitemia levels of mice challenged in this trial however were influenced by the administration of bicistronic vectors, regardless of the lack of survival. Figure 7(A.i-C.i) shows the percentage parasitemia measured from day 6 post infection.

No significant differences were found in the peak parasitemia between BC construct 1 IM and the empty vector IM control at day 9 post infection (Fig.7A.i). However, 4 out of 6 control mice reached their peak parasitemia at day 9, with the two remaining mice continuing to rise in parasitemia until day 11 (Fig.7A.i). This is reflected in the BC construct 1 IM vaccinated survival curve, with 33% of IM control mice dead at 10, compared to the first deaths occurring at day 11 for BC construct 1 IM (Fig.7A). There was also a sharp drop in parasitemia by day 12 for the two surviving BC construct 1 IM mice, which outlived empty vector IM control mice and had almost resolved parasitemia before death at day 13 (Fig.7A.i).
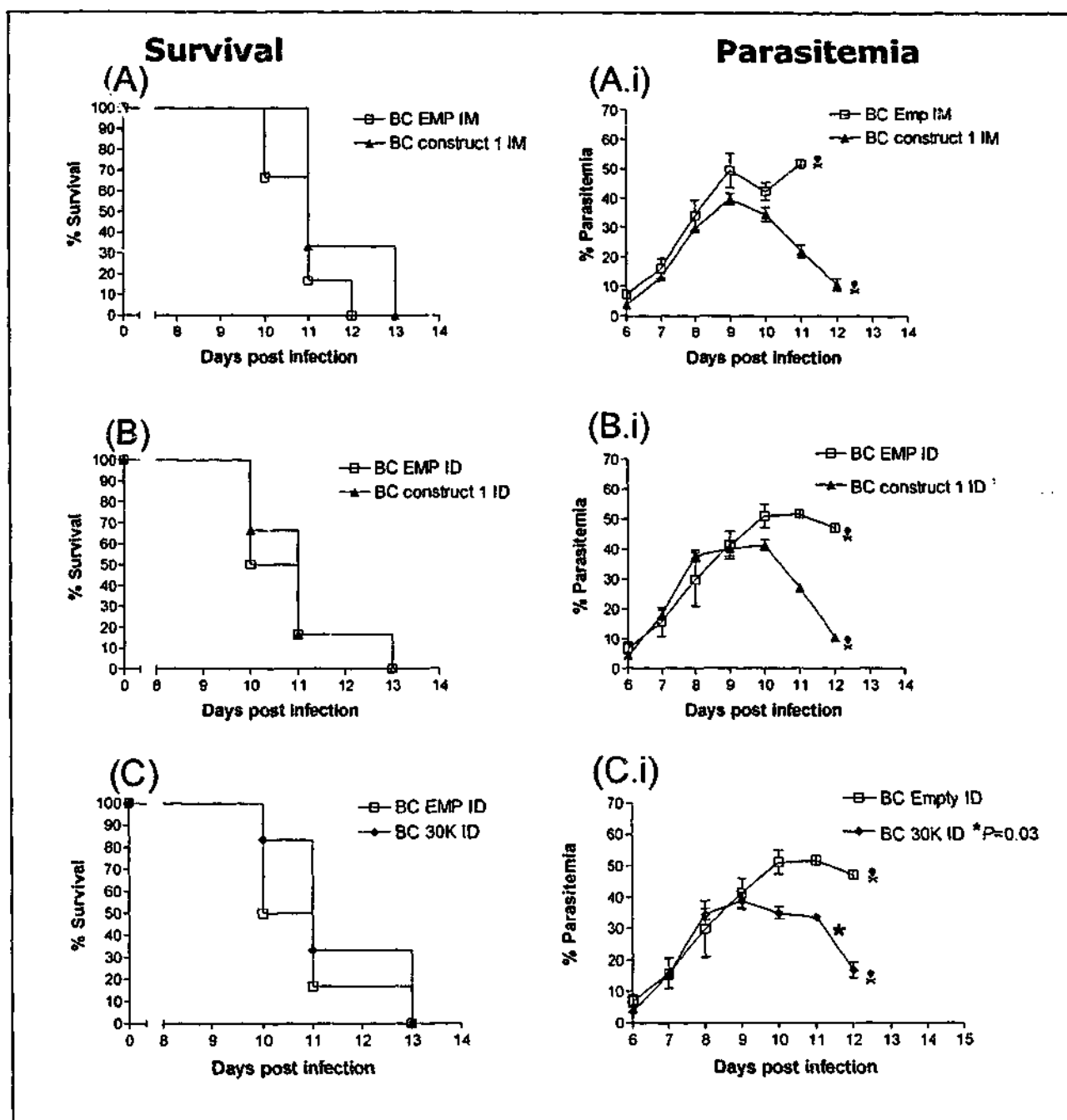
**Figure 7. (A-C)** Bicistronic Trial 1 survival curves. Six mice per group were vaccinated IM by injection with 100 μg of DNA or 1 μg via gene-gun three times at two week intervals. Mice were challenged with 100,000 *P.c.adami* DS IRBC. There were no significant differences between survival curves of control mice versus vaccinates, as determined by the areas under the curves using the Mantel-Haenszel test for comparing survival curves. **(A.i-C.i)** Bicistronic Trial 1 parasitemia curves. Smears were taken from individual mice (six mice per group) from day 6 post-infection, with 300-400 cells counted per smear. The death of a group is represented by a ☠. Significant differences between peak parasitemias are indicated by an * (Student *t*-test).

Vaccination with BC construct 1 ID did not result in a significant reduction in peak parasitemia when compared to the empty vector control (Fig.7B.i; $P=0.05$). This experiment was repeated in Figure 8 (Fig.8B & B.i), with no significant differences in survival or parasitemia.

The use of BC 30K appeared to result in a decrease in peak parasitemia relative to the empty vector ID control group (Fig.7C.i). By day 10, only a single death was recorded in the BC 30K group, compared to five deaths in the control group on day 10 post infection (Fig.7C). Parasitemia peaked in all BC 30K mice on day 9 post infection, while control mice continued to rise in parasitemia (Fig.7C.i). As with BC construct 1 (IM & ID), the parasitemia in the BC 30K vaccinated group was also resolved earlier in the surviving mice relative to mice in the empty vector control groups; however, this did not prevent death of all BC 30K mice by day 13 post infection.

### Vaccine efficacy: Bicistronic DNA vaccine challenge Trial 2

In order to confirm the results from Trial 1, bicistronic Trial 2 involved the same groups as for Trial 1, however a BC 30K IM group was included. Again, animals were vaccinated and challenged as in Trial 1.

Figure 8 shows the survival (and parasitemia) curves of bicistronic Trial 2. For comparison, Figure 9 shows VR1020/AMA-1 control experiment, while Figure 10 shows an MCP-3/MSP4/5 control experiment. These control experiments were performed using different vector backbones (not the BC vector backbone), but are included as examples of how monocistronic DNA vaccines with AMA-1 and MSP4/5

antigens perform (in terms of parasitemia and survival) when the *P.c.adami* DS model is used in our laboratory.

Empty vector control mice in bicistronic Trial 2 followed the same pattern of survival, with all mice dying by day 11 post challenge regardless of vaccination route. Mice vaccinated with BC construct 1 IM showed a significantly enhanced survival compared to vaccination with the empty vector control group (Fig.8A; $P=0.03$). There was no significant difference in survival when BC construct 1 was delivered via the ID route when compared to the empty vector control group (Fig.8B), although 2 out of 6 mice survived. Vaccination via the ID route using VR1020/AMA-1 (containing the TPA/AMA-1 sequence as used in BC construct 1) has no effect on survival (Fig.9). The survival found after BC construct 1 ID delivery is comparable to that seen using MCP-3/MSP4/5 (the same sequence contained in BC construct 1 in position 2) delivered ID as a monocistronic construct (Fig.10) (159).

Vaccination of mice using the BC 30K produced a significant delay in death when delivered ID (Fig.8D; $P=0.004$), with all mice surviving until day 11 when they suddenly died. This was not the case when BC 30K was delivered IM, as there was no significant difference when compared to the empty vector control IM survival curve (Fig.8C).
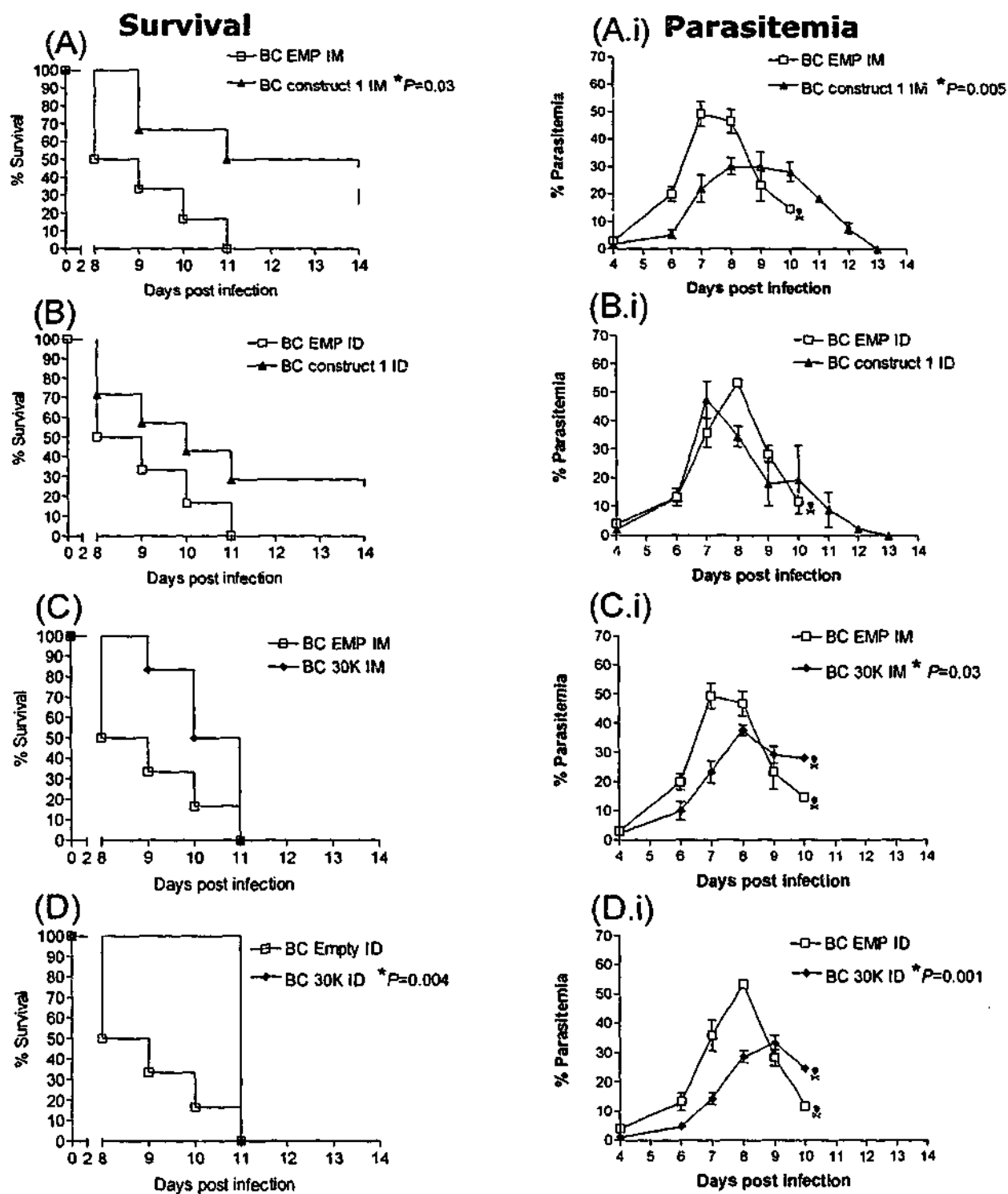
**Figure 8. (A-C)** *Bicistronic Trial 2 survival curves.* Six mice per group were vaccinated IM by injection with 100 µg of DNA or 1 µg via gene-gun three times at two week intervals. Mice were challenged with 100,000 *P.c.adami* DS IRBC. Significant differences between survival curves of control mice versus vaccinates were determined by the areas under the curves using the Mantel-Haenszel test for comparing survival curves. **(A.i-C.i)** *Bicistronic Trial 2 parasitemia curves.* Smears were taken from individual mice (six mice per group) from day 4 post-infection, with 300-400 cells counted per smear. The death of a group is represented by a ☠. Significant differences between peak parasitemias are indicated by an * (Student *t*-test).

The parasitemia of mice vaccinated with BC construct 1 IM was significantly reduced compared to IM delivery of the empty control vector (Fig.8A.i; $P=0.005$). This does not occur when the antigens are delivered as monocistronic constructs using either VR1020/AMA-1 (Fig.9), or MCP-3/MSP4/5 (Fig.10). Vaccination via the ID route however did not have any effect in reducing the parasitemia, with the BC construct 1 group peaking before the empty vector control group (Fig 8B.i). When vaccinating with MCP-3/MSP4/5 ID using the *P.c.adami* DS model, there is no effect on parasitemia, although survival is increased relative to empty vector control groups (Fig.10) (159). In these preliminary experiments, combining AMA-1 (secreted) and MCP-3/MSP4/5 together in a bicistronic DNA vaccine construct delivered IM appears to reduce parasitemia when compared to monocistronic delivery of these antigens.

Mice vaccinated with BC 30K did not survive, however the parasitemia in both IM and ID groups was significantly reduced at days 6-8 post infection (Figs.8C.i & D.i). Vaccination with BC 30K via the IM or ID route resulted in this group reaching peak parasitemia a day later than the empty vector control, as well as a significant reduction in peak parasitemia (Fig.8C.i; $P=0.03$; Fig.8D.i; $P=0.001$). Although the interpretation of these data is complicated by the death of mice in the control group, the data does suggest a significant effect of the vaccine on parasitemia.
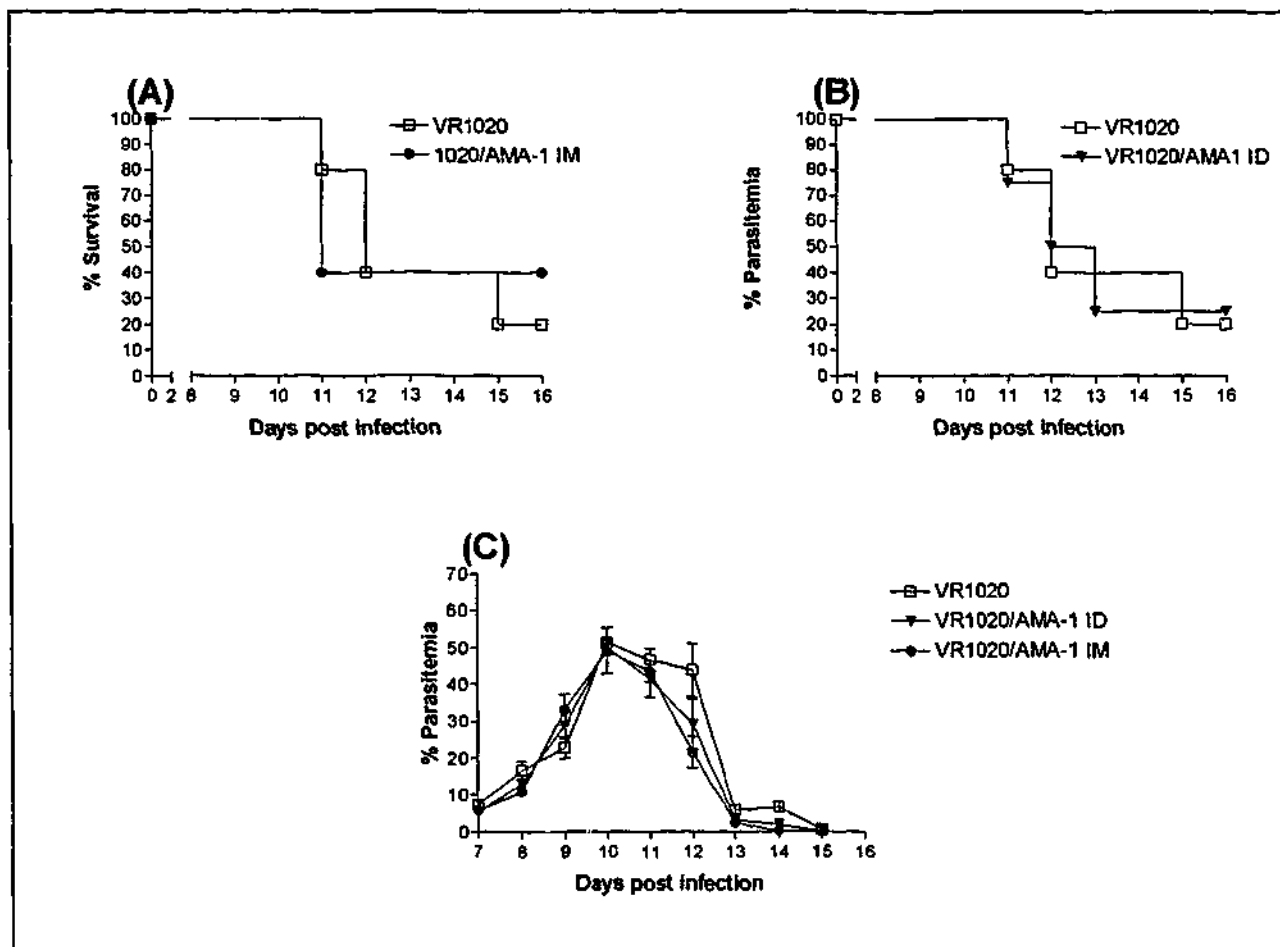
**Figure 9.** Survival curves and parasitemia data after VR1020/AMA-1 vaccination in mice. Five mice per group were vaccinated with DNA three times at two week intervals via IM (**A**) injection (100 µg) or ID (**B**) via gene-gun (1 µg), and challenged with 100,000 *P.c.adami* DS IRBC. There were no significant differences between survival curves, or parasitemia data (**C**).
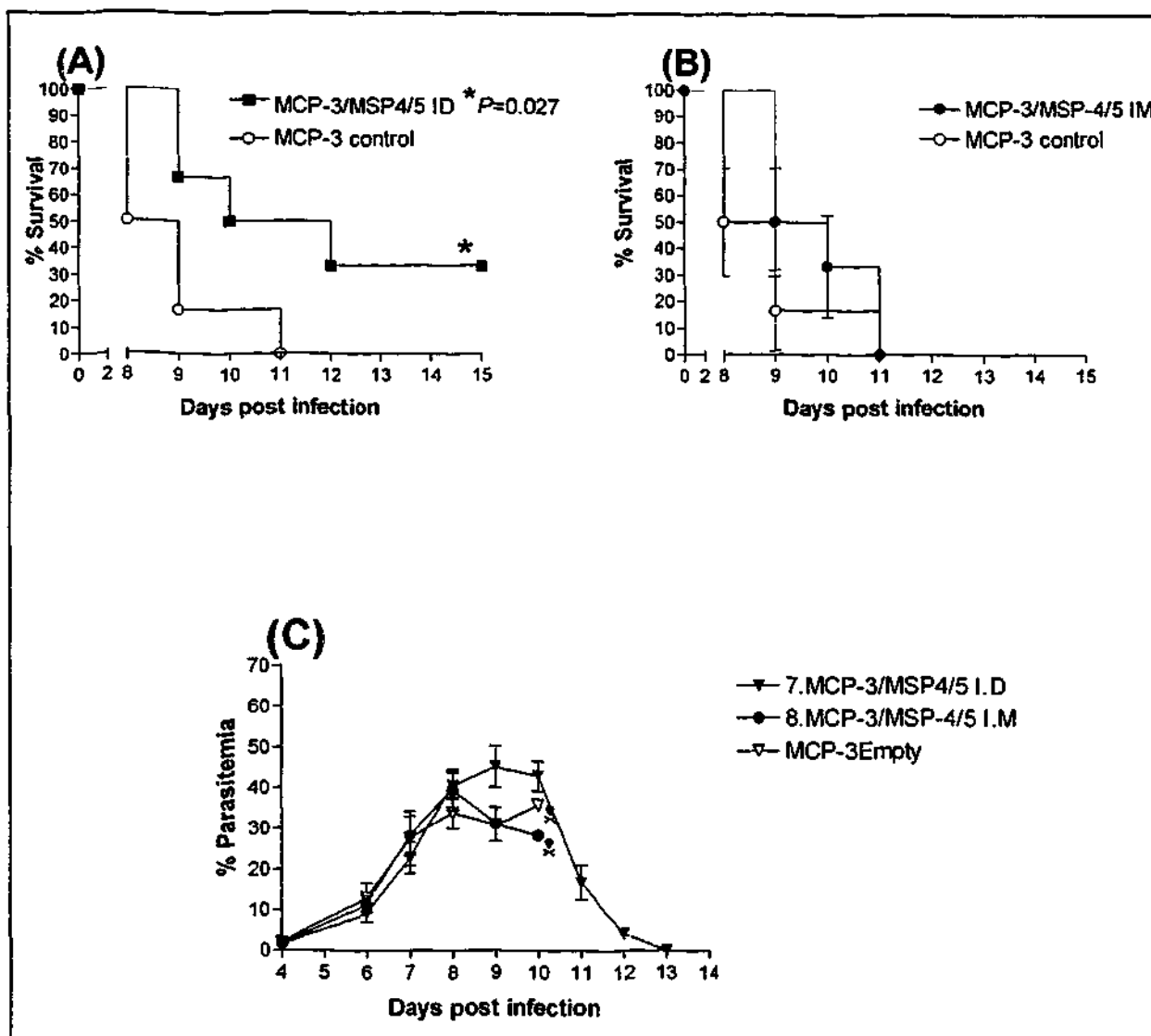
**Figure 10.** Survival curves and parasitemia data after MCP-3/MSP4/5 vaccination of mice. Six mice per group were vaccinated with DNA three times at two week intervals via IM injection (100 µg) or ID via gene-gun (1 µg), and challenged with 100,000 *P.c.adami* DS IRBC. (A) A significant difference between survival curves of MCP-3/MSP4/5 ID and the empty vector control was found ($P=0.027$; Mantel-Haenszel test). There were no other significant differences between survival curves for mice vaccinated IM (**B**) or parasitemia data (**C**).

## 4.4) Discussion

In this preliminary study it has been shown that bicistronic vectors used as DNA vaccines can evoke humoral and cellular responses, reduce parasitemia, and partially protect against lethal *P.c.adami* DS challenge in mice. The aim of this study was not to directly compare the efficacy of bicistronic versus monocistronic vectors, but to evaluate the potential of bicistronic vectors as components of a multivalent vaccine against malaria. To directly compare monocistronic and bicistronic vectors in future studies, the experimental design must be refined. The inclusion of candidate antigen sequences (TPA/AMA-1 and MCP-3/MSP4/5) as monocistronic constructs in separate bicistronic vector backbones will be required. This will account for any possible different CpG motifs in the VR1020 (AMA-1) and VR1012 (MCP-3/MSP4/5) vectors, compared to the empty BC construct (Fig.1iii) used in the present experiment. Vaccination with these monocistronic constructs using two separate gene-gun bullet cartridges (or co-precipitation of monocistronic constructs onto gold particles) into a single site on the mouse should be tested to confirm that bicistronic delivery is superior to co-delivery of 2 separate antigens in a malaria model. However, delivery of monocistronic vectors containing candidate antigens in hepatitis models as DNA vaccines, was shown to be less efficient at stimulating cellular and humoral responses than delivery in bicistronic vectors (34, 35, 43). This enhanced efficiency with bicistronic delivery was believed to be due to expression of antigens (or cytokines) contained in bicistronic constructs from a single cell, and therefore an increased likelihood that both would be encountered by circulating APCs (34, 35). To date, there are no studies evaluating the potential of bicistronic vectors to deliver

malarial antigens, even though a multistage and multiantigen malarial vaccine is believed to be optimal to protect against malaria (54, 113).

Delivery of combinations of malarial antigens can evoke enhanced immune responses, and protect to a greater extent than a single antigen alone (55, 100, 109, 197). The delivery of multiple antigens by a single vaccine construct would also have many practical advantages. The construction of a single plasmid for vaccine delivery would reduce problems associated with production costs, manufacturing time, quality control, and use in a clinical setting (179).

In the present study, bicistronic vector construction was aimed at delivering two targeted malarial antigens using the chemoattractant MCP-3. An MCP-3 vector has been found to enhance the survival of mice using MSP4/5 as a vaccine after DNA vaccination relative to a VR1020 construct (159). It was hypothesised that the fusion of the MCP-3 sequence to both AMA-1 (position 1 of BC construct 2) and MSP4/5 (position 2 of BC construct 2) would recruit dendritic cells to the site of antigen expression, and enhance priming of naïve T cells by both antigens (reviewed in 127). Transfection of bicistronic plasmids into COS 7 cells resulted in both antigens fused to MCP-3 being secreted into the supernatant and were detected via Western Blot. However after vaccination of mice, a humoral response to MCP-3/AMA-1 could not be detected *in vivo* even though secretion was found possible *in vitro*. This does not discount the possibility that a cellular response may have been evoked in the absence of antibody *in vivo* (reviewed in 52). One possibility is that a homologous recombination event may have occurred *in vivo* due to the identical MCP-3 leader sequences in two positions in the construct (reviewed in 43). However, this would have had to occur in all plasmids after vaccination to account for the lack of responsiveness. This does not explain why an antibody response was generated

towards MSP4/5 after BC construct 2 vaccination. It has been found that the nucleotide composition of genes contained within a bicistronic construct has a significant effect upon IRES promoted translation (84). As there have been no results published at this time involving the use of malarial antigens in bicistronic DNA vaccines, the effects *in vivo* using BC construct 2 could not be predicted.

The transfection of BC construct 1 (containing a TPA leader secretion signal in place of the MCP-3 gene in Position 1) into COS 7 cells resulted in the secretion of both the AMA1 and MSP4/5 antigens *in vitro*. Expression of MCP-3/MSP4/5 (Position 2 of BC construct 1) by COS 7 cells was markedly reduced compared to AMA-1 in position 1 of this construct. This was supported by the observation that after vaccination of mice using BC construct 1 (via ID and IM routes), only low MSP4/5 antibody titres were detected, relative to an enhanced AMA-1 antibody response. Again, this emphasizes that the dominant determinant of translation efficiency is the arrangement and nature of coding sequences on the mRNA after bicistronic vaccination (84).

Vaccination with bicistronic vectors, containing rodent malaria homologues of human candidate malarial antigens AMA-1, MSP4 and MSP5, as well as a genomic library, expressed proteins that were recognised by the rodent immune system. The route of delivery (IM or ID) did not have any significant effects upon antibody titres, nor did it have any effect on enhancing or reducing T-cell proliferation when T cells were stimulated with AMA-1 or MSP4/5 antigens. It was therefore possible for the bicistronic malarial DNA vaccine to prime the immune system with both antigens simultaneously regardless of route. B-cell deficient mice can control erythrocytic stage malaria infections by limiting parasite growth, emphasizing the importance of T-cell mediated immunity (194, 195). The generation of CD4^{+} T-cell responses

against *P. falciparum* erythrocytic infection is believed to be of primary importance, by acting as T helper cells for antibody responses and effector cells by limiting parasite growth via antibody-independent cell-mediated immunity (reviewed in 70, 71). To analyse the precise characteristics of the splenic T-cell response generated in the present study, further experiments using fluorescent automated cell sorting (FACS) could be implemented. Initial characterisation of splenocyte populations using antibodies directed at $CD4^+$ and $CD8^+$ cells would be a logical starting point, as the IFN-γ and IL-4 cytokine analysis performed in these preliminary experiments was not useful.

The use of the BC 30K construct promoted significant T-cell responses to native *P.c.adami* DS IRBC. It is difficult to determine whether the reaction to the IRBC by splenocytes was due to MSP4/5 priming (as a result of the MCP-3/MSP4/5 sequence in Position 2 of BC construct 1) or due to the genomic DNA inserts delivering multiple novel antigens. Immunofluorescence studies have shown that anti-MSP4/5 sera does not react with early ring stage parasites, but do react with the trophozoite stage, schizonts, and free merozoites where MSP4/5 is abundantly expressed (18). The IRBC preparations used in the present experiment ranged from ring stage parasites to late stage schizonts. The different *P.c.adami* DS erythrocytic stages were used to maximise the probability that any splenic T cells primed by BC 30K genomic vaccination would proliferate, due to exposure by a range of erythrocytic stage antigens. Preliminary studies in our laboratory have shown that MSP4/5 DNA vaccinated mice can also react to IRBC at low levels. A pure ring stage *P.c.adami* DS IRBC preparation would be effective at eliminating the bias of priming with MSP4/5, but would also eliminate any other reaction to later erythrocytic stages primed by the genomic DNA contained in BC 30K. One factor that can be used to distinguish the

effects of responses to antigens expressed from the genomic inserts in BC 30K, from responses due to MSP4/5 alone (159), is the reduction in parasitemia found across both challenge trials. The BC 30K vaccine induced significant reductions in parasitemia whereas the MCP3/ MSP4/5 vaccine alone does not effect parasitemias (159).

The use of MCP-3/MSP4/5 as a DNA vaccine alone does not promote a significant reduction in parasitemia, by either ID or IM routes, and the same result is obtained with MSP4/5 using VR1020 and CTLA4 monocistronic vectors for the *P.c.adami* DS model (159). However, when MCP3/MSP4/5 is included with genomic DNA in a bicistronic vector, it was found that the *P.c.adami* DS induced parasitemia was indeed reduced after vaccination. An effect (albeit partial) of reducing parasitemia has been found in our laboratory after vaccination with other DNA expression libraries (prepared using the same *P.c.adami* DS genomic DNA stock used to construct the present BC 30K library) (181). Again, these observations provide indirect evidence that vaccination with the BC 30K construct resulted in the expression of novel peptides/antigens *in vivo*, resulting in a reduction in parasitemia. Division of the BC 30K library into smaller pools of 3000 plasmids or less, may help to enhance synergistic effects between MSP4/5 and any protective peptides/antigens contained within the library due to an increased copy number of protective plasmids in the smaller pool: however, it is also possible that protective sequences will be also lost as pool size is reduced. It is possible that this technique may lead to the discovery of additional protective MSP4/5-peptide/antigen pairs.

Survival after bicistronic vaccination, following challenge with lethal *P.c.adami* DS, did not correlate with significant reduction in parasitemia observed in both challenge trials. However, in bicistronically vaccinated groups across both challenge

trials, peak parasitemia was either significantly reduced, or the parasitemia reduced markedly compared to control animals before death. Trial 2 in particular resulted in a significant delay in death in mice vaccinated with either the BC construct 1 IM or BC 30K ID vaccine, as well as significant reductions in peak parasitemia.

Several approaches could be used to optimise the bicistronic vaccination strategy and enhance survival and further reduce parasitemia. The gene sequences used in BC construct 1 (AMA-1 and MSP4/5) were the native *P.c.adami* DS gene sequences, which like the *P.falciparum* genome is highly A/T rich. Hoffman *et al* (89) have optimised the codon usage of *P.falciparum* genes to more closely reflect codon usage in mammalian genes, resulting in a 5-40 fold enhancement of *in vitro* expression in mammalian cells, and 5-100 fold higher antibody titres in outbred mice (52, 89). This is one method of enhancement that could improve BC construct 1 efficacy. The use of a non-lethal strain of rodent malaria (such as *P.c.adami* DK) (117) would allow a single parameter, that of parasitemia to be measured in the absence of the complications due to pathology: this was found to be a key effect in the present study. Indeed, our laboratory has recently found that vaccination of BALB/c mice with a *P.c.adami* DS VR1020/30K library can significantly augment differences in parasitemia responses between vaccinate and control animals, resulting after challenge with non-lethal *P.c.adami* DK infected erythrocytes (unpublished data). This would simplify studies by removing the parameter of survival, which has been shown in this thesis to generate results that are very difficult to replicate. The measurement of parasitemia alone as an indicator of vaccine efficacy would facilitate division of the genomic library into smaller pools of plasmids for protective peptide/antigen discovery. The importance of reducing parasitemia has implications

for the reduction of morbidity (and potential mortality) in human malaria, as this is the stage of the disease that results in pathogenesis (138).

The ability of bicistronic constructs in the present study to produce malarial antigens simultaneously *in vitro* and *in vivo* after vaccination, allows for many more possibilities in malarial vaccine design. The inclusion of MCP-3 in the second position of the bicistronic construct may have increased antigen presentation of untargeted antigens in BC 30K and BC construct 1. The migration of dendritic cells (due to MCP-3 expression) to a single site of bicistronic antigen production may have enhanced the efficiency of the vaccine (as both bicistronically expressed antigens were in the same cellular location), leading to significant reductions in parasitemia. The bicistronic delivery of the cytokine GM-CSF in other systems has resulted in significant enhancements to DNA vaccination. It has been shown that using a hepatitis C bicistronic DNA vaccine, the delivery of a bicistronic plasmid containing hepatitis antigens and GM-CSF can significantly enhance T-cell proliferation and antibody responses, compared to vaccination with two separate plasmids (34). This has also been shown for hepatitis B DNA vaccination (35). However, the co-delivery of GM-CSF with 3000 plasmids from the VR1020 *P.c.adami* DS genomic library as monocistronic constructs is not effective at reducing peak parasitemia after erythrocytic stage challenge with *P.c.adami* DS (181). Monocistronic co-administration of *P.yoelii* CSP DNA plasmids and plasmids containing GM-CSF has been shown to result in increased $CD4^+$ and $CD8^+$ T cells, antibody production, and protection against sporozoite challenge in murine studies (203). The use of GM-CSF and $MSP1_{42}$ as a DNA vaccine in rhesus monkeys resulted in a rapid induction of antibodies after the first dose, but had no effect on the T-cell response (114). The delivery of cytokines such as GM-CSF, along with malarial candidate antigens or

genomic libraries in bicistronic vectors, may allow for a more efficient vaccine delivery system as seen in hepatitis models. However, the efficiency of immune responses to combinations of gene pairs within bicistronic constructs vary markedly between different constructs (84). Whether the enhancement of DNA vaccines by cytokines can be applied to bicistronic malarial erythrocytic stage vaccines still remains to be tested.

Bicistronic delivery of DNA vaccines is a relatively new concept. It is believed that a successful malaria vaccine will include as many as 15 antigens from many different stages of the life-cycle (54, 114). The induction of both antibody and T-cell responses, as well as the reduction in parasitemia against lethal *P.c.adami* DS challenge in mice demonstrates that the delivery of malarial antigens via bicistronic vectors is feasible in the murine model. The optimisation and refinement of bicistronic DNA vaccines for malaria will now need to occur. This may include codon optimisation, co-delivery of cytokines, and testing in different murine malaria models.

# Chapter 5

## Summary & Conclusions

This thesis evaluated new methods of DNA vaccination against malaria involving targeting and bicistronic vectors using known malarial candidate antigens, and genomic libraries expressing *P.c.adami* DS peptides. This thesis also attempted to further enhance and characterise immune responses induced by ELI against *P.c.adami* DS malaria.

Chapter 2 "The protective efficacy of MSP4/5 against lethal *P.chabaudi adami* DS challenge is dependent on the type of DNA vaccine vector and vaccination protocol" (159) described the optimal DNA vaccine vector type and route of vaccination against *P.c.adami* DS erythrocytic stage challenge using the recently discovered MSP4/5 sequence as a model antigen. Priming the mouse immune system using the non-targeted VR1020 vector containing MSP4/5, followed by MSP4/5 recombinant protein boosting, resulted in enhanced protection against lethal *P.c.adami* DS challenge. Protein boosting alone is not as commonly used to enhance the immune system as are viral vectors. DNA priming and pox virus boosting is now being tested in humans (reviewed in 113). Boosting strategies will benefit malaria naïve individuals although DNA vaccination alone could be a useful and inexpensive way to prime the immune system of individuals in malaria endemic areas (89).

The use of the VR1020/MSP4/5 construct alone did not protect mice, nor did MSP4/5 fused to CTLA4 when delivered as DNA vaccines. It was established that the ID route of DNA vaccination using a gene-gun was optimal for the generation of a protective immune response against lethal infection in mice, using a vector containing the chemoattractant MCP-3 fused to MSP4/5. However, this protection was not associated with a reduction in parasitemia using the *P.c.adami* DS model. At present,

it is not known why vaccination with the MCP-3/MSP4/5 ID vaccine promoted survival in the absence of a reduction in parasitemia.

Chemokines are known to induce inflammatory responses in most organs, such as the brain, skin, spleen and blood vessels (125). Cells that contain receptors for MCP-3 include eosinophils, basophils, monocytes, T-lymphocytes and dendritic cells (166). To elucidate why there was no reduction in parasitemia after MCP-3/MSP4/5 vaccination, a crucial experiment would be first to ascertain which of these cell types is predominant in the spleen (using FACS analysis) after vaccination, as the spleen is a primary site of erythrocytic stage parasite clearance (reviewed in 122). It is also an organ in which MCP-3 is known to induce an inflammatory response (reviewed in 125).

Identification of specific immune cell types (and cytokines they produce) induced in the spleen after MCP-3/MSP4/5 vaccination and erythrocytic stage challenge, may be a first step to explain why no reduction in parasitemia was found. Protection after gene-gun administration of MCP-3/MSP4/5 induced a Th2 antibody response (159). MCP-3 can bind to CCR3 receptors, which is preferentially expressed on lymphocytes of the Th2 phenotype (125). It has been shown that IL-10 produced by Th2 cells, reduces inflammatory responses produced after infection of mice with *P.c.chabaudi*, such as enhancement of TNF-α levels associated with pathology (reviewed in 117). Anaemia and hypoglycaemia are major causes of pathology in mouse malaria infections, and treatment of mice with anti-TNF-α antibodies helps to reduce these features (117). Enhanced TNF-α responses can lead to severe disease and increased mortality without an increase in parasitemia, although recombinant TNF-α administered at the beginning of infection can protect susceptible stains of mice in lethal *P.c.chabaudi* infections (reviewed in 122). This may help to explain why

parasitemia levels between gene-gun vaccinated MCP-3/MSP4/5 and control mice were not significantly different. It is possible that MCP-3/MSP4/5 specific vaccination resulted in IL-10 production, thereby controlling enhanced TNF-α levels in mice after lethal infection. The levels of TNF-α and IL-10 produced after vaccination with MCP-3/MSP4/5, followed by lethal *P.c.adami* DS infection, needs to be investigated in the future. Cytokine secretion of the predominant cell type taken from the spleen also needs to be investigated, and this then correlated with survival.

The use of chemokines to target erythrocytic stage malarial antigens as DNA vaccines had not been reported prior to this work (159). In experimental cerebral *P.berghei* ANKA malaria in mice, chemokine receptors and chemokines have been implicated in rendering mice susceptible to infection (80). Recently however, a dendritic cell-specific chemokine, Dendritic Cell-Derived CC Chemokine 1 (DC-CK1), enhanced protective cell-mediated immunity against *P.yoelii* sporozoite challenge when co-delivered (as a recombinant protein) with *P.yoelii* circumsporozoite protein (in a recombinant adenovirus vector) (27). Chemokines can therefore be potentially used for both pre-erythrocytic and erythrocytic stage malaria vaccines. Based on genomic information, it has been estimated that there may be as many as 40 to 50 human chemokines (reviewed in 166), of which MCP-3 is but one. Future testing of these ligands may allow the enhancement of malaria vaccines, and improve the efficacy of DNA vaccination in general.

Concomitant with the experiments in Chapter 2, Chapter 3 entitled "Induction of specific T-cell responses, opsonising antibodies and protection against *P.chabaudi adami* DS malaria in mice vaccinated with genomic expression libraries expressed from targeted and secretory DNA vectors" (158), involved the construction and testing of genomic expression libraries. The libraries were in either VR1020

(secretory), MCP-3 (chemoattractant), or CTLA4 (lymph node targeting) vectors delivered via the ID route using a gene-gun. It had previously been established that the use of a malarial genomic library in the VR1020 vector, when vaccinating with 30,000 plasmids, could significantly reduce parasitemia after lethal *P.c.adami* DS infection (181). Reduction in parasitemia in humans infected with malaria is associated with a reduction in clinical illness (138). It was hypothesised that the efficacy of the genomic expression library would be enhanced using the MCP-3 vector, as seen when this vector was used to deliver the MSP4/5 sequence as a DNA vaccine. However, this was not the case and the MCP3-30K library vaccine did not enhance efficacy over the protection observed with the VR1020/30K library (discussed below). The VR1020/30K plasmid pool produced significantly greater T-cell responses (including IFN-$\gamma$, IL-4 and proliferative responses) in mice in response to native antigens expressed by infected erythrocytes, when compared to empty vector controls. The MCP-3/30K pool also resulted in significant enhancements to T-cell responses but to a lesser extent than the VR1020/30K pool, and T cells from mice vaccinated with the CTLA4/30K pool did not respond to infected erythrocytes.

The delivery of VR1020/MSP4/5 ID described in Chapter 2 did not afford any protection against erythrocytic stage challenge with *P.c.adami* DS, while vaccination with the VR1020/30K library results in significant protection (158, 181). Antigen dependent protection using this vector appears therefore to contribute to efficacy. However, antigen dependent protection could not be attributed to the CTLA4 vector, as protection against erythrocytic stage *P.c.adami* DS was not seen using either the MSP4/5 candidate antigen or the CTLA4/30K library. Protective efficacy using a CTLA4 construct has been demonstrated against *Corynebacterium pseudotuberculosis* after vaccination in sheep (121). CTLA4 constructs have also

been shown to significantly reduce viral titres in mice using a hemagglutinin-based influenza DNA vaccine (45), and enhanced antibody efficacy was demonstrated against *Taenia ovis* antigens in sheep (57). The results generated in this thesis clearly demonstrate that using CTLA4 DNA vaccine targeting can not be applied as a general DNA vaccine enhancement strategy to a pathogen of choice. Careful consideration must therefore be given to immunological mechanisms in the context of CTLA4 expression and function.

After malaria infection, CTLA4 is expressed on CD4$^+$ T cells of humans (172) and mice (99). The levels of CTLA4 expression on resting T cells are low, and CTLA4 appears following T-cell activation (168). After activation, CTLA4 provides an essential inhibitory function by regulating the T-cell response to maintain self-tolerance and prevent organ destruction (168). Schlotmann *et al* (172) have shown in human *P.falciparum* erythrocytic stage infections, that CTLA4 expression on CD4$^+$ T ($\alpha\beta$) cells was positively correlated with disease severity. In mice infected with *Leishmania*, CTLA4 expression has been found to suppress cellular immunity (68). However, it has been shown that mice infected with erythrocytic stage *P.berghei* display high parasitemia (<60%), severe anaemia, and a 50% reduction of CD4$^+$ T cells in the spleen, but using this model only 20% of mice die (99). Blockage of CTLA4 binding to B7 using a monoclonal antibody exacerbates the disease, resulting in cerebral malaria and death of all mice (99). It is possible that the presence of CTLA4 fusion proteins used as DNA vaccines in this thesis may have contributed to blockage by acting as a competitor for B7 (CD80) on APCs. Natural CTLA4 and B7 binding interactions between T cells and APCs results in complex cellular signalling, and direct cell-cell contact is required to regulate T-cell inhibition (reviewed in 168). The use of the CTLA4 DNA vaccines in this thesis did not allow for direct cell-cell

contact, and hence CTLA4/antigen binding to B7 on APCs may have reduced available APCs to suppress T cells after malaria infection. The amount of CTLA4/antigen fusions actually expressed by individual cells after DNA vaccination must obviously be taken into account, but determination of this would be technically difficult. Possible future experiments to quantify CTLA4 expression levels after gene-gun DNA vaccination would be to vaccinate with a green fluorescent protein/CTLA4 fusion sequence, and perform T-cell binding assays and FACS analysis using cell suspensions from the area of vaccination and lymph nodes (described in 167).

The results using CTLA4 DNA vaccines in this thesis however clearly show a rapid decline in survival after *P.c.adami* DS challenge compared to groups vaccinated with DNA vaccines not utilising CTLA4. The abrogation of optimal cell-cell contact between APCs and T cells is one hypothesis as to why disease was enhanced in CTLA4 vaccinated mice.

An important result of the experiments conducted in Chapter 3 was the demonstration that vaccination with genomic libraries could indeed induce specific immune responses: T cells that react to native *P.c.adami* DS antigen and opsonising antibodies (158). These data showed that the protection and reduction in parasitemia induced by the genomic vaccine, observed here and earlier (181), was not due to non-specific stimulation of the murine immune system by genomic 'non-coding' DNA. This data will now allow further work to be conducted in the Spithill laboratory to discover protective epitopes contained within genomic 30K libraries.

The experiments in Chapter 3 established that the delivery of multiple sequences contained in a genomic library was optimal using the non-targeted secretory vector VR1020, but not the MCP-3 or CTLA4 targeting vectors. It was found that the

libraries had similar characteristics such as average insert size, average size of encoded peptides, and the percentage of reading frames expressing peptides (158). Fusion of small peptides to the larger moieties was either detrimental (in the case of the CTLA4/30K library), or did not enhance protection (as with the MCP-3/30K library). It is possible that any protective peptides expressed in the MCP-3 and CTLA4 libraries were obscured by the larger moieties; however the immunological considerations described for the CTLA4 vector above help to explain its lack of efficacy. The lack of enhanced efficacy when using the MCP-3/30K library may have indeed been due to the larger MCP-3 moiety (approximately 100 amino acids), fused to smaller peptides (average size 32 amino acids). It has been shown that the delivery of DNA encoding separate MCP-3 and tumor (16) or HIV antigens (15) does not enhance the efficacy of DNA vaccines. The physical linkage of MCP-3 and antigen as a fusion protein is required for enhanced vaccine efficacy (15). This suggests that chemokine receptor-mediated uptake of antigen by APCs is responsible for enhanced efficacy, rather than simple recruitment of APCs to sites of infection (16). A high proportion of the peptides contained in the *P.c.adami* DS genomic libraries described in this thesis are less than 20 amino acids in length. Fusion to MCP-3 may obscure recognition of small peptide sequences after uptake into APCs, resulting in the lack of enhanced efficacy. However, fusion of genomic sequences to MCP-3 was not completely ineffective, since significant proliferation of T cells was found after exposure to IRBCs. This suggests a limiting size for effective APC processing, as a lack of protective efficacy was not observed when MCP-3 was fused to the larger MSP4/5 sequence (159). The expression of peptides from the VR1020/30K library (and potentially protective epitopes) were not obscured by any large moieties, allowing effective uptake and processing by APCs.

The simplest interpretation of the protection observed after ELI is that the genomic libraries express epitopes/polypeptides which mediate the protection and reduction in parasitemia seen in the challenge trials. The sequencing of 664 plasmids from the VR1020/30K library showed that the methods used to construct genomic libraries in this thesis results in a high proportion (71%) of sequences expressed that are less than 20 amino acids (Fig.1, Chapter 3.5). It was hypothesised that these smaller peptides were mediating protection. The eventual outcome of division of the protective plasmid pools may be the discovery of small protective epitopes which may form part of, or whole antigens, rather than the expression of large open reading frames. This is currently being performed in Professor Spithill's laboratory. The use of ELI in this thesis can be best described as a "bottom-up" approach to the discovery of protective epitopes; from the genomic level to the protein level. Epitopes contained in erythrocytic stage antigens are being investigated by others, and have the potential to be exploited as malaria vaccines in the future (205). This approach, utilising known antigens, can be described as "top-down", with the end point being the elucidation of protective epitopes. The common feature of both methods is the discovery and use of epitopes to aid in malaria vaccine design. Important epitopes derived from antigens, rather than whole antigens, may be under less immune pressure than entire antigens. These would make attractive vaccine targets in the future.

The idea of including epitopes derived from known antigens in malaria vaccine design is not a new concept. Identification of protective epitopes on pre-erythrocytic stage antigens such as the circumsporozoite protein have been investigated (79, 174, 185, 211). An example of the potential for epitopes to be exploited for erythrocytic stage vaccine design is a region contained within the variant erythrocytic stage antigen PfEMP1, that is highly conserved between *Plasmodium* strains (13).

Monoclonal antibodies directed at a functionally conserved region of PfEMP1 (the cysteine-rich interdomain region 1, mediating adhesion of infected erythrocytes to CD36), have been found to cross-react with epitopes on multiple parasite strains (65). This region of PfEMP1 contains highly conserved residues (particularly cysteine) between parasite strains that form a conserved CD36 binding domain (13). The CD36 binding domain of PfEMP1 is poorly immunogenic in natural human *P.falciparum* infections (12), and this would lead to less immune pressure and therefore possibly less variation in these epitopes, making this region a potentially effective epitope vaccine target. Multiple T-cell epitopes have also been found in $MSP1_{33}$, and peptides derived from these epitopes are able to protect mice against lethal *P.yoelii* YM erythrocytic stage challenge (205). It has also been shown that DNA vaccination of single epitopes (derived from a mutant p53 sequence) was found to induce anti-tumor immunity and protective CTL responses in mice (36). These results help to justify the use of ELI for the discovery of protective epitopes for use in the development of malaria vaccines.

The use of genomic expression libraries in this thesis, rather than cDNA libraries, can allow for the eventual discovery of protective sequences from all stages of the malaria life cycle. Successful genomic ELI was demonstrated by Piedrafita *et al* (156) using another protozoan parasite, *Leishmania major*. Subdivision of pools led to incremental improvement of the protective effect induced after *L.major* challenge in mice. These protective effects were also attributed to epitopes rather than full-length proteins using the genomic approach.

The use of cDNA ELI, rather than genomic ELI, is better suited for the discovery of full-length proteins. Using the *L.donovani* disease model, Melby *et al* (134) used a cDNA library constructed from RNA found in the amastigote stage of the parasite that

persists (and confers immunity) in *Leishmania* infection. Immunisation of mice with plasmid DNA from 15 cDNA pools (approximately 2000 cDNAs per pool) significantly reduced hepatic parasite burden. It was found that in one of the cDNA pools, a set of nine novel cDNAs and one group of five cDNAs encoding *L.donovani* histone proteins were attributed to protection. The use of direct expression library immunisation (DELI) (Chapter 3.5) however, would also be suited to the discovery of large open reading frames. The advantage of using a genomic library over a cDNA library is that no assumptions are made as to what sequences are protective, all stages of the malaria life-cycle are represented.

The DELI technique was used to attempt to discover large open reading frames contained within the VR1020/30K library, which could be potentially used in future DNA vaccine trials in Professor Spithill's laboratory. The DELI technique has been successfully used in the *Mycoplasma hyopneumoniae* disease model in pigs (140). This section of Chapter 3 described a pilot study conducted to evaluate DELI involving 1-3 kilobase sequences taken from the VR1020/30K library. After screening a random sample of 36 colonies using DELI, a large 22 kDa peptide with similarities to a *P.falciparum* and *P.yoelii* nuclear splicing protein was found. This was the largest peptide ever found in our laboratory using DNA from the VR1020/30K library. A smaller uncharacterised 3 kDa peptide was also discovered. Importantly, no false positive colonies were selected out of this preliminary evaluation of the technique when applied to a large genome. This technique has the potential to successfully discover peptides contained within genomic libraries, and future studies using DELI are necessary. The DELI DNA vectors allow the insertion of the genomic sequences contained within the VR1020/30K library. The 1-3 Kbp fragments in the VR1020/30K library can be easily excised using restriction enzymes

and ligated into the DELI vectors. This would allow the discovery of large open reading frames already within the library, while the genomic ELI approach will eventually allow the discovery of small protective epitopes. As the specificity of ELI using the VR1020/30K library is now attributed to the expression of peptides from *P.c.adami* DS genomic DNA (and not non-specific effects of non-coding DNA) (158), it is now possible to subdivide pools in an effort to discover groups of protective epitopes.

Chapter 4 was entitled "Evaluation of bicistronic DNA vaccines against *P.chabaudi adami* DS malaria". With the knowledge gained from data contained in Chapters 2 and 3, a multi-valent delivery system evaluating bicistronic vectors against lethal erythrocytic stage challenge was implemented. The protective sequence MCP-3/MSP4/5 found in Chapter 2 was inserted into the second position of a bicistronic vector. The Tissue Plasminogen Activated (TPA) secretion signal from VR1020 was inserted into the first position of the bicistronic vector, as non-targeted secretion of the VR1020/30K library had proven to be reliable in protecting and reducing parasitemia across many animal trials (181). Two bicistronic vector types were constructed: BC construct 1 contained a TPA sequence and cloning site (in Position 1) with MCP-3/MSP4/5 (in Position 2), and BC construct 2 contained an MCP-3 leader sequence and cloning site (in Position 1) with MCP-3/MSP4/5 (in Position 2). The candidate antigen AMA-1 was inserted into Position 1 of both types of bicistronic vector constructs. A genomic library was inserted into the first position of BC construct 1 (replacing AMA-1), which was designated BC 30K. This was constructed in an attempt to produce synergistic responses between epitopes/antigens contained in the genomic library and MSP4/5 which was contained in the second position of the bicistronic vector. Malarial antigens, in specific combinations, are known to enhance

immune responses to the antigens used (55, 73, 100, 197). It is the synergistic property of malarial antigens which was attempted to be exploited using bicistronic vectors.

As the bicistronic expression of malarial antigens had not been reported, an evaluation of immunogenicity *in vitro* and *in vivo* was required. Transfection of COS 7 cells *in vitro* using BC construct 1 (containing AMA-1 and MCP-3/MSP4/5) resulted in antigen expression *in vitro*, as well as IgG responses, and T-cell responses to both antigens. BC 30K also produced IgG responses to MSP4/5 recombinant protein, and T-cell responses to antigen shed by infected erythrocytes and MSP4/5. Survival of animals after lethal *P.c.adami* DS challenge was variable using bicistronic vectors; however, a significant reduction in peak parasitemia was found for BC30K and BC construct 1. Bicistronic Trial 2 in particular resulted in the most obvious reductions in peak parasitemia. The first bicistronic trial did result in significant reductions in peak parasitemia, but the number of mice surviving at the peak reduced the power of this analysis. DNA vaccination using MCP-3/MSP4/5 (159) and VR1020/AMA-1 monocistronic vectors followed by *P.c.adami* DS challenge does not result in a reduction in parasitemia in challenge trials conducted in our laboratory. Further bicistronic trials must be conducted with larger sample sizes, and a less virulent parasite strain (described below) to confirm this promising preliminary data. To definitively determine whether bicistronic DNA delivery is better than monocistronic delivery of malarial DNA vaccines, delivery of single antigens (AMA-1 and MCP-3/MSP4/5) in the bicistronic vector backbone will be required.

These results suggest that bicistronic delivery of malarial antigens may be an efficient method for delivering multiple antigens required to protect against the complex life-cycle of malaria. Enhanced efficacy is seen in hepatitis DNA vaccine

models using bicistronic compared to monocistronic vectors (34, 35, 120). When applied to malaria, given that combinations of malarial antigens can enhance the immunogenicity of antigens over either antigen alone, the development of bicistronic DNA priming may be an efficient way to induce synergistic immune responses to multiple malarial antigens.

Further studies using a less virulent strain of malaria would be of benefit. During malaria vaccine trials conducted in our laboratory, many animals in vaccinated groups would die before a peak parasitemia was reached after erythrocytic stage challenge with *P.c.adami* DS. Using a less virulent strain of parasite, such as *P.c.adami* DK (isolate 556 KA) would allow the study of immune mechanisms and pathogenesis without premature death (117). This will allow a better assessment of the differences in parasitemia between vaccinated and control animals. It will also be of particular use for future investigation into bicistronic vaccination. Although reductions in peak parasitemia appeared to be obtained in bicistronic Trial 1, the deaths of animals in both control and vaccinated groups reduced the power of statistical tests. The use of a less virulent malaria strain would remove the variable of survival and allow the parameter of parasitemia to be focussed upon, one of the indicators of vaccine efficacy in human malaria vaccine trials (112, 133).

DNA vaccines on their own may be adequate to enhance the immune responses of individuals already exposed and at risk of malaria in endemic regions. A model of malaria vaccine development by Doolan and Hoffman (89) proposes two vaccine types: 1) a vaccine to prevent all clinical manifestations (for malaria naïve individuals); and 2) a vaccine to reduce mortality and severe disease.

Results from the first human trial for the first type of vaccine have recently been published. The trial involved priming with plasmid DNA encoding the pre-

erythrocytic antigen thrombospondin-related adhesion protein (TRAP) linked to a codon optimised string of epitopes (derived from pre-erythrocytic stage antigens covering multiple HLA types) and boosting with recombinant vaccinia virus (133). Significant delays in parasitemia were found after challenge with *P. falciparum* sporozoites as well as enhanced T-cell responses. Human T-cell responses were found against multiple epitopes contained within the 20 selected sequences, indicating that they were successfully processed and presented after DNA vaccination. New epitopes discovered using genomic ELI could one day be included in an erythrocytic stage epitope string.

It is the second vaccine type that DNA vaccines alone would be most suited to. In mice infected with *Mycobacterium tuberculosis*, it has been shown that a DNA vaccine initially designed to prevent infection, can switch the immune response from Th2 to Th1, and kill the bacteria (124). DNA vaccination of humans with previous exposure to malaria would focus the immune system on important antigens or epitopes to reduce parasite burden and morbidity.

This thesis shows that careful consideration must be given to the type of DNA vaccine vector used. The generation of high antibody responses did not correlate with protection seen in mice, particularly when using the CTLA4 vector. The use of the MCP-3/MSP4/5 DNA vaccine did not reduce parasitemia, and if such a construct was used on its own in a human setting in malaria endemic regions, would presumably do nothing to reduce the incidence of morbidity associated with severe disease. Delivery of the MCP-3/MSP4/5 fusion antigen when combined with protein expression from genomic sequences, or delivered with AMA-1 in a bicistronic vector, however, does have an effect on parasitemia in mice. The eventual discovery and inclusion of new

epitopes from genomic ELI will add to the immunogenicity of these vaccines. This preliminary data needs to be further explored.

Experts in the field of malaria research agree that a malaria vaccine is a minimum of 15 years away (Dr Michael Good, personal communication; Dr Denise Doolan, personal communication). A greater understanding of the host immune interactions with malaria, and using this information, an optimisation of DNA vaccine technology against malaria will be required to produce a vaccine capable of protecting genetically diverse human populations in this timeframe.

# Appendix I

The protective efficacy of MSP4/5 against lethal *P.chabaudi adami* challenge is dependent on the type of DNA vaccine vector and vaccination protocol

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# The protective efficacy of MSP4/5 against lethal *Plasmodium chabaudi adami* challenge is dependent on the type of DNA vaccine vector and vaccination protocol

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## Abstract

The enhancement of immunogenicity of malarial DNA vaccines is important if they are to have practical application in protecting against blood-stage malaria. Here we describe three different DNA vaccine vector types used in conjunction with the blood-stage merozoite surface protein 4/5 (MSP4/5), the murine homologue of *Plasmodium falciparum* MSP4 and MSP5, in an attempt to enhance survival against lethal *Plasmodium chabaudi adami* DS blood-stage challenge. MSP4/5 was inserted into VR1020 (secretory), monocyte-chemotactic protein-3 (MCP-3) (chemoattractant), and cytotoxic T-lymphocyte antigen 4 (CTLA4) (lymph node targeting) vectors. Mice were immunized intradermally via gene-gun, IM injection, or boosting with recombinant MSP4/5 protein. Antibody responses after boosting were predominantly of the IgG1 and IgE isotypes, with low avidity antibodies produced in DNA primed groups. Despite antibody responses comparable to recombinant protein immunization, boosting mice primed with antigens encoded by MCP-3 and CTLA4 vectors did not enhance survival compared to vector control groups. Gene-gun vaccination using VR1020/MSP4/5 followed by recombinant MSP4/5 boosting, or gene-gun DNA vaccination alone using MCP-3/MSP4/5, resulted in enhanced survival compared to empty vector control mice. The results suggest that the enhancement of survival against lethal blood-stage malaria challenge after utilizing MSP4/5 DNA vaccination is therefore highly dependent on the route and type of vaccine vector employed.
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*Keywords:* DNA vaccine; Prime boost; Gene-gun; Malaria; MSP4/5; MCP-3

## 1. Introduction

Malaria infection is one of the major causes of disease and death in the developing world and is an important barrier to economic progress in endemic countries. Measures to contain malaria such as vector control by insecticides or drug treatment of active infection have become decreasingly effective and new methods such as vaccines against the various malaria life cycle stages are needed. Protection in humans against asexual blood stages of malaria is believed to include mechanisms such as antibodies that block merozoite entry and inhibit parasite development within erythrocytes (reviewed in [1]).

A number of vaccine delivery methods are being studied including that of DNA immunization. Improvements in the immunogenicity of malarial antigens delivered by DNA vaccines are important if there is to be any possible practical application of this technology to provide protection against blood-stage malaria. It has been demonstrated that immunization strategies using malarial antigen through priming with DNA plasmids followed by boosting with recombinant protein or viral constructs, can result in enhanced immunogenicity against malaria when compared with DNA vaccination alone (reviewed in [1]). Enhancement of antibody titre in malarial prime/boost (p/b) studies has relied heavily on administration of recombinant poxvirus expressing

malarial antigen, and such studies have also focussed on inducing CD8 T-cell immunogenicity against liver-stage malaria ([2], reviewed in [3]). In order to help resolve blood-stage malarial infection however, antibody and the activation of CD4 T-cells has been found to be required when vaccinating with recombinant candidate blood-stage antigens such as apical membrane antigen-1 (AMA-1) [4,5] and merozoite surface protein-$1_{19}$ (MSP$1_{19}$) [6,7]. Enhanced humoral responses to Pf155/RESA after priming with a DNA vaccine, and boosting with recombinant protein in adjuvant has been demonstrated in mice [8]. Protection of *Aotus* monkeys against *Plasmodium falciparum* after DNA priming using the blood-stage antigen EBA-175, followed by protein boosting in adjuvant has been observed [9]. Priming *Rhesus* monkeys with *P. falciparum* MSP$1_{42}$ DNA vaccine and boosting with recombinant MSP$1_{19}$ has also been shown to enhance antibody titres [10].

It has been suggested that on their own, first-generation DNA vaccines may not be adequate to protect against malaria (reviewed in [11]). Antigen presenting cells (APCs), in particular dendritic cells, have been shown to be potent initiators of immune responses following DNA vaccination, and are important in the uptake of antigen expressed from cells transfected by a DNA vaccine (reviewed in [12]). Improvement of DNA vaccine efficacy by targeting antigen to APCs may be required if DNA priming alone is to be sufficient to provide a significant level of protection against blood-stage malaria. The use of the targeting ligand cytotoxic T lymphocyte antigen 4 (CTLA4) in DNA vaccination has been shown to improve the magnitude and speed of the antibody response [13–15]. Chemokines such as monocyte chemotactic protein-3 (MCP-3) have also been shown to enhance protective efficacy after DNA vaccination in a tumor challenge model [16].

The mouse homologue of *P. falciparum* MSP-4 and MSP-5 was first identified in 1999 from *Plasmodium chabaudi* [17]. Since then, MSP4/5 mouse homologues have also been identified for *Plasmodium berghei* and *Plasmodium yoelii* [18]. Vaccination of mice using recombinant MSP4/5 protein of *P. yoelii* has been shown to protect mice from lethal challenge, with the greatest protection observed in mice producing the highest antibody levels prior to challenge [19]. To date, protection studies using MSP4/5 in the *P. chabaudi* model system have not been reported and there is no information about the efficacy of DNA immunization with this sequence either alone or in combination with protein boosting in any challenge system.

The present study investigates the potential of MSP4/5 DNA vaccination to protect mice against blood-stage malarial challenge, and compares the efficacy of MSP4/5 vaccines when delivered by different vectors and protocols. The use of three different DNA vaccine constructs [VR1020/MSP4/5 (secretory vector), CTLA4/MSP4/5 (lymph node targeting), and MCP-3/MSP4/5 (chemoattractant)] was used to prime mice via IM or ID (gene-gun) routes. Antibody responses were assessed in terms of titre, isotype, and avidity. Recombinant MSP4/5 protein was also used to boost antibody responses previously primed via different DNA vectors containing MSP4/5. The protective efficacy of vaccine combinations was evaluated against lethal blood-stage challenge using the *P. chabaudi adami* DS-BALB/c mouse model which is a stringent model for evaluating vaccine efficacy.

## 2. Materials and methods

### 2.1. Creation of plasmids

The *P. c. adami* DS MSP4/5 sequence (lacking the predicted hydrophobic signal and GPI anchor sequences) was amplified by PCR from the plasmid pTrcHis-A/MSP4/5 (described in [17]) using the oligonucleotides 5′-GGAGGCACGCGTATGAAGATCGCAAATTAT (containing an *Mlu*I restriction site) and 5′-CTCTAGAGATT-AATATAATAATGCTATTAT (containing an *Xba*I restriction site). The CTLA4 vector [13] was prepared by digestion with *Mlu*I and *Xba*I and the PCR amplified MSP4/5 fragment was inserted to produce CTLA4/MSP4/5. Again, the MSP4/5 sequence was amplified from the pTrcHis-A/MSP4/5 plasmid using the oligonucleotides 5′-ATGATGGGATC-CATGAAGATCGCAAAT (containing a *Bam*HI site) and 5′-GAAGTAGATCTTTATGAATCTGCACTGAG (containing a *Bgl*II site). VICAL vector VR1020 [20] was prepared by digestion with *Bam*HI and *Bgl*II and the amplified MSP4/5 sequence inserted to produce VR1020/MSP4/5.

Murine MCP-3 cDNA was supplied by Dr. Harshall Nandurkar (Monash University). The oligonucleotides 5′-TATTATTAGCGGCCGCATGAGGATCTCTGCCACGCTT (containing a *Not*I site) and 5′-TATGGATCCTCCACCT-CCACCTCCAGGCTTTGGAGTTGGGGT (containing sequence encoding 6xGly aa followed by a *Bam*HI site) were used to amplify MCP-3 by PCR. The MCP-3 PCR fragment was digested with *Not*I and *Bam*HI, and inserted into the VR1012 vector (VICAL, San Diego, CA, USA) digested with the same enzymes to produce the MCP-3 vector (A. Cody and P. Smooker, unpublished). MSP4/5 was amplified by PCR from pTrcHis-A/MSP4/5 using the oligonucleotide 5′-ATGATGGGATCCATGAAGATCGCAAAT (containing a *Bam*HI site) and 5′-CTACTGGATCCTTATGAATCTGC-ACTGAG (containing a *Bam*HI site), then inserted into the *Bam*HI site of the completed MCP-3 vector.

Ligation reactions were transformed into *Escherichia coli* DH5α and selected on solid media containing 50 µg/ml kanamycin for VR1020/MSP4/5 and MCP-3/MSP4/5 vectors, and 100 µg/ml ampicillin for the CTLA4 vector.

### 2.2. Expression and purification of recombinant proteins

Expression and purification of MSP4/5 ectodomain recombinant protein was performed as described in [17]. Briefly, the pTrcHis-A/MSP4/5 vector was transfected into

*E. coli* BL21 (DE3) (Novagen, Milwaukee, WI, USA) for expression of recombinant protein. Large scale purification of the recombinant protein was performed using TALON metal affinity resin (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

### 2.3. Mammalian cell transfection with MSP4/5 DNA plasmids

Plasmid constructs were tested for expression in COS7 cells prior to use in animals. Freshly grown COS7 cells were seeded at $2 \times 10^5$ cells per 35 mm tissue culture dish. Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. COS7 cells were then incubated in 5% $CO_2$ until 80% confluent. One microgram of plasmid DNA was used to transfect COS7 cells using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Media was changed 24 h after transfection. After incubation for a further 2 days at 37 °C the cells were washed with PBS, and media was replaced with serum free media to remove FCS that may have interfered protein detection, and cells were grown for a further 24 h. The supernatant was then collected and subjected to SDS-PAGE and Western blotting.

### 2.4. SDS-PAGE and western blotting

Protein and COS7 supernatants were fractionated by SDS-PAGE on 12% (v/v) polyacrylamide gels under reducing conditions and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked in 5% milk powder overnight at 4 °C. The membranes were probed using an α-MSP4/5 rabbit antibody, followed by an α-rabbit Ig conjugated to horseradish peroxidase (HRP) (Silenus Laboratories, Melbourne, Australia). The reactive antibodies were then visualized by Enhanced Chemiluminescence (Amersham, Piscataway, NJ, USA).

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Antibody reactivity with recombinant MSP4/5 protein was measured by ELISA. Microtitre plates were coated with 0.1 ml/well of recombinant MSP4/5 (1 μg/ml) overnight at 4 °C using carbonate-bicarbonate buffer pH 9.6. Plates were washed with PBS and 0.05% TWEEN 20 (Sigma, St. Louis, MO, USA) (PBS-T), followed by blocking overnight at 4 °C in 5% skim milk powder and PBS-T. Plates were again washed and diluted sera incubated at 37 °C for 2 h. After washing plates again using PBS-T, total humoral responses were obtained using HRP-conjugated sheep α-mouse Ig (Silenus) diluted 1:2000 and incubated for 1 h, followed by washing and addition of substrate. For detection of antibody isotypes, non-conjugated sheep α-mouse IgG1, IgG2a, IgG2b, IgG3 and IgE (The Binding Site, Birmingham, UK) was diluted to 1:1000 and incubated for 1 h at 37 °C. This

was followed by washing and addition of HRP-conjugated donkey α-sheep Ig (Silenus) diluted to 1:1000 and incubated for 1 h at 37 °C. After final washing, the ELISA was developed by addition of substrate 3,3′,5,5′-tetramethylbenzidine (Sigma). Plates were read at 450nm and titres defined as the highest dilution required to give an absorbance of 0.2.

### 2.6. Estimation of antibody avidity

The avidity of the antibody response was performed as described [21], with the following modifications. Briefly, MSP4/5 was used to coat microtitre plates and blocked as described for ELISA. Sera were diluted in the following concentrations of guanidine–HCl: 0, 1, 2, 3, 3.5, 4, 4.25, 4.5, and 5 M. Sera were then incubated on the plates for 1 h at 37 °C. After extensive washing, total humoral responses were obtained using HRP-conjugated sheep α-mouse Ig (Silenus) and developed as described for ELISA. The antibody titre was described as the dilution for sera that gave, on the linear portion of the dilution curve, an OD of 50% of the maximum OD observed. Titres were then normalized for comparison by setting the titre observed in the absence of guanidine at 100%. Sera from individual mice were analyzed separately and data represented as the mean titre for each group.

### 2.7. Isolation of plasmid DNA and construction of vaccination cartridges

Plasmid preparation and endotoxin removal was performed as described by Boyle et al. (1998). Briefly, DNA was purified from a cleared lysate by PEG8000 precipitation and endotoxin was removed by three extractions with Triton-X114. Purified DNA was precipitated onto gold microcarriers and these attached to plastic supports as per manufacturer's recommendations (Bio-Rad Laboratories, Hercules, CA, USA). DNA was combined with carriers at a ratio of 100 μg DNA/50 mg carriers. Each cartridge contains approximately 1 μg DNA.

### 2.8. Mice and vaccinations

All mice were BALB/c, female, and 5–6 weeks of age at the time of first vaccination.

Groups of mice were vaccinated with MSP4/5 protein according to [19], with the following modifications. The MSP4/5 protein group mice were initially vaccinated intraperitoneally (IP) with 25 μg of MSP4/5 protein in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA), followed by two booster immunizations IP of 25 μg of MSP4/5 protein in incomplete adjuvant at 2-week intervals. The Freund's adjuvant control group received the same protocol minus MSP4/5 protein. DNA vaccinated mice received three immunizations at 2-week intervals. For intradermal (ID) DNA vaccination the abdominal region was shaven and particles containing 1μg of DNA were delivered by the Helios gene-gun (Bio-Rad Laboratories,

USA) with a pulse of helium gas at 400 psi. Intramuscular (IM) DNA plasmids were delivered into the *tibialis anterior* muscle (100 μg total) in PBS. All prime/boost mice (including vector controls) initially received three immunisations with plasmid DNA at 2-week intervals (via ID or IM routes), and were then boosted with a single dose of 10 μg of MSP4/5 IP in incomplete Freund's adjuvant 2 weeks after the final DNA vaccination.

## 2.9. Infection of mice, blood sampling and parasitemia measurements

Blood from an infected mouse with a known parasitemia (1–10%) was taken and immediately diluted in PBS to give the required dosage ($1 \times 10^5$ infected RBC per dose). Mice were infected by intraperitoneal injection at day 0, and parasitemia assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels and day to peak parasitemia were compared using the Mann–Whitney non-parametric *t*-test.

## 2.10. Analysis of survival curves

Survival curves for vaccinated and control mice were compared using the Mantel–Haenszel test. Statistical analysis was performed using Prism 3.02 software (GraphPad, San Diego, CA, USA).

## 3. Results

### 3.1. Expression of protein encoded by DNA vaccine plasmids in vitro

The ability of mammalian cells to secrete MSP4/5 after transfection with DNA vaccine plasmids was tested in COS cells. Proteins encoded by three constructs were secreted into the culture supernatant in vitro (Fig. 1). CTLA4/MSP4/5 (≈95 kDa) and MCP-3/MSP4/5 (≈50 kDa) fusion proteins, as well as MSP4/5 (≈36 kDa) (secreted by virtue of the TPA signal sequence contained in VR1020), were detected in the cell free supernatant after being probed with anti-MSP4/5 rabbit sera by Western blot. Supernatants collected from COS cells after transfection with DNA vaccine vectors not containing MSP4/5 inserts did not react with anti-MSP4/5 rabbit sera (data not shown).

### 3.2. IgG responses in mice vaccinated with MSP4/5 DNA vaccine constructs

The plasmids VR1020/MSP4/5, CTLA4/MSP4/5, and MCP-3/MSP4/5 were used to vaccinate groups of four mice either IM by injection or ID using the gene-gun. A group of mice vaccinated with *E. coli* derived MSP4/5 *P.*
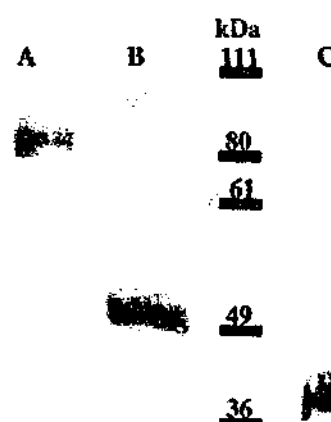


Fig. 1. Western blot of supernatants taken from COS7 cells transfected with plasmid vectors containing MSP4/5 inserts. The Western blot was probed with α MSP4/5 rabbit sera. Fusion proteins expressed by each construct are secreted into the culture supernatant (A) CTLA4/MSP4/5. (B) MCP-3/MSP4/5, and (C) VR1020/MSP4/5. Control vectors (CTLA4, MCP-3, and VR1020) not containing MSP4/5 inserts did not react with α MSP4/5 rabbit sera (not shown). Molecular mass standards are shown.

*chabaudi adami* DS protein formulation in Freund's adjuvant was included as a positive control. The resulting antibody responses were measured by ELISA using recombinant MSP4/5 protein. Fig. 2 shows the titre of IgG antibodies produced by each group over a 15 week period. Immunization using MSP4/5 protein or the CTLA4/MSP4/5 constructs, regardless of route, induced a high antibody response 2 weeks after the initial dose which peaked by week 4. The VR1020/MSP4/5 and MCP-3/MSP4/5 ID immunized constructs induced antibodies at a slower rate, with titres peaking at week 9 after the initial dose (Fig. 2). However, a slower and poorer induction of IgG was observed when both of these constructs were delivered IM relative to their ID counterparts (Fig. 2). Immunization using the three empty DNA vaccine vectors did not produce a detectable antibody response by ELISA to MSP4/5 protein (data not shown).

### 3.3. IgG responses of mice receiving the MSP4/5 prime/boost vaccine protocol

After receiving three priming DNA vaccinations, four mice in each group received a boosting immunization with 10 μg of MSP4/5 protein in incomplete Freund's adjuvant IP at week 13. Fig. 3 shows a dramatic increase in IgG antibody titre detected at week 15 in each vaccine group, 2 weeks after MSP4/5 protein boosting. A single dose of 10 μg of MSP4/5 increased IgG titres to 1/64,000 in control mice primed with either CTLA4, MCP-3, or VR1020 negative control vectors. Boosting the VR1020/MSP4/5 IM or MCP-3/MSP4/5 IM mice achieved a similar titre of IgG. However, boosting with MSP4/5 protein after priming with VR1020/MSP4/5 ID resulted in an antibody response
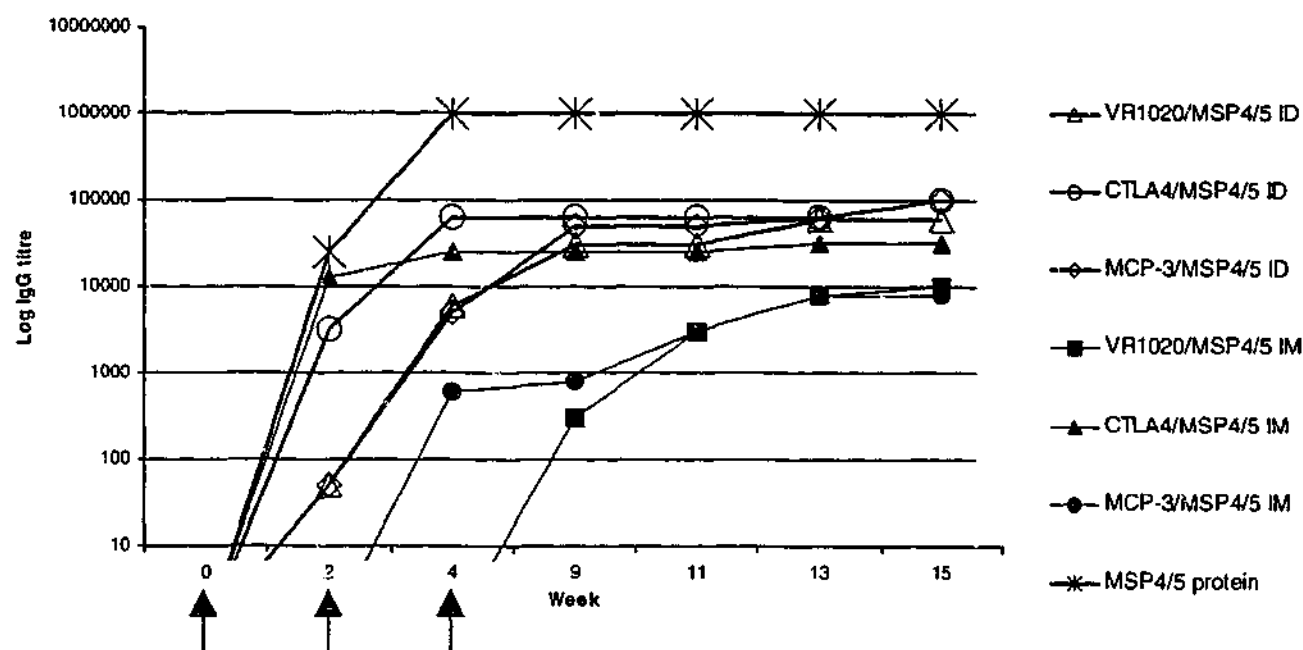
Fig. 2. IgG responses in mice primed with MSP4/5 DNA or protein vaccines. Pooled mouse IgG antibody responses were measured by ELISA. Vaccines were delivered using either 1 μg of DNA ID (gene-gun), or 100 μg of DNA IM using vectors containing an MSP4/5 insert. MSP4/5 protein (25 μg) was administered IP into control animals according to the protocol described in the methods. Arrows indicate a vaccination point. Vaccination with control vectors CTLA4, MCP-3, and MSP4/5 did not induce an antibody response (not shown).
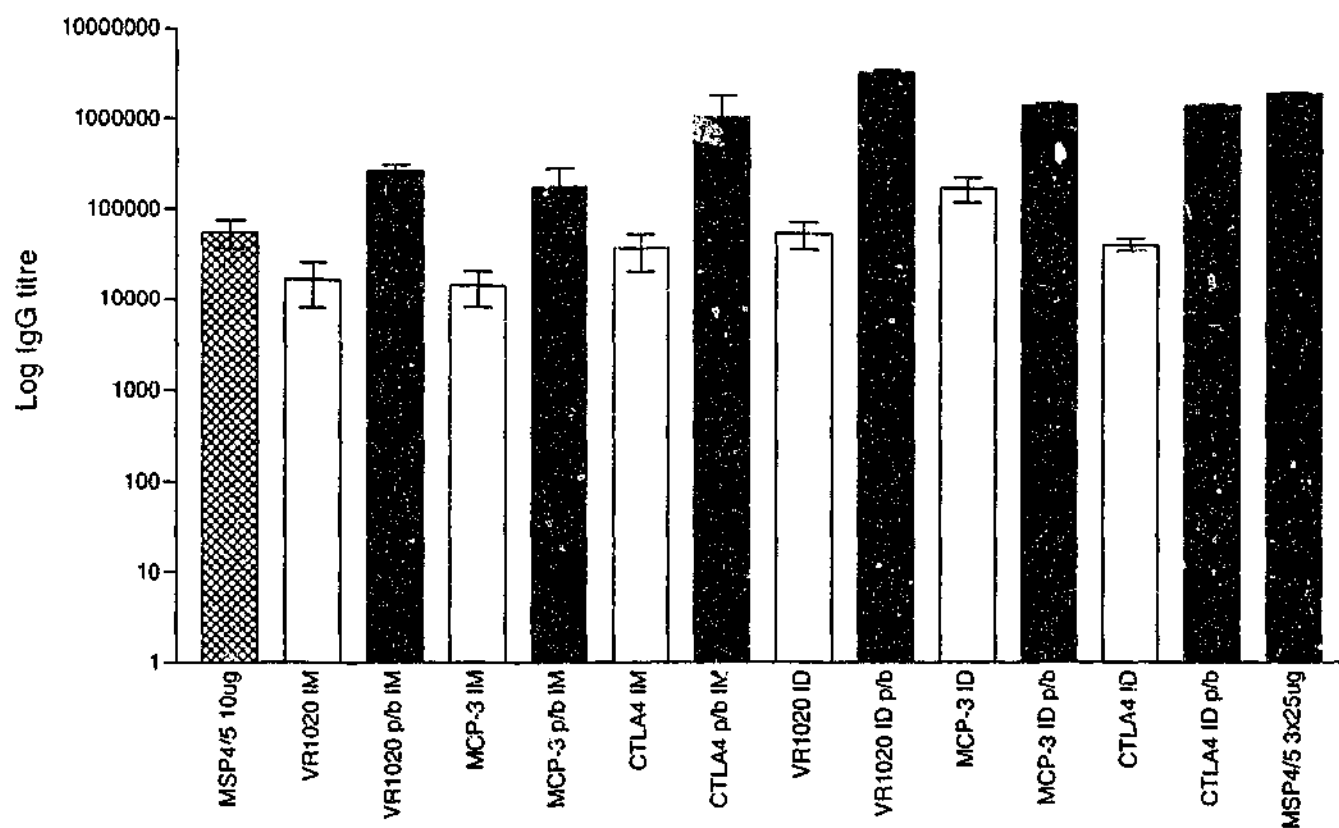


Fig. 3. IgG responses in mice receiving a prime/boost vaccine protocol. Mouse IgG antibody titres in response to MSP4/5 vaccination in each vector are shown at week 13 after three priming doses of plasmid DNA (empty bars), or at week 15 following boosting with 10 μg of MSP4/5 IP in incomplete Freund's adjuvant (shaded bars). Empty vector primed control groups (hatched bar) received 10 μg of MSP4/5 protein in incomplete Freund's adjuvant. The MSP4/5 protein control group was vaccinated with three doses of protein according to the standard MSP4/5 protein protocol described in methods (black bar). Standard error is shown.

90 times greater than ID priming alone. Boosting of mice vaccinated with CTLA4/MSP4/5 and MCP-3/MSP4/5 constructs, regardless of vaccination route also resulted in a marked increase of IgG antibodies, which approached the levels observed in the MSP4/5 protein vaccinated control group.

### 3.4. Isotype analysis of pooled Ig responses in boosted mice

Since the isotype of the humoral response may be important in determining the efficacy of malaria vaccines by promoting appropriate ADCI/ADCC reactions (reviewed in [22]), we analysed the isotypes of Ig responses in mice vaccinated using the different protocols and boosted with MSP4/5 protein. Analysis of isotype responses after boosting mice revealed a dominance of IgG1 and IgE isotypes in all vaccine groups. The level of IgG1 and IgE responses are comparable to the total IgG observed (Fig. 3). A Th-2-type immune response regardless of IM or ID gene-gun vaccination, is evidenced by the ratio of IgG1/IgG2a responses, and the level of the IgE responses to the vaccine (Fig. 4). Mice primed only with plasmid DNA encoding MSP4/5 revealed a Th-2 like response, with levels of IgG1 and IgE antibodies present and very low levels of IgG2a, IgG2b and IgG3 (data not shown). Boosting DNA primed mice with MSP4/5
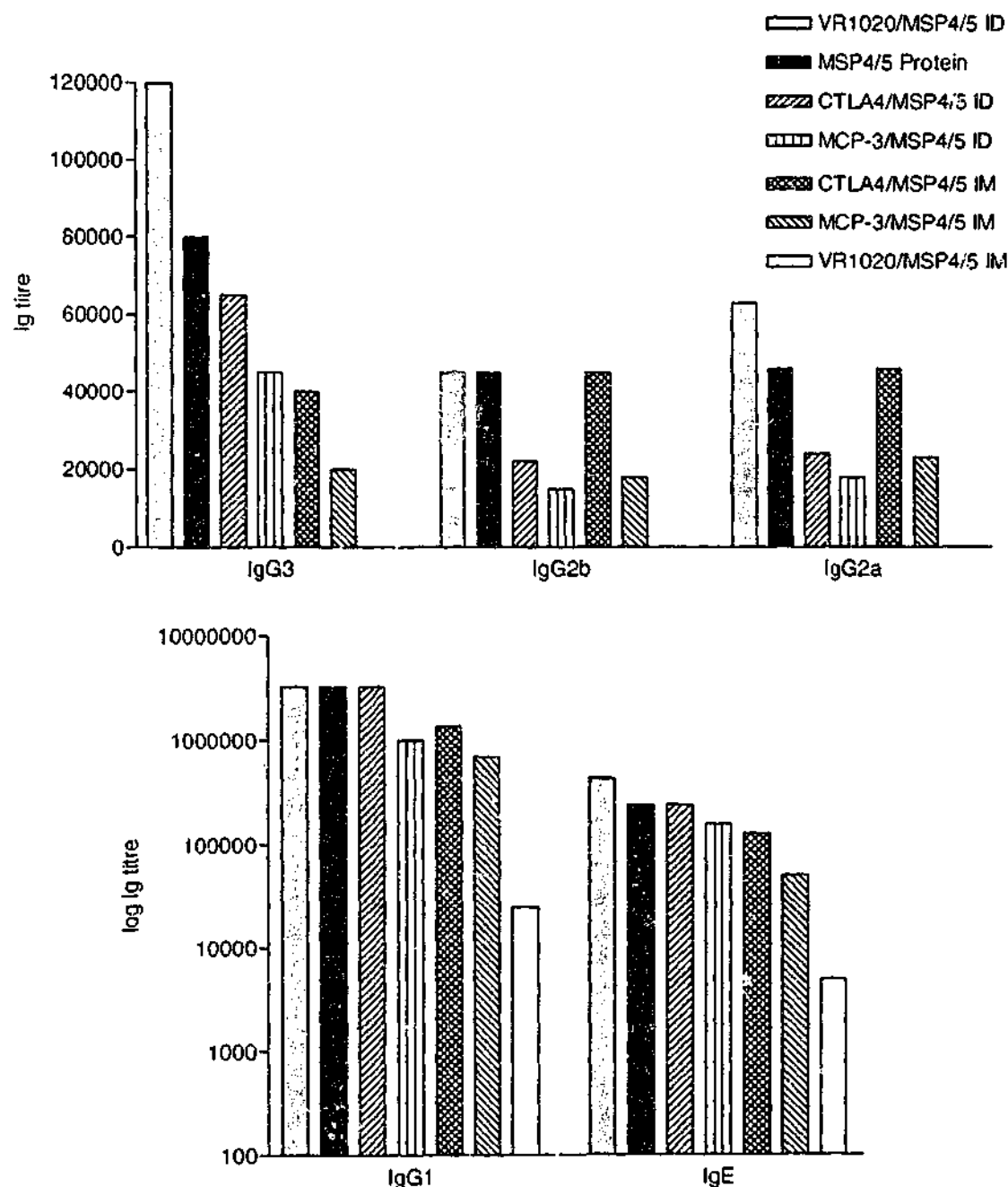


Fig. 4. ELISA analysis of Ig responses at week 15 in pooled sera from MSP4/5 boosted mice. Mice were vaccinated using protein alone, by gene-gun ID, or by IM injection using 100 μg of plasmid DNA. All DNA primed groups were boosted at week 13 with 10 μg of MSP4/5 in IFA IP. Sera were obtained at week 15 and pooled for analysis.
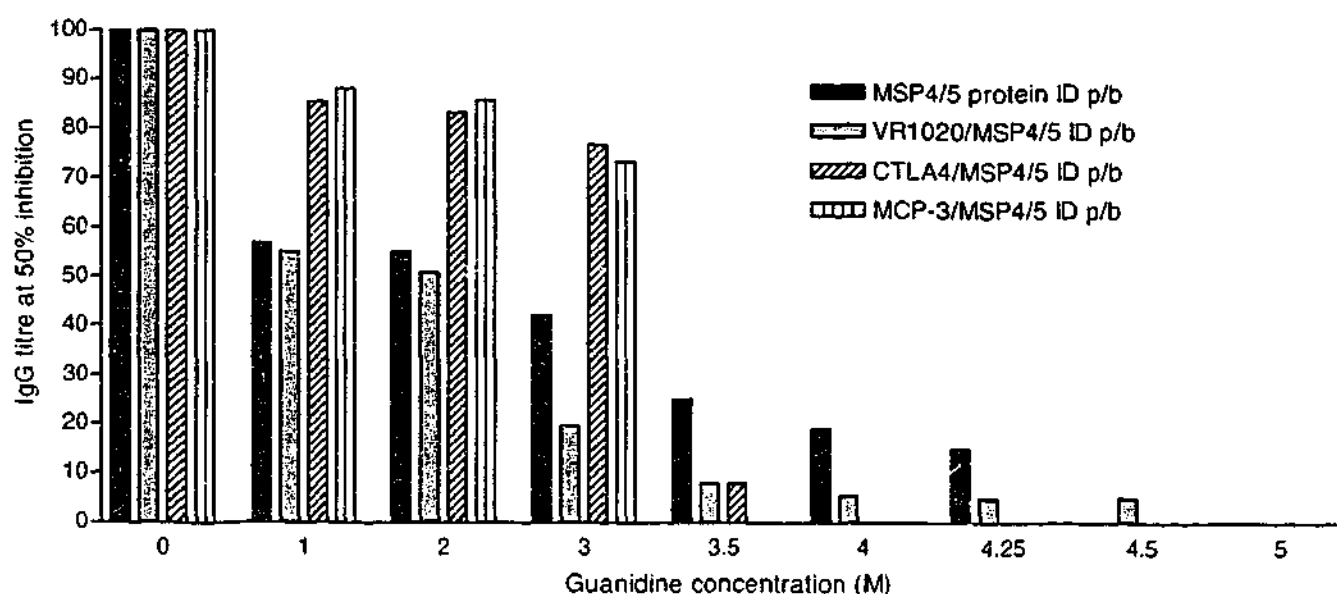
Fig. 5. Avidity of the antibody response to MSP4/5 in vaccinated mice. ELISAs were performed on sera over a wide range of dilutions (1/1000-1/10⁶) using sera from MSP4/5 protein vaccinated mice or mice given an ID p/b vaccination. Duplicate ELISAs using different concentrations of guanidine–HCl (0-5M) were performed over the same dilution range. A logarithmic trend line was calculated for the dilution curve. The intersection of the trend line which corresponded to a 50% reduction in OD (in the linear portion of the dilution curve) was used to determine antibody titre. The titre observed in the absence of guanidine–HCl was normalised to 100%. The titres observed in the presence of differing concentrations of guanidine–HCl were compared to the untreated titre and expressed as a percentage of this figure.

protein did however enhance IgG2a, IgG2b, and IgG3 antibody subclasses (Fig. 4).

## 3.5. Avidity of IgG immune response in MSP4/5 DNA and protein vaccinated mice

The avidity of the antibody response is an important determinant of the antibody-mediated effector response [22,23]. The average affinity of the antibodies generated by DNA prime/boost (p/b) or protein immunization was determined by incubation with varying concentrations of the chaotropic agent guanidine–HCl. To determine avidity of the antibody response, the concentration of guanidine–HCl required to disrupt MSP4/5 antibody interactions by 50% was calculated. For each guanidine–HCl concentration the antibody titre at the midpoint of the linear portion of the ELISA curve was estimated [21]. The antibody titre observed in the absence of guanidine–HCl was normalised to 100%, and the corresponding groups were compared to this figure at differing concentrations of guanidine–HCl. Fig. 5 shows that a higher guanidine–HCl concentration (>4 M) was required to completely disrupt MSP4/5 IgG interactions in the group vaccinated with MSP4/5 protein, relative to the groups primed ID with MSP4/5 DNA in all three vector types and boosted with recombinant protein. In the concentration range of 1–3 M guanidine–HCl, the sera for the CTLA4/MSP4/5 and MCP-3/MSP4/5 vaccines showed a higher avidity than MSP4/5 protein or VR1020/MSP4/5 groups. Above 3M guanidine–HCl this difference was lost, suggesting that there may be two populations of IgG present in sera for the CTLA4/MSP4/5 and MCP-3/MSP4/5 groups.

## 3.6. Vaccine efficacy

### 3.6.1. MSP4/5 P. chabaudi adami DS Pilot study

The P. chabaudi adami DS mouse model is a stringent test for vaccine efficacy due to the high virulence of this DS strain [24,25]. In order to determine the protective efficacy of P. chabaudi adami DS MSP4/5 against a virulent challenge, a pilot study containing eight BALB/c mice per group was conducted. The study involved a Freund's adjuvant control group and an MSP4/5 protein group. Mice received an initial IP vaccination of either CFA (control) or 25 μg of MSP4/5 in CFA, and two subsequent vaccinations at 2-week intervals with either IFA (control) or 25 μg of MSP4/5 protein in IFA. Mice were then challenged with 100,000 IRBC 2 weeks after the final vaccination. A significant difference between Freund's control mice and MSP4/5 vaccinated mice was found, with 75% survival in the vaccinated mice ($P = 0.015$; Fig. 6, panel i), comparable to the protection found with P. yoelii MSP4/5 protein vaccination [19].

### 3.6.2. Challenge trial 1

The first challenge trial initially contained eight mice per group. Mice were vaccinated IM (100 μg) by injection or ID (1 μg) using the gene-gun. Mice were primed a total of three times at 2-week intervals for both IM and ID groups (Table 1). The control groups consisted of mice vaccinated with recombinant MSP4/5 in Freund's adjuvant and a group vaccinated with Freund's adjuvant alone (Table 1, groups 13 and 14, respectively). Protein control mice were injected IP with 25 μg of MSP4/5 protein three times at 2-week
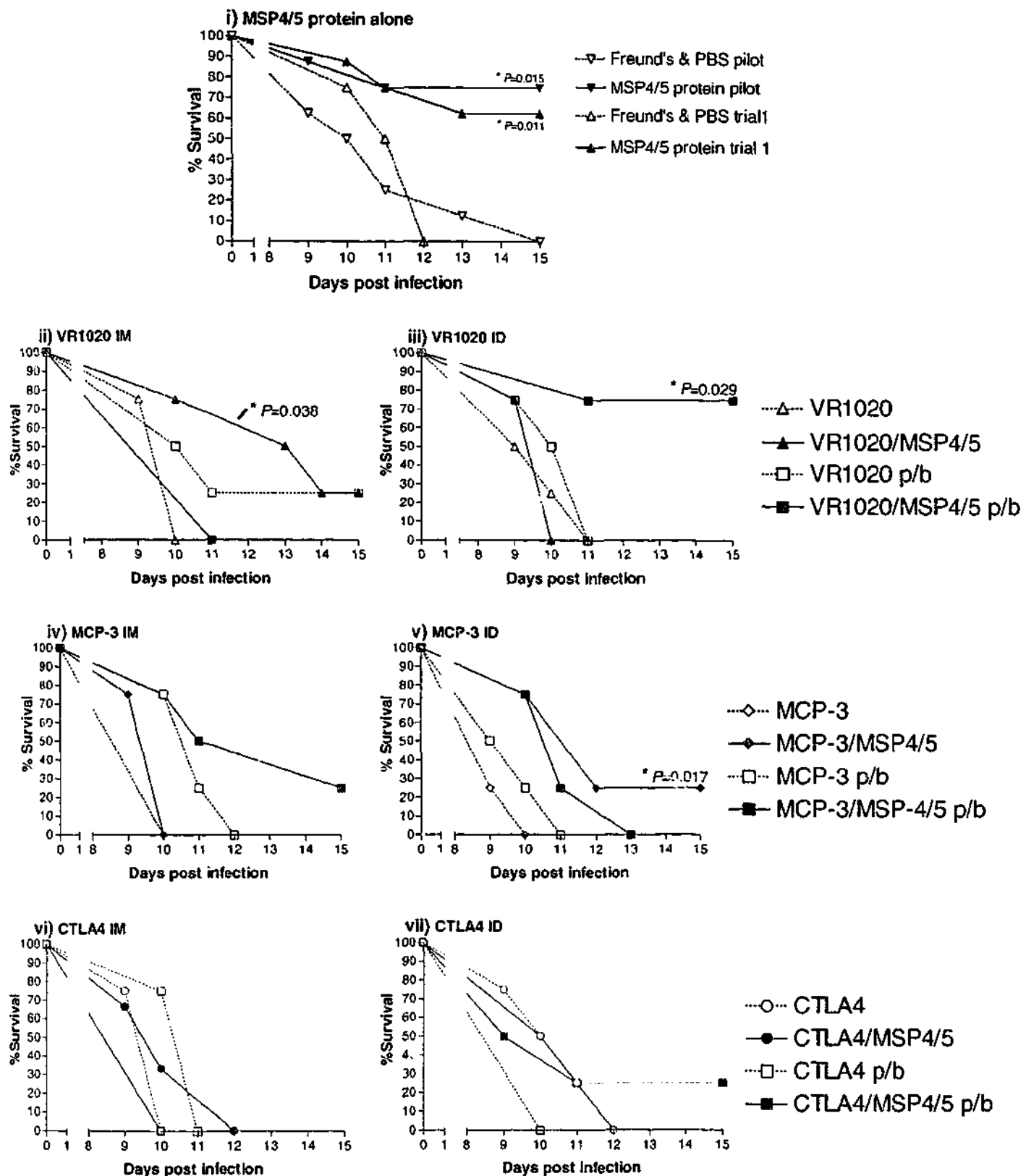
Fig. 6. Survival curves of mice vaccinated using IM or ID protocols and challenged with *P. chabaudi adami* DS. Panels ii–vii depict trial 1 groups vaccinated IM by injection with 100 μg of DNA, or ID using the gene-gun. Groups were split in half at week 13, with one group receiving a 10 μg of MSP4/5 protein boost in IFA IP. MSP4/5 protein control groups (presented for clarity in panel i) initially received 25 μg of MSP4/5 in CFA IP, followed by two extra doses of 25 μg of MSP4/5 in IFA at 2-week intervals. Control groups in all panels are represented by a dashed survival curve, vaccinates are represented in black. Significant differences between controls and vaccinates are indicated with an (*).

Table 1

Protocol, parasitemia measurements, and survival curve P-values (delay in death) of mice challenged with lethal *P. chabaudi adami* DS over 3 challenge trials

| Group | Peak % parasitemia[a] | DNA prime only | | | | MSP4/5 ± DNA prime | | |
|---|---|---|---|---|---|---|---|---|
| | | Route | No. mice | Delay in death (P-values) | Surviving (%) | No. mice | Delay in death P | Surviving (%) |
| MSP4/5 protein pilot | | | | | | | | |
| 1. MSP4/5 protein | 27 ± 10 | – | – | – | – | 8 | 0.015 | 75 |
| 2. Freund's adjuvant alone | 32 ± 7 | – | – | – | – | 8 | ns[b] | 13 |
| Challenge trial 1 | | | | | | | | |
| 1. VR1020 vector (control) | 32 ± 10 | ID | 4 | – | 0 | 4 | – | 0 |
| 2. CTLA4 vector (control) | 34 ± 6 | ID | 4 | – | 0 | 4 | – | 0 |
| 3. MCP-3 vector (control) | 33 ± 6 | ID | 4 | – | 0 | 4 | – | 0 |
| 4. VR1020 vector (control) | 29 ± 4 | IM | 4 | – | 0 | 4 | – | 25 |
| 5. CTLA4 vector (control) | 35 ± 9 | IM | 4 | – | 0 | 4 | – | 0 |
| 6. MCP-3 vector (control) | 32 ± 6 | IM | 4 | – | 0 | 4 | – | 0 |
| 7. VR1020/MSP4/5 | 38 ± 6 | ID | 4 | ns | 0 | 4 | 0.029 | 75 |
| 8. CTLA4/MSP4/5 | 33 ± 6 | ID | 4 | ns | 0 | 4 | ns | 25 |
| 9. MCP-3/MSP4/5 | 37 ± 9 | ID | 4 | 0.017 | 25 | 4 | ns | 0 |
| 10. VR1020/MSP4/5 | 34 ± 10 | IM | 4 | 0.038 | 25 | 4 | ns | 0 |
| 11. CTLA4/MSP4/5 | 33 ± 5 | IM | 4 | ns | 0 | 4 | ns | 0 |
| 12. MCP-3/MSP4/5 | 35 ± 5 | IM | 4 | ns | 0 | 4 | ns | 25 |
| 13. MSP4/5 protein | 36 ± 6 | – | – | – | – | 8 | 0.011 | 63 |
| 14. Freund's adjuvant alone | 39 ± 4 | – | – | – | – | 8 | ns | 0 |
| Challenge trial 2 | | | | | | | | |
| 1. VR1020 vector (control) | 45 ± 11 | ID | 3 | – | 0 | 3 | – | 0 |
| 2. MCP-3 vector (control) | 36 ± 13 | ID | 3 | – | 0 | 3 | – | 0 |
| 3. CTLA-4 vector (control) | 36 ± 15 | ID | 3 | – | 0 | 3 | – | 0 |
| 4. VR1020/MSP4/5 | 34 ± 4 | ID | 6 | ns | 0 | – | – | – |
| 5. MCP-3/MSP4/5 | 28 ± 7 | ID | 6 | 0.021 | 50 | – | – | – |
| 6. CTLA-4/MSP4/5 | 30 ± 17 | ID | 6 | ns | 0 | – | – | – |
| 7. VR1020/MSP4/5 (p/b) | 32 ± 4* | ID | – | – | – | 6 | 0.009 | 0 |
| 8. MCP-3/MSP4/5 (p/b) | 28 ± 5 | ID | – | – | – | 6 | ns | 33 |
| 9. CTLA-4/MSP4/5 (p/b) | 34 ± 8 | ID | – | – | – | 6 | ns | 0 |
| Challenge trial 3 | | | | | | | | |
| 1. VR1020 vector (control) | 44 ± 8.7 | ID | – | – | – | 6 | – | 0 |
| 2. VR1020/MSP4/5 (p/b) | 37 ± 9.7 | ID | – | – | – | 6 | 0.009 | 33 |
| 3. MCP-3 vector (control) | 34 ± 6.7 | ID | 6 | – | 0 | – | – | – |
| 4. MCP-3/MSP4/5 | 45 ± 10.1 | ID | 6 | 0.03 | 33 | – | – | – |

Survival curves between vector control mice and corresponding vaccinates were compared using the Mantel–Haenszel test to determine the significance of a delay in death. MSP4/5 protein group mice received 25 μg of MSP4/5 protein three times at 2-week intervals as described in the methods. DNA prime and prime/boost mice all received a total of three DNA vaccinations at 2-week intervals. Prime/boost mice (including vector controls) received 10 μg of MSP4/5 in IFA 2 weeks after the final DNA priming vaccination.

[a] There were no differences in peak parasitemia between DNA prime and MSP4/5 prime boost mice in challenge trial 1, therefore the peak parasitemia was combined for all animals in these groups. (±) show S.D.

[b] ns: not significant.

* $P = 0.03$, Mann–Whitney test compared with VR1020 vector (control).

intervals. At week 13, groups 1–12 (Table 1) were divided into two groups of four mice, with one group receiving 10 μg of MSP4/5 protein in incomplete Freund's adjuvant as a boost. Control mice primed with empty vector also received 10 μg of MSP4/5 in incomplete Freund's adjuvant to control for the effects of a single dose MSP4/5, and any possible synergy with the priming plasmid DNA. The IgG antibody kinetics was followed until week 15 (Fig. 3) and then all mice were challenged with 100,000 infected *P. chabaudi adami* DS red blood cells.

Fig. 6 shows survival data for both IM and ID vaccination using each vector type. Mice vaccinated ID using

VR1020/MSP4/5 p/b were protected against lethal challenge (75% survival) compared with the VR1020 vector ID p/b control ($P = 0.029$; Fig. 6, panel iii). A significant delay in death after administration of VR1020/MSP4/5 IM compared with the VR1020 vector IM control was also detected ($P = 0.038$; Fig. 6, panel ii), but not with the VR1020/MSP4/5 IM p/b protocol (Fig. 6, panel ii). Vaccination with the MCP-3/MSP4/5 ID construct also resulted in a significant delay in death compared with the MCP-3 ID vector control ($P = 0.017$; Fig. 6, panel v), although vaccination using the MCP-3/MSP4/5 ID p/b protocol did not enhance protection ($P = 0.11$; Fig. 6, panel v). No

significant differences in survival between groups vaccinated using MCP-3/MSP4/5 IM compared to its MCP-3 IM vector control (Fig. 6, panel iv), or CTLA4/MSP4/5 constructs administered via either route compared to CTLA4 control vectors were detected (Fig. 6, panels vi and vii).

Vaccination using MSP4/5 protein alone resulted in 63% protection of mice in the MSP4/5 protein control group ($P = 0.011$; Fig. 6, panel i). There were no significant differences between survival curves of mice that received MSP4/5 protein alone, when compared against survival curves of other statistically significant groups, namely VR1020/MSP4/5 ID p/b ($P = 0.94$), MCP-3/MSP4/5 ID ($P = 0.19$), or VR1020/MSP4/5 IM ($P = 0.17$).

### 3.6.3. Challenge trial 2

In order to confirm the protection (primarily in ID vaccinated groups) found in trial 1, further challenge experiments were performed testing the efficacy of the MSP4/5 vaccine delivered by an ID and p/b protocol, with several modifications made to the protocol. For trial 2, each group contained six mice, and all mice were primed three times ID at 2-week intervals. Mice in groups 7–9 (trial 2, Table 1) were boosted using 10 µg of MSP4/5 protein in incomplete Freund's adjuvant at week 6, 2 weeks after the final priming vaccination. Half of the vector control groups (1–3, trial

2, Table 1) were similarly boosted with 10 µg of MSP4/5 protein in incomplete Freund's adjuvant as controls for the protein boost. Significant differences in the efficacy of the vaccines were observed between ID and ID p/b groups, as seen in trial 1. A significant difference between mice vaccinated with VR1020 vector versus VR1020/MSP4/5 p/b was detected; although mice vaccinated with the p/b formulation did not survive in this experiment, there was a significant delay in death ($P = 0.009$; Fig. 7, panel ii). Vaccination using MCP-3/MSP4/5 plasmid DNA alone resulted in a significant delay in death as well as survival of 50% of animals compared with the MCP-3 vector alone ($P = 0.021$; Fig. 7, panel i) confirming the results of trial 1. Boosting of the MCP-3/MSP4/5 primed animals did not enhance this protection, and no significant difference was found between survival curves when compared to the MCP-3 control vector ($P = 0.37$; Fig. 7, panel ii).

As observed in the first trial, there were no significant differences between survival curves of the VR1020 control vector and VR1020/MSP4/5 prime only (Fig. 7, panel i), or any CTLA4 control vector and CTLA4/MSP4/5 vaccinated groups (Fig. 7, panels i and ii). Analysis of parasitemia levels also showed no statistically significant differences between groups vaccinated with vectors containing MSP4/5 or control mice, except for VR1020/MSP4/5 p/b versus VR1020
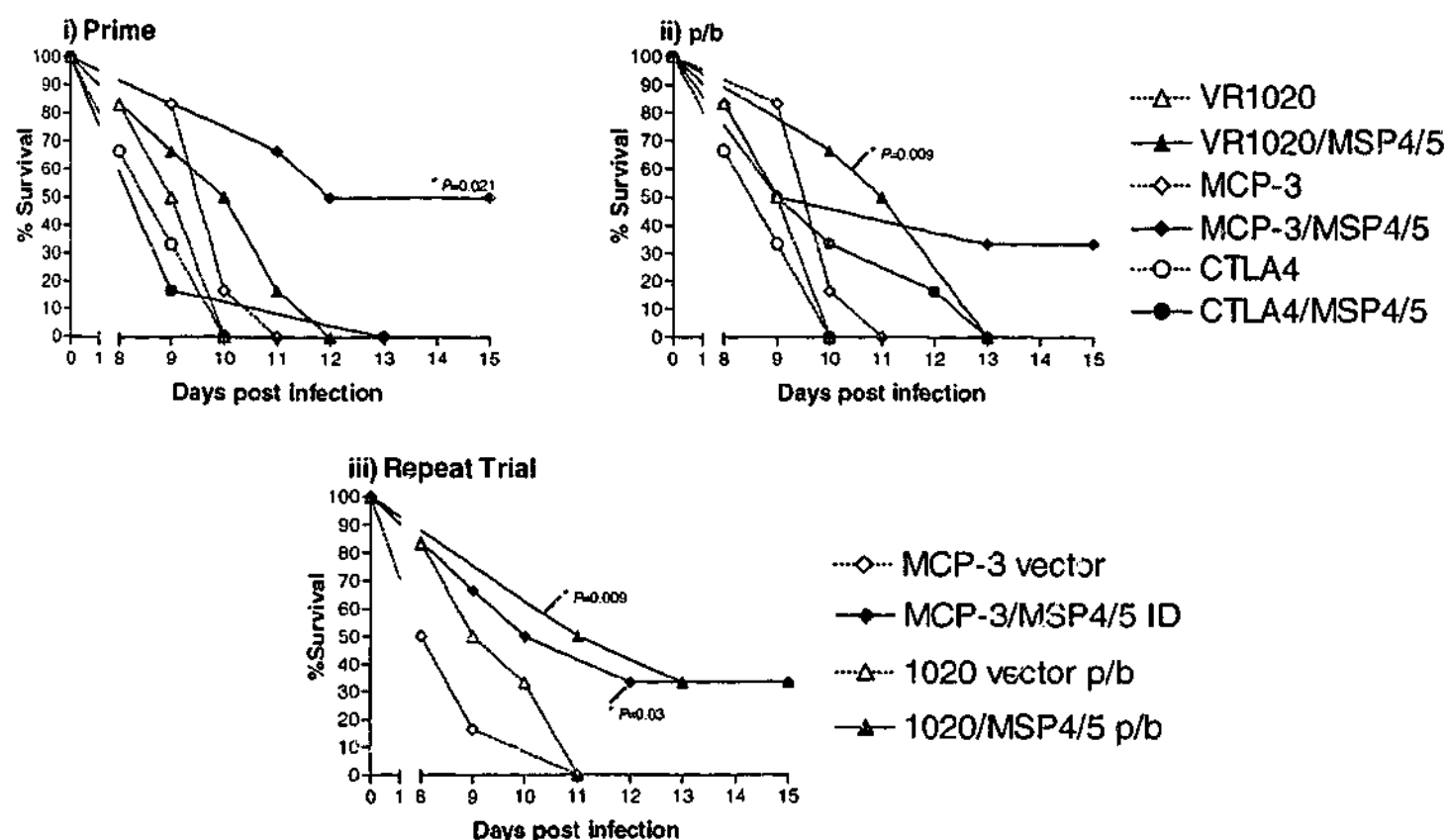


Fig. 7. Survival of mice vaccinated by different protocols and challenged with *P. chabaudi adami* DS. Panels i (DNA prime only) and ii (p/b groups) depict survival curves from challenge trial 2; panel iii depicts survival data from challenge trial 3, representing MCP-3/MSP4/5 ID and VR1020/MSP4/5 p/b groups. No significant differences in survival curves were found between control animals receiving 10 µg of MSP4/5 protein in incomplete Freund's adjuvant versus control vector primed mice (panels ii and iii). Survival curves for vector and vector/protein boost were combined and represented as one survival curve (panel ii). Control groups in all panels are represented by a dashed survival curve, vaccinates are represented in black. Significant differences between control and vaccinates are indicated with an (*).

vector alone ($P = 0.03$, Mann–Whitney non-parametric
$t$-test) (Table 1).

### 3.6.4. Challenge trial 3

The final trial involved confirming observations from
groups with survival curves significantly different from
their control groups, namely MCP-3/MSP4/5 ID and
VR1020/MSP4/5 p/b (trial 3, Table 1). For trial 3, each
group contained six mice, and all mice were primed three
times ID at 2-week intervals. Mice in groups 1 and 2
(Table 1, trial 3) were boosted using 10 μg of MSP4/5 pro-
tein in incomplete Freund's adjuvant at week 6, 2 weeks
after the final priming vaccination.

Again, a significant difference in kinetics of survival
between mice vaccinated with the VR1020 control vector
versus VR1020/MSP4/5 p/b was detected ($P = 0.009$),
with 33% survival (Fig. 7, panel iii). Vaccination using
MCP-3/MSP4/5 plasmid DNA alone resulted in a signif-
icant delay in death, as well as 33% survival of animals
compared with the MCP-3 vector alone ($P = 0.03$; Fig. 7,
panel iii). Analysis of parasitemia showed no statistically
significant differences between groups vaccinated with vec-
tors containing MSP4/5 or control mice (Mann–Whitney
non-parametric $t$-test).

## 4. Discussion

In this study we have shown that immune and protective
responses generated, using the rodent malaria homologue of
the human candidate malarial antigens MSP-4 and MSP-5,
is dependent on the route and type of DNA vector used for
vaccination. Intradermal gene-gun vaccination using a single
malarial antigen and a vaccine-targeting approach, namely
the MCP-3/MSP4/5 construct, produced a significant differ-
ence in protective efficacy compared with vaccination using
the MCP-3 vector alone after lethal blood-stage challenge.
We have also shown that generation of antibodies after boost-
ing MSP4/5 DNA primed mice with recombinant MSP4/5
does not always correlate with protection against lethal P.
chabaudi adami DS blood-stage challenge. Boosting with
recombinant MSP4/5 protein however did enhance protec-
tive efficacy after priming with the untargeted secretory vec-
tor construct VR1020/MSP4/5, but not with MCP-3/MSP4/5
and CTLA4/MSP4/5 constructs. Although boosting with
MSP4/5 recombinant protein can enhance the generation
of antibodies (particularly after gene-gun priming), the
avidity of these antibodies varied between the vaccine
groups.

Enhanced antibody generation 2 weeks after the initial
vaccination was produced by the CTLA4/MSP4/5 construct,
regardless of route. This is presumably due to the CTLA4
ligand binding directly to antigen-presenting cells to in-
crease the likelihood of initiating an immune response [13].
This rapid response is in contrast to administration of the
VR1020 and MCP-3 MSP4/5 constructs which followed a

slower pattern of kinetics after ID administration only. The
blood-stage malarial antigen apical membrane antigen-1
(AMA-1) is a leading candidate vaccine antigen [1]. Lew
et al. [14] demonstrated that vaccination of mice using
AMA-1 of P. chabaudi inserted into the CTLA4 vaccine
vector (the same vector type used in the present experiment)
could also enhance antibody levels at just 2 weeks after
the initial vaccination. The use of the CTLA4/MSP4/5 con-
struct did not enhance survival regardless of administration
route or recombinant protein boosting. Unfortunately, pro-
tection data using the CTLA4/AMA-1 construct, or CTLA4
applied to a malarial model has not been previously pub-
lished. However, high protective efficacy using a CTLA4
construct has been demonstrated against Corynebacterium
pseudotuberculosis after vaccination in sheep [14]. CTLA4
constructs have also been shown to significantly reduce
viral titres in mice using a haemagglutinin-based influenza
DNA vaccine [26]. The lack of protective efficacy observed
in the present experiment with CTLA4/MSP4/5 constructs
appears to be due to the CTLA4 targeting strategy, given
that significant protection was observed when this antigen
was delivered using the two other vaccine vectors.

The efficacy of the chemoattractant MCP-3 when ap-
plied to malaria DNA vaccines has not been previously
described. MCP-3 fusion DNA vaccines have however been
successfully applied to induce T-cell dependent antitumor
immunity in mice [16], and MCP-3 itself is believed to be
a potential inhibitor of HIV-1 infection (reviewed in [27]).
When used ID via gene-gun delivery, the MCP-3/MSP4/5
targeting vector construct provided a significant degree of
survival against lethal P. chabaudi blood-stage challenge
without the need for protein boosting. The use of the
gene-gun to transfect dendritic cells such as Langerhans
cells, which are abundant in skin, may provide an envi-
ronment whereby MCP-3 is most useful. MCP-3 has been
shown to bind to chemokine receptors CCR1, CCR2, and
CCR3 which are all expressed on immune cells such as
monocytes, T-lymphocytes, eosinophils, basophils and den-
dritic cells (reviewed in [27]). Dendritic cells in particular
have been shown to play a major role inducing protection
by processing and presenting antigen to naïve T cells after
DNA vaccination ([12,28], reviewed in [29]). It is possible
that priming with MCP-3/MSP4/5 ID is enhancing T-cell
production, however further work is required to confirm this.

The use of the untargeted secretory DNA vaccine con-
struct VR1020/MSP4/5 resulted in an enhanced antibody
titre, as well as significant protection upon protein boosting
when compared to priming with control vector DNA alone
and boosting. The use of the VR1020/MSP4/5 DNA vaccine
construct alone however did not enhance survival. Secretion
of MSP4/5 protein from VR1020/MSP4/5 transfected cells
is also likely to represent a form of the protein that more
closely resembles the recombinant boosting protein. The fu-
sion of CTLA4 to MSP4/5, when vaccinating with this con-
struct, may affect the tertiary structure of the final secretory
protein, and thus affect immunogenicity. The CTLA4/human

Ig moiety (approximately 60 kDa) is considerably larger than the MSP4/5 fusion partner (approximately 36 kDa). It is possible that some protective epitopes on MSP4/5 may have been obscured upon folding of the CTLA4 moiety. Further studies evaluating the ability of sera generated by the CTLA4/MSP-4/5 and MCP-3/MSP4/5 constructs to react with specific MSP4/5 protein domains (which may or may not have been obscured by the CTLA4 or MCP-3 moieties) are required to confirm this. The production of overlapping *P. c. adami* MSP4/5 domains would be useful in determining any differences in the binding specificities of sera generated using each of the three MSP4/5 vector constructs.

In the present study, enhanced antibody levels after boosting were at levels comparable to three doses of *P. chabaudi* MSP4/5 protein, particularly in the gene-gun primed groups. Gene-gun vaccination itself has been found to bias the immune response to produce IgG1 and IL-4, a Th2-like response, in other DNA vaccine systems [30–32]. MSP4/5 DNA vaccination in this study biased the immune response towards producing IgG1 and IgE isotypes, regardless of vaccine route or boosting. Vaccinating with DNA ID using the C-terminal region of MSP1 of *P. yoelii* has also been found to produce predominantly IgG1 antibodies [23]. Vaccination in the present study with MSP4/5 *P. chabaudi* protein IP produced a Th2 like IgG1 and IgE response and high levels of protection. Protection after vaccination with *P. yoelii* MSP4/5 protein in female BALB/c mice however has been shown to correlate with high levels of IgG2a and IgG2b isotypes [33]. This suggests that there may be major differences in the isotype response induced by the vaccine when using the analogous antigen from two malaria species. This is not surprising given that MSP4/5 for these two species share only 52% amino acid identity. It is therefore difficult to make comparisons of protective efficacy in relation to antibody isotype between these two malarial blood-stage challenge systems. Using *P. chabaudi chabaudi*, it has been shown that IgG1 and IgG2a antibodies from hyperimmune mice coat the surface of schizont infected erythrocytes, and that either of these isotypes can prevent reinvasion of erythrocytes by inhibiting generation of new ring forms [34].

It has been reported that BALB/c mice immunized with ovalbumin via injection with DNA can produce antibodies with a higher avidity, particularly via the ID route, than mice injected by the same route with ovalbumin protein [35]. In light of this, results from the present study show that avidity may be antigen specific, and that priming via the ID route does not always enhance the avidity of antibody at high concentrations of guanidine–HCl. A similar pattern of antibody avidity was has been found elsewhere [23]. DNA vaccination of three different mouse strains via ID or IM routes using the C-terminal region of *P. yoelii* MSP1, also produced lower avidity antibodies than that observed in protein vaccinated mice. This was attributed to a lack of affinity maturation of antibodies that were induced by DNA vaccination, with low avidity antibodies still detected at up to 32 weeks after the initial vaccination. Low avidity antibody were also believed to be a contributing factor in the lack of protection found after lethal blood-stage challenge [23]. In the present study however the production of low avidity antibodies after ID vaccination did not result in a lack of protection after lethal blood-stage challenge, as significant survival was observed in prime/boost mice vaccinated using the VR1020/MSP4/5 construct when compared with control mice.

This study demonstrates for the first time that vaccination using MSP4/5 from *P. chabaudi adami* can protect against a highly virulent blood-stage challenge when given as a protein only, DNA only (MCP-3 vector), or as a prime/boost vaccine (VR1020 vector). The targeting vector MCP-3/MSP4/5 when administered ID, results in significant protection against death in mice without the need for protein boosting. Priming with DNA, followed by boosting with recombinant protein, however, does have an effect on survival when using the untargeted VR1020/MSP4/5 vector. Although the level of humoral responses and predominant antibody isotypes were similar between DNA primed and boosted groups vaccinated using different vectors, use of the CTLA4/MSP4/5 vector did not enhance survival against blood-stage challenge despite the high levels of antibody obtained. This suggests that the fine specificity of the antibody response is important in determining vaccine efficacy in this model. The results also suggest that the enhancement of survival against blood-stage malaria challenge after utilizing MSP4/5 DNA vaccination is therefore highly dependent on the route and type of vaccine vector employed.

## Acknowledgements

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## Appendix II

**Induction of specific T-cell responses, opsonising antibodies and protection against *Plasmodium chabaudi adami* malaria in mice vaccinated with genomic expression libraries expressed in targeted and secretory DNA vectors.**

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# Induction of Specific T-Cell Responses, Opsonizing Antibodies, and Protection against *Plasmodium chabaudi adami* Infection in Mice Vaccinated with Genomic Expression Libraries Expressed in Targeted and Secretory DNA Vectors

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It has been proposed that a multivalent malaria vaccine is necessary to mimic the naturally acquired resistance to this disease observed in humans. A major experimental challenge is to identify the optimal components to be used in such a multivalent vaccine. Expression library immunization (ELI) is a method for screening genomes of a pathogen to identify novel combinations of vaccine sequences. Here we describe immune responses associated with, and the protective efficacy of, genomic *Plasmodium chabaudi adami* DS expression libraries constructed in VR1020 (secretory), monocyte chemotactic protein-3 (chemoattractant), and cytotoxic T lymphocyte antigen 4 (lymph node-targeting) DNA vaccine vectors. With splenocytes from vaccinated mice, specific T-cell responses, as well as gamma interferon and interleukin-4 production, were observed after stimulation with *P. chabaudi adami*-infected erythrocytes, demonstrating the specificity of genomic library vaccination for two of the three libraries constructed. Sera obtained from mice vaccinated with genomic libraries promoted the opsonization of *P. chabaudi adami*-infected erythrocytes by murine macrophages in vitro, further demonstrating the induction of malaria-specific immune responses following ELI. Over three vaccine trials using biolistic delivery of the three libraries, protection after lethal challenge with *P. chabaudi adami* DS ranged from 33 to 50%. These results show that protective epitopes or antigens are expressed within the libraries and that ELI induces responses specific to *P. chabaudi adami* malaria. This study further demonstrates that ELI is a suitable approach for screening the malaria genome to identify the components of multivalent vaccines.

Malaria is one of the major causes of death in the developing world and is an important barrier to economic progress in countries where the disease is endemic (reviewed in reference 37). Measures to contain malaria such as vector control by use of insecticides and drug treatment of active infection have become decreasingly effective, and new methods such as the use of vaccines against malaria-causing parasites at various life cycle stages are needed to control this disease (reviewed in reference 14). Although there are several candidate antigens under development to combat malaria, there is no effective single-stage malarial vaccine yet available (reviewed in reference 37). There is a general consensus that a multivalent vaccine is necessary to mimic naturally acquired resistance in humans. A major challenge is to identify the best antigen components to be used in such a multivalent vaccine (12, 20).

Protection in humans against asexual blood stages of malaria-causing parasites is believed to include mechanisms such as antibodies that block merozoite entry into erythrocytes and inhibit parasite development (reviewed in reference 20). In order to help resolve blood-stage malarial infection, induction of antibody and the activation of CD4 T cells are required

when vaccinating with recombinant candidate blood-stage antigens such as apical membrane antigen-1 (18, 40) and merozoite surface protein-1$_{19}$ (10, 16). In addition, other studies have shown that malaria parasite-specific T cells can adoptively transfer resistance in mice against challenge with *Plasmodium yoelii* and *P. chabaudi*, indicating that CD4$^+$ T cells alone may also play a role in vaccine-induced immunity (39).

Improvements in the immunogenicity of malarial antigens delivered by DNA vaccines are important if there is to be any possible practical application of this technology to provide protection against blood-stage malaria. It has been suggested that DNA vaccines alone may not be adequate to protect against malaria (reviewed in reference 19). Antigen-presenting cells, in particular dendritic cells, have been shown to be potent initiators of immune responses following DNA vaccination and are important in the uptake of antigen expressed from cells transfected by a DNA vaccine (reviewed in reference 27). Improvement of DNA vaccine efficacy by targeting antigen to antigen-presenting cells may be required if DNA priming alone is to be sufficient to provide a significant level of protection against malaria. The use of the targeting ligand cytotoxic T lymphocyte antigen 4 (CTLA4) in DNA vaccination has been shown to improve the magnitude and speed of the antibody response (6, 13, 22, 29). Fusion of antigens to the chemokine monocyte chemotactic protein-3 (MCP-3) has also been shown to enhance protective efficacy after DNA vaccina-

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tion in a tumor challenge model (5), to induce cytotoxic T lymphocytes and neutralizing antibodies to human immunodeficiency virus type 1 envelope proteins (4), and to induce antibody responses to the *P. chabaudi adami* merozoite surface protein 4/5 (MSP4/5) antigen in mice (29); in the latter case, the MCP-3/MSP4/5 DNA vaccine protected mice against lethal challenge with *P. chabaudi adami.*

Expression library immunization (ELI) enables screening of a pathogen's genome and eventual discovery of potential vaccine candidates. ELI has been applied to bacterial (3, 7) and parasitic (1, 24, 28, 31) infections and recently to simian immunodeficiency virus infection (32). Genomic ELI libraries encode antigens from all stages of the life cycle, potentially allowing the discovery of antigens from particular stages of a life cycle. A primary focus for malaria vaccine development is the blood stage, which is responsible for the morbidity and mortality associated with malaria (reviewed in reference 37).

It was previously reported that ELI with a *P. chabaudi adami* genomic library significantly protects mice against blood-stage malaria caused by the lethal *P. chabaudi adami* DS strain (31), although the protective mechanism(s) induced by this multivalent genomic vaccine remained to be elucidated. Here we extend these observations and report the DNA vaccination of mice with three different libraries of *P. chabaudi adami* genomic DNA, expressed in the VR1020, MCP-3, or CTLA4 vector. Protection after library vaccination was observed only with the VR1020 and MCP-3 libraries and was found to be associated with T-cell responses of splenocytes from vaccinated mice that were specific to native malarial antigens or epitopes produced in *P. chabaudi adami* DS-infected blood. Murine macrophages incubated with sera, obtained after genomic library vaccination, also possessed the ability to opsonize *P. chabaudi adami* DS-infected red blood cells (*P. chabaudi adami* DS-IRBCs) in vitro, providing evidence that genomic library vaccination enhances humoral effector responses.

## MATERIALS AND METHODS

**Creation of plasmid pools.** The creation of a VR1020 genomic expression library has been described previously (31). Briefly, *P. chabaudi adami* DS genomic DNA was isolated from Ficoll-purified erythrocytes of infected BALB/c mice (parasitemia, 20 to 30%). The purified DNA was partially digested with *Tsp*509I (5 U for 90 s at 65°C), and the digested fraction between 1 and 3 kbp was isolated by gel elution. Vector VR1020 (VICAL, San Diego, Calif.) was prepared by digestion with *Bam*HI and *Bgl*II and the insertion of an *Eco*RI linker constructed from the oligonucleotides 5'GATCCGGGAATTCAA and 5'GATCTT GAATTCCCG. Once obtained, the new vector was digested with *Eco*RI, treated with alkaline phosphatase, and ligated with *Tsp*509I-digested *P. chabaudi adami* genomic DNA. Ligation mixes were transformed into *Escherichia coli* DH5α, and colonies were selected on solid medium containing 50 μg of kanamycin/ml. After overnight growth, *E. coli* colonies were combined into pools. A total of 10 pools (termed 3KA to 3KJ), each comprising approximately 3,000 individual clones, were constructed and stored at −80°C as glycerol stocks. The pool of 30,000 clones (termed 30K) was obtained by combining each of the 10 pools of 3,000 clones. Murine cDNA was supplied by Harshall Nandurkar (Monash University, Clayton, Australia). The oligonucleotides 5'TATTATTAGCGGCCGCATGAG GATCTCTGCCACGCTT (containing a *Not*I site) and 5'TATGGATCCTCCA CCTCCACCTCCAGGCTTTGGAGTTGGGGT (encoding six glycine residues followed by a *Bam*HI site) were used to amplify the MCP-3 gene by PCR. The MCP-3 PCR fragment was digested with *Not*I and *Bam*HI and inserted into the VR1012 vector (VICAL) digested with the same enzymes to produce the MCP-3 vector (29). The CTLA4 vector (6) was a gift from Andrew Lew (Walter and Eliza Hall Institute, Melb,Australia). Libraries in MCP-3 and CTLA4 vectors were constructed as described for the VR1020 vector by using the same stock of

*P. chabaudi adami* genomic DNA. Ligation reaction mixtures were transformed into *E. coli* DH5α, and colonies were selected on solid media containing 50 μg of kanamycin/ml for the MCP-3 vector and 100 μg of ampicillin/ml for the CTLA4 vector.

**Isolation of plasmid DNA and construction of vaccination cartridges.** Library pools stored as glycerol stocks were grown to confluence on solid medium prior to inoculation into liquid medium. Cells from five to ten confluent plates were used to inoculate 1 liter of Luria broth and grown with shaking at 37°C for 6 h prior to harvest. Plasmid preparation and endotoxin removal were performed by using an endotoxin-free plasmid giga kit according to the instructions of the manufacturer (QIAGEN inc, Valencia, Calif.). DNA purified under endotoxin-free conditions was precipitated onto gold microcarriers which were attached to plastic supports as per the recommendations of the manufacturer (Bio-Rad Laboratories, Hercules, Calif.). DNA was combined with carriers at a ratio of 100 μg of DNA/50 mg of carriers. Each projectile used for vaccination contained approximately 1 μg of DNA.

**Mice and vaccinations.** All mice were BALB/c, female, and 4 to 6 weeks of age at the time of first vaccination. For vaccination, the abdominal regions were shaved and particles were delivered via the intraepidermal (i.d.) route by using a Helios gene gun (Bio-Rad Laboratories) with a pulse of helium gas at 400 lb/in².

**Infection of mice, blood sampling, and parasitemia measurements.** Blood from an infected mouse with a known parasitemia level (1 to 10%) was taken and immediately diluted in phosphate-buffered saline (PBS) to give the required dosage (10⁵ IRBCs/dose). Mice were infected by the intraperitoneal route at day 0, and parasitemia was assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels and the numbers of days to peak parasitemia were compared by using the Student *t* test. The Mantel-Haenszel (or log rank) test was used to measure the parameter of survival. This method involves calculating a *P* value and testing the null hypothesis that the survival curves are identical between vaccinated and control groups. This analysis was performed with Prism 3 software (GraphPad, San Diego, Calif.).

**In vitro spleen cell proliferation.** Ten days after the final DNA vaccination, mice were sacrificed and single-cell suspensions of splenocytes were obtained by crushing whole spleens with a 5-ml syringe barrel. The splenocytes were then resuspended in complete RPMI 1640 medium (Invitrogen Corporation, Grand Island, N.Y.) supplemented with 10% fetal calf serum, 2 mM ʟ-amine (Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St Louis, Mo.), and penicillin-streptomycin (100 U/ml; Invitrogen). Suspensions were then passed through a 100-μm-pore-size nylon cell strainer (Becton Dickinson, Franklin Lakes, N.J.) and exposed for 3 min to erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrogen carbonate, 0.1 mM EDTA [pH 7.4]). Splenocytes were again washed and resuspended in RPMI 1640 medium. Cell viability was greater than 80% as determined by trypan blue exclusion (Invitrogen). Splenocytes were stimulated with 2 × 10⁶ *P. chabaudi adami* DS-IRBCs, freshly extracted from an infected mouse, or 2 × 10⁶ freshly extracted red blood cells (RBCs) as a control. As a control for cell viability, splenocytes were stimulated with concanavalin A (Sigma) at a final concentration of 2.5 μg/ml. Splenocytes were cultured for 96 h in flat-bottom microtiter plates in triplicate at a final concentration of 5 × 10⁶ cells/ml (10⁶ cells/well) and pulsed with 1 μCi of [³H]thymidine (Amersham Biosciences Corp., Piscataway, N.J.)/well 18 h before harvesting. The splenocytes were harvested onto fiberglass filter mats (Saktron Instruments inc., Sterling, Va.) with an automated cell harvester (Saktron), and incorporated radioactivity was measured with a liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, Mass.).

**ELISA for IFN-γ and IL-4.** Spleen cells from vaccinated and control mice were incubated for 72 h in the presence of 2 × 10⁶ *P. chabaudi adami* DS-IRBCs or 2 × 10⁶ control RBCs, and supernatants were harvested after 72 h and stored at −20°C until cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). Gamma interferon (IFN-γ) and interleukin (IL-4) ELISAs were carried out according to the instructions of the manufacturer (Endogen, Woburn, Mass.). Briefly, Maxisorp ELISA plates (Nunc, Kamstrupvej, Denmark) were coated with anti-mouse IFN-γ monoclonal antibody (Endogen) or anti-mouse IL-4 monoclonal antibody (Endogen) and incubated overnight at room temperature. The ELISA plates were then blocked in assay buffer (PBS with 4% bovine serum albumin, pH 7.4). The supernatants obtained from the splenocyte cultures were tested in duplicate against serial dilutions of recombinant IFN-γ (starting at 5 ng) and IL-4 (starting at 1 ng) standards. After incubation at room temperature for 1 h, anti-mouse IL-4 biotin-labeled monoclonal antibodies (Endogen) or anti-mouse IFN-γ biotin-labeled monoclonal antibodies (Endogen) were added, and the plates were incubated for 1 h at room temperature. After the plates were washed three times, horseradish peroxidase-

TABLE 1. Features of *P. chabaudi adami* library clones

| Library[a] | No. of plasmid clones examined by restriction digestion[b] | No. (%) with inserts | No. of plasmid clones examined by sequencing | Avg insert size (kb) | No. (%) with reading frames expressing peptides[c] | Size range (aa) of encoded peptides | No. (%) of peptides longer than: | | Avg size (aa) of encoded peptides |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 20 aa | 50 aa | |
| VR1020/30K[d] | 58 | 58 (100) | 24 | 1.5 | 18 (75) | 1–115 | 10 (42) | 3 (13) | 25 |
| MCP-3/30K | 32 | 32 (100) | 22 | 1.3 | 20 (90) | 3–113 | 8 (36) | 3 (14) | 32 |
| CTLA4/30K | 45 | 41 (93) | 33 | 1.5 | 23 (70) | 2–139 | 11 (33) | 6 (18) | 36 |

[a] Ten plasmid pools, designated 3KA to 3KJ and each containing approximately 3,000 individual clones, were combined to create pool 30K, with 30,000 clones.
[b] All clones examined were from pool 3KA from each library.
[c] Number in frame with the TPA signal sequence (VR1020/30K), the MCP-3 sequence (MCP-3/30K), and the CTLA4 sequence (CTLA4/30K).
[d] The VR1020/30K library is described in reference 31.

conjugated streptavidin (Amersham) was added at 1:16,000, and the plates were incubated for 30 min at room temperature. The plates were again washed three times. The reaction was developed with TMB substrate solution (Sigma) and stopped with 0.18 M $H_2SO_4$, and the absorbance was read at 450 nm.

**Phagocytosis assays.** Phagocytosis assays were performed according to the method described in reference 26, with the following modifications. Macrophages were obtained from BALB/c mice by peritoneal lavage with 9 ml of ice-cold 0.34 M sucrose. The cells were then centrifuged at 1,200 rpm for 10 min at 4°C. Peritoneal cell exudates were then resuspended in complete RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma), and penicillin-streptomycin (100 U/ml; Invitrogen) to a final concentration of $2 \times 10^6$ cells/ml. Eight-well chamber slides (Nalge Nunc International Corp. Naperville, Ill.) were used, with $4 \times 10^5$ macrophages added to each well. The macrophages were allowed to adhere for 2 h at 37°C with 5% $CO_2$. After 2 h, nonadherent cells were removed by careful washing with 1 ml of 37°C RPMI 1640 medium. Fresh complete RPMI 1640 medium was added, and macrophages were left to adhere for a further 2 h. During this time, fresh *P. chabaudi adami* DS-IRBCs ($10^8$ IRBCs/ml containing trophozoites and schizonts at approximately 40 to 50% parasitemia) in complete RPMI 1640 medium were purified with Ficoll (Amersham). The IRBCs were washed twice with complete RPMI 1640 medium. After washing, IRBC pellets were placed into 1.5-ml centrifuge tubes in 15-µl aliquots. PBS (30 µl) and 1 µl of sera obtained from groups vaccinated with the genomic libraries, or sera from mice vaccinated with empty vector, were then added to the IRBC pellets and incubated for 1.5 h at 37°C with shaking. After 1.5 h, IRBCs and sera were added to adherent macrophages and incubated for 2 h at 37°C with 5% $CO_2$. The eight-well slides were then washed four times with PBS to remove nonadherent macrophages and noningested IRBCs. Noningested but adherent IRBCs were then lysed by incubation of the slides with cold water for 20 s, followed by washing with PBS. The eight-well slides were then fixed and stained with Kwik Diff staining solution (Temo Shandon, Pittsburgh, Pa.). The number of IRBCs taken up by 200 macrophages per individual sample was then quantified by using light microscopy.

**Blockage of macrophage Fcγ receptors to determine opsonization specificity.** Macrophages from BALB/c mice were prepared in eight-well chamber slides (Nalge Nunc International Corp ) as described for the phagocytosis assay. Macrophages were washed three times with warm RPMI 1640 medium (Invitrogen) to remove nonadherent cells. The cells were incubated in 200 µl of complete RPMI 1640 medium plus rat anti-mouse CD16/32 (Fcγ receptor)-blocking antibody (clone 93; Southern Biotech, Birmingham, Ala.) at a concentration of 100 µg/ml for 60 min at 37°C. The excess blocking antibody was removed by two washes with warm RPMI 1640 medium. IRBCs and sera from all three genomic libraries were prepared and incubated with macrophages (treated with and without the blocking antibody) as described for the phagocytosis assay.

## RESULTS

**Characterization of library clones.** All libraries were constructed by using the same stock of a partial digest of *P. chabaudi adami* DS genomic DNA in order to minimize any bias among the libraries in insert sizes after enzymatic digestion of the genomic DNA. A partial enzymatic digest of DNA (described in Materials and Methods and in reference 31) with *Tsp*509I was required as this enzyme recognizes AATT se-

quences and the *P. chabaudi adami* genome is more than 80% adenosine and thymidine. Table 1 shows the characteristics of random samples of 32 to 58 plasmid clones taken from the VR1020/30K (31), MCP-3/30K, and CTLA4/30K genomic libraries. The DNA inserts in selected clones were sequenced, and the sequences were compared against *P. falciparum* and *P. yoelii* genomic databases found at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov /PMGifs/Genomes/plasmodium.html), which confirmed the presence of *P. chabaudi adami* genomic DNA (data not shown). The DNA sequences were also translated and compared against sequences in *P. yoelii* and *P. falciparum* protein databases (data not shown), which revealed in-frame peptides predicted to be encoded by the insert DNA, as shown in Table 1. The construction method resulted in three libraries that were almost uniform in terms of numbers of recombinant plasmids, mean sizes of DNA inserts and encoded peptides, and percentages of encoded peptides of >50 amino acids (aa) in length. This uniformity was important in order to make comparisons between the three different vectors. The average size of the inserts among the three libraries was 1.43 kbp. The *P. chabaudi adami* genome is predicted to be approximately 23 Mbp in size (see the National Center for Biotechnology Information website at www.ncbi.nlm.nih.gov/projects/Malaria /Rodent/chabaudi.html). Therefore, the 30,000 plasmids contained in each of the genomic libraries represent 43 Mbp of DNA, resulting in an overall coverage of approximately two times the size of the *P. chabaudi adami* genome for each library.

We also estimated the proportion of clones that are predicted to encode peptides. Out of the 30,000 clones, about 50% will include a reading frame (since 50% of the genome comprises exons) (8). Of the remaining 15,000 clones, only 50% will be oriented correctly (since cloning into the vector is not directional), and of these 7,500 clones, only 1:3 will be in frame with the signal sequence: this leaves approximately 2,500 clones (or about 8%) potentially encoding an in-frame peptide. From the sequence analysis, it was found that 13 to 18% of the clones from all the libraries actually encoded in-frame peptides longer than 50 aa (we assume anything longer than 50 aa is likely to be a real peptide due to the high AT content of malaria parasite DNA, which creates stop codons at high frequency). Thus, in the 30K genomic vaccines, approximately 13 to 18% of clones (3,900 to 5,400) should potentially be able to encode peptides.
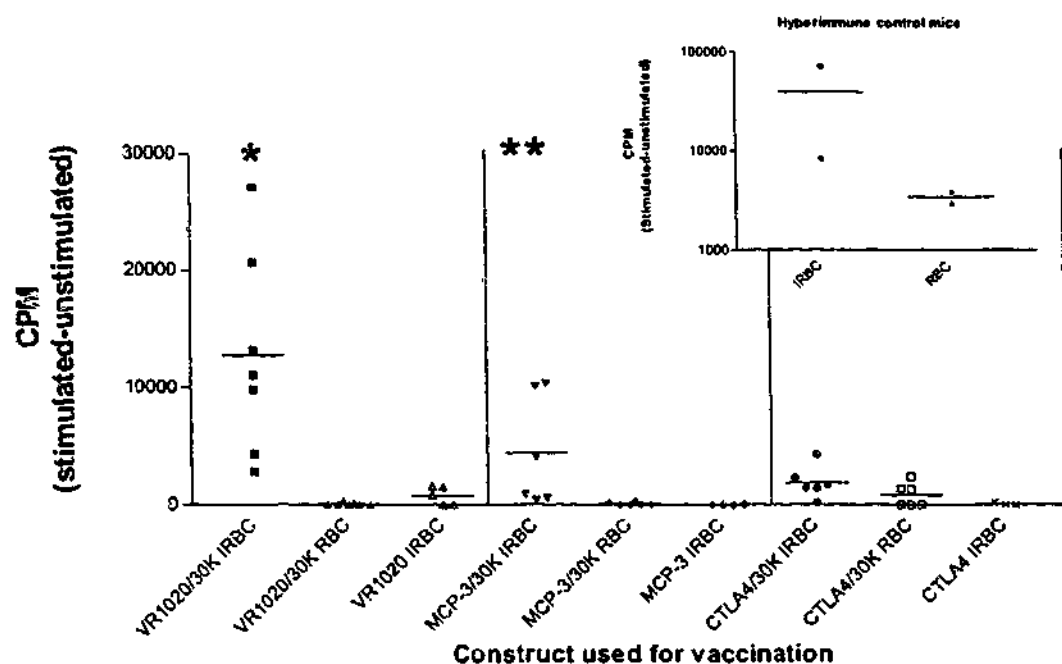
FIG. 1. In vitro proliferation of splenocytes from individual BALB/c mice vaccinated i.d. with the gene gun. Mice were vaccinated with VR1020/30K, MCP-3/30K, and CTLA4/30K three times at 2-week intervals. Splenocytes were harvested 10 days after the final vaccination. Following 72 h of stimulation with $2 \times 10^6$ IRBCs or $2 \times 10^6$ RBCs, [³H]thymidine was added for 18 h. Numbers of counts per minute of radioactivity incorporated by cells were then recorded. Splenocytes vaccinated with empty vector control DNA (VR1020, MCP-3, or CTLA4) were also stimulated with $2 \times 10^6$ RBCs, but no incorporation of [³H]thymidine was observed (data not shown). Splenocytes from all individual splenocyte cultures responded to concanavalin A stimulation (data not shown). The mean number of counts per minute (bars) is shown. *, $P <$ 0.01; **, $P < 0.05$. The insert shows the response of splenocytes from hyperimmune control mice (mice that had survived *P. chabaudi adami* infection) that were used as a positive control for IRBCs.

**Antigen-specific cellular immune responses induced by ELI DNA vaccination with three different vectors.** Groups of mice were vaccinated i.d. with either the VR1020/30K, MCP-3/30K, or CTLA4/30K genomic library or with the corresponding empty plasmid DNA control vectors, according to the procedure described in Materials and Methods. To evaluate cellular responses to native *P. chabaudi adami* antigens induced by each of the genomic libraries (and responses of corresponding empty vector-vaccinated control mice), spleens were removed from mice 10 days after the final i.d. vaccination and cell suspensions from individual mice were stimulated by using $2 \times 10^6$ *P. chabaudi adami*-IRBCs or $2 \times 10^6$ RBCs (prepared from naïve BALB/c mice) as a control.

Significant proliferation of spleen cells taken from VR1020/30K-vaccinated mice ($P < 0.01$) and MCP-3/30K-vaccinated mice ($P < 0.05$) was detected upon stimulation with *P. chabaudi adami*-IRBCs compared to that of spleen cells taken from VR1020/30K- and MCP-3/30K-vaccinated mice and stimulated with control RBCs (Fig. 1). In contrast, significant levels of proliferation were not detected with spleen cells obtained from CTLA4/30K-vaccinated mice upon stimulation with IRBCs ($P = 0.17$) compared to that of spleen cells obtained from CTLA4/30K-vaccinated mice and stimulated with control RBCs. Spleen cells obtained from control mice vaccinated with the MCP-3 or CTLA4 vector alone and stimulated with IRBCs (Fig. 1) or RBCs (data not shown) showed no detectable proliferation. However, spleen cells obtained from VR1020 vector-vaccinated control mice did proliferate to a very low extent above background levels upon stimulation with IRBCs (but not RBCs), but the levels of proliferation observed were signifi-

cantly lower than those of IRBC-stimulated spleen cells from VR1020/30K-vaccinated mice ($P = 0.0128$) (Fig. 1).

Splenocytes taken from VR1020/30K-vaccinated mice and stimulated with IRBCs produced significantly higher levels of IFN-γ than splenocytes from control mice vaccinated with vector DNA ($P < 0.05$) (Fig. 2A). When stimulated with IRBCs, spleen cells obtained from MCP-3/30K-vaccinated mice also produced detectable levels of IFN-γ in the culture supernatant ($P < 0.05$) (Fig. 2A). No IFN-γ was detected in culture supernatants of splenocytes taken from CTLA4/30K-vaccinated mice and stimulated with IRBCs (Fig. 2A) or in culture supernatants of splenocytes taken from control mice vaccinated with VR1020, MCP-3, and CTLA4 vector DNA and stimulated with IRBCs (Fig. 2A) or RBCs (data not shown).

IL-4 was detected in three out of five culture supernatants from spleen cells primed with the VR1020/30K and MCP-3/30K genomic libraries and stimulated with IRBCs (Fig. 2B). No IL-4 was detected with CTLA4/30K-primed IRBC-stimulated splenocytes or in supernatants of splenocytes from control mice vaccinated with VR1020, MCP-3, and CTLA4 vector DNA that were stimulated with IRBCs or RBCs (data not shown).

**Phagocytosis by macrophages of *P. chabaudi adami* DS-IRBCs after incubation with sera from mice vaccinated with each of the genomic libraries.** i.d. DNA vaccination with any of the genomic expression libraries did not produce humoral responses that were detectable when sera were evaluated by ELISA using soluble blood-stage *P. chabaudi adami* lysate as the antigen (data not shown). However, the method of gene gun vaccination has been shown to produce a strong humoral
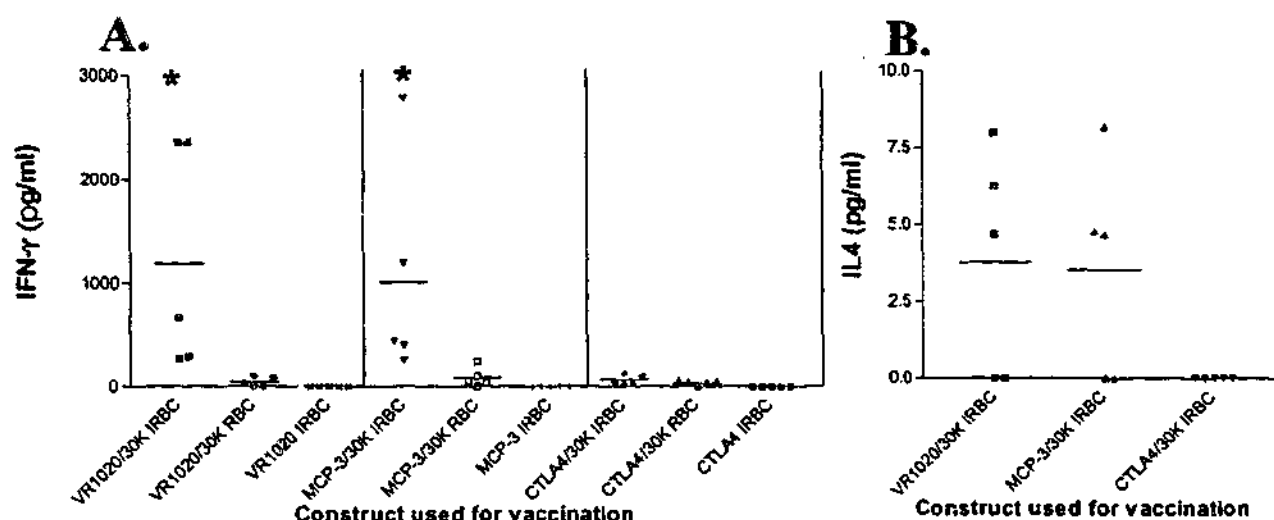
FIG. 2. IFN-γ (A) and IL-4 (B) production by splenocytes from BALB/c mice vaccinated with the VR1020/30K, MCP-3/30K, and CTLA4/30K libraries. Splenocytes from individual mice were cultured as described in the legend to Fig. 1. After 72 h of stimulation with $2 \times 10^6$ IRBCs or 2 $\times 10^6$ RBCs, supernatants were harvested and analyzed by ELISA for the presence of IFN-γ and IL-4. Splenocytes from mice vaccinated with empty vector control DNA (VR1020, MCP-3, or CTLA4) were also stimulated with $2 \times 10^6$ RBCs, but IFN-γ and IL-4 responses were not observed (data not shown). Unstimulated splenocytes did not produce detectable IFN-γ or IL-4. The mean level of IFN-γ or IL-4 production is shown (bars). The raw data were $\log_{10}$ transformed and compared by using the Student $t$ test. *, $P < 0.05$.

response after delivery of plasmids containing known antigens in many different vaccine models (reviewed in reference 27). Antibody-mediated opsonization of IRBCs, and subsequent internalization and destruction by macrophages, has been shown to be a factor contributing to a reduction in parasitemia during crisis in the *P. chabaudi* mouse model (25, 26).

To determine whether vaccination with the *P. chabaudi adami* genomic expression libraries could induce opsonizing antibodies, sera were taken before challenge from mice vaccinated with each library. The prechallenge sera were tested for the ability to promote opsonization of *P. chabaudi adami*-IRBCs by BALB/c macrophages. Figure 3 shows that sera obtained from individual mice vaccinated with each of the genomic libraries have the ability to enhance uptake and promote destruction of IRBCs by macrophages. The percentage of macrophages ingesting IRBCs was significantly higher in incubation mixtures containing macrophages treated with sera taken from mice vaccinated with VR1020/30K ($P < 0.01$), MCP-3/30K ($P < 0.001$), and CTLA4/30K ($P < 0.05$) than in incubation mixtures containing sera from control mice vaccinated with vector DNA (Fig. 3A). For comparison, Fig. 3B shows the percentages of macrophages ingesting IRBCs after incubation with a pool of sera from six mice vaccinated with the malarial blood-stage antigen MSP4/5 (fused to the MCP-3 vector) used as a positive control. We have shown that this construct can significantly enhance antibody responses to MSP4/5 (29).

To confirm that macrophage uptake of IRBCs was antibody-mediated, CD16/Fcγ II and CD32/Fcγ III receptors on macrophages were blocked by incubation of macrophages with the specific monoclonal antibody MAb93 (as described in Materials and Methods); antibodies directed against the CD16/Fcγ II and CD32/Fcγ III receptors have been shown to block Fc receptor binding by immunoglobulin G (IgG) antibodies and inhibit effector functions (17, 34, 35). Figure 3C shows that incubation of macrophages with the anti-Fcγ antibody MAb93

significantly inhibits the percentage of macrophages ingesting IRBCs opsonized with sera from mice vaccinated with the genomic libraries (VR1020/30K, MCP-3/30K, and CTLA4/30K). This result demonstrates that opsonizing antibodies are indeed present in sera of mice after genomic library vaccination and that these antibodies are the major component in the sera responsible for macrophage uptake of IRBCs.

**Efficacy of protection induced by DNA vaccination with genomic expression libraries against lethal challenge with blood-stage *P. chabaudi adami* DS.** The *P. chabaudi adami* DS mouse model is a stringent test for vaccine efficacy due to the high virulence of the DS strain (2, 9, 31). In order to determine the protective efficacy of the VR1020/30K, MCP-3/30K, and CTLA4/30K genomic libraries against a virulent challenge, three separate challenge trials were conducted with BALB/c mice (Table 2). It has been previously shown with the *P. chabaudi adami* DS BALB/c mouse model that the VR1020/30K library induces significant (albeit partial) protection against lethal challenge (31).

The first challenge trial included 10 mice per group. Mice were vaccinated i.d. with a gene gun a total of three times at 2-week intervals and challenged 2 weeks after the final vaccination with $10^5$ *P. chabaudi adami*-IRBCs. Trial 1 involved a comparison of the CTLA4/30K and VR1020/30K libraries. The VR1020/30K library was used as a positive control based on previous experiments where this vaccine has induced 30 to 60% protection against lethal challenge (31). Although there was a significant reduction in peak parasitemia in mice vaccinated with both the VR1020/30K and CTLA4/30K libraries compared to those vaccinated with the empty CTLA4 control vector (Table 2), this result was not reproduced in trial 3 (see below). While there were no significant differences between survival curves (i.e., delays in death) of control and vaccinated mice, increased survival was observed in the group vaccinated with the VR1020/30K library (Fig. 4A), and this result was
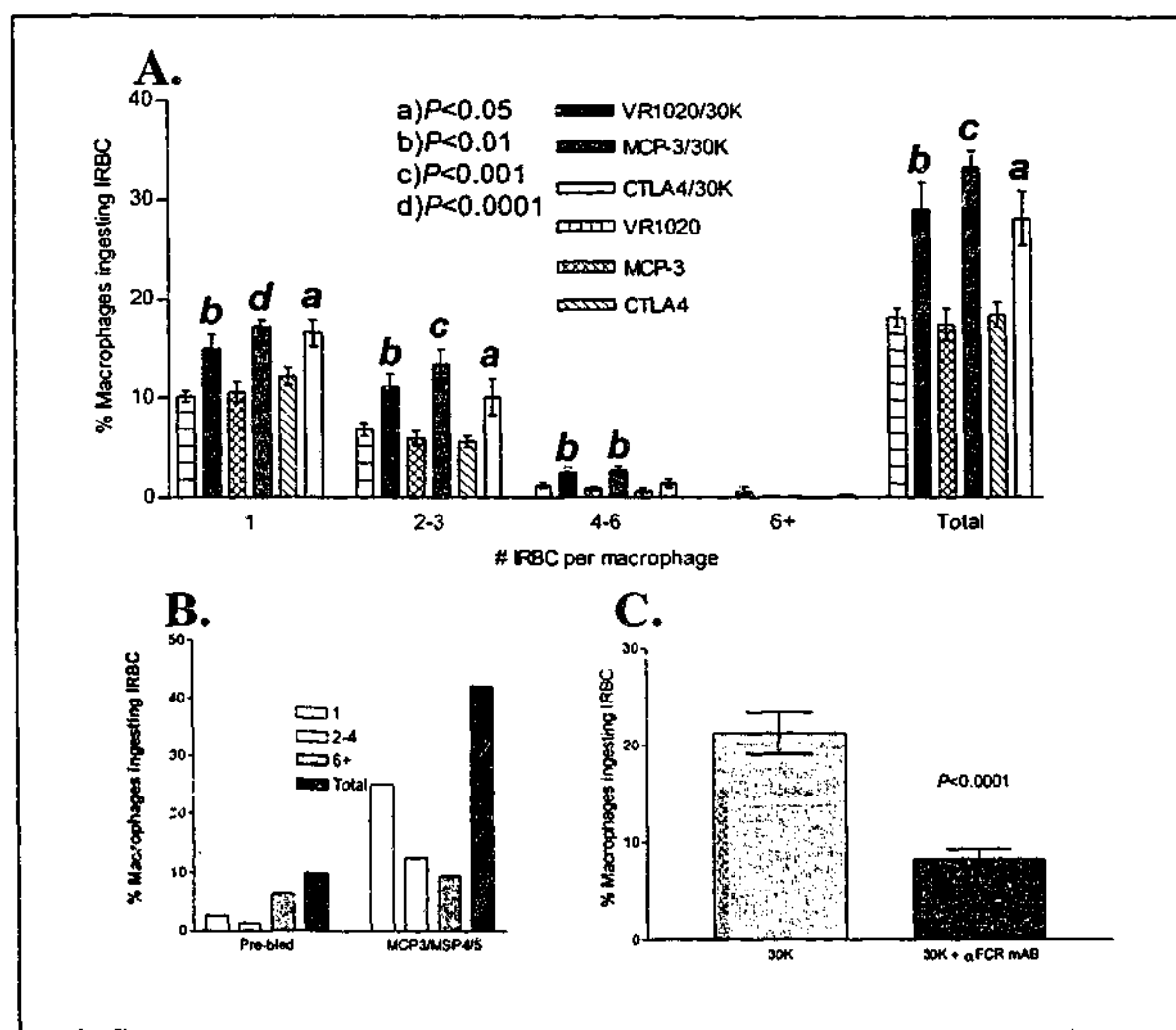
FIG. 3. Phagocytosis of *P. chabaudi adami*-IRBCs preincubated with sera from mice vaccinated with VR1020/30K, MCP-3/30K, and CTLA4/30K (and control vector DNA). (A) Percentage of macrophages phagocytosing IRBCs after preincubation with sera. All six groups contained 10 BALB/c mice each. The values are expressed as percentages of macrophages containing IRBCs out of a total of 200 counted. The number of IRBCs contained within an individual macrophage for each vaccine group is also shown. Data are expressed as means ± standard errors of the means. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$; d, $P < 0.0001$. (B) Percentage of macrophages ingesting IRBCs after incubation with prebleed pooled sera ($n = 6$) and pooled sera from mice vaccinated with MCP-3/MSP4/5 DNA ($n = 6$) as a positive control (29). (C) Incubation of macrophages with a monoclonal antibody specific for Fcγ II and Fcγ III receptors inhibits phagocytosis of IRBCs. Macrophages were treated with a α-Fcγ monoclonal antibody, followed by incubation with IRBCs previously exposed to sera from mice vaccinated with each genomic library (30K + α-FCR mAB), or they were left untreated and incubated with IRBCs exposed to sera from vaccinated mice as a positive control (30K). The data shown are expressed as means ± standard errors of the means of the percentages of macrophages ingesting IRBCs. Five serum samples from mice vaccinated with each of the VR1020/30K, MCP-3/30K, and CTLA4/30K libraries were tested, giving a total of 15 individual samples. Treated (30K + α-FCR mAB) and untreated (30K) groups were compared by using the Student *t* test.

repeated in trial 3 (Fig. 4Ci). In contrast, the CTLA4/30K vaccine did not induce increased survival (Fig. 4A).

The second trial involved the evaluation of the MCP-3/30K genomic library. Although there was a drop in mean peak parasitemia level in the MCP-3/30K group compared to that in the MCP-3 vector control group, the difference was not statistically significant due to the large variation in parasitemia levels between individual mice (Table 2). A significant delay in death was observed in mice vaccinated with the MCP-3/30K library compared to that in mice vaccinated with the MCP-3 control vector ($P = 0.05$) (Fig. 4B). Half of the mice vaccinated with the MCP-3/30K library survived until day 12 postinfection, with two mice surviving to the conclusion of the trial at day 14.

No MCP-3 vector control mice survived, with all mice dying by day 10 postinfection.

The third challenge trial directly compared the efficacy of the VR1020/30K, MCP-3/30K, and CTLA4/30K genomic libraries. Although there was a reduction in the mean peak parasitemia level for all mice vaccinated with the genomic libraries compared with that of their respective controls (mice vaccinated with vector-only DNA), the difference among groups was not statistically significant (Table 2). However, there was a significant reduction in peak parasitemia in surviving mice (in the MCP-3/30K- and VR1020/30K-vaccinated groups) compared with that in nonsurviving mice (Table 2). Of the mice vaccinated with the VR1020/30K and MCP-3/30K

TABLE 2. Protocol and parasitemia measurements for mice challenged with lethal *P. chabaudi adami* DS and percentages of survivors over three challenge trials

| Group | No. in group | Mean peak parasitemia (%) ± SD | No. of survivors (%) |
|---|---|---|---|
| Trial 1 | | | |
| CTLA4 | 10 | 42 ± 6 | 3 (30) |
| CTLA4/30K | 10 | 35 ± 7[a] | 3 (30) |
| VR1020/30K | 10 | 35 ± 4[a] | 5 (50) |
| Nonsurvivors | 19 | 36 ± 8 | |
| Survivors | 11 | 37 ± 5 | |
| Trial 2 | | | |
| MCP-3 | 6 | 40 ± 13 | 0 (0) |
| MCP-3/30K | 6 | 31 ± 15 | 2 (33)[b] |
| Nonsurvivors | 10 | 39 ± 12 | |
| Survivors | 2 | 34 ± 2 | |
| Trial 3 | | | |
| VR1020 | 6 | 45 ± 11 | 0 (0) |
| VR1020/30K | 6 | 37 ± 5 | 3 (50)[c] |
| MCP-3 | 6 | 43 ± 7 | 0 (0) |
| MCP-3/30K | 6 | 29 ± 18 | 3 (50) |
| CTLA4 | 6 | 41 ± 7 | 0 (0) |
| CTLA4/30K | 6 | 38 ± 6 | 0 (0) |
| Nonsurvivors | 30 | 39 ± 11 | |
| Survivors | 6 | 25 ± 14[a] | |

[a] $P < 0.05$; Student's $t$ test.
[b] $P = 0.05$; Mantel-Haenszel test for comparison with MCP-3 vector.
[c] $P < 0.01$; Mantel-Haenszel test for comparison with VR1020 vector.

libraries, 50% survived, and there was a significant delay in death ($P < 0.05$) in the VR1020/30K-vaccinated group versus that in the VR1020 vector control group (Fig. 4Ci and Cii). In contrast, the CTLA4/30K library did not protect mice against lethal *P. chabaudi adami* challenge (Fig. 4Ciii).

## DISCUSSION

Protection of mice against lethal *P. chabaudi adami* DS challenge by using ELI has been previously demonstrated (31). The aim of the present study was to determine whether the efficacy of ELI could be enhanced by using targeting vectors and to assess the immune responses induced by ELI in mice in order to confirm that ELI induces an antigen-specific response. Both humoral and cellular responses have been found to be required for resolution of infection with *P. chabaudi* in mice (reviewed in references 21 and 36). The data presented demonstrate that genomic ELI can induce cellular as well as humoral responses to native *P. chabaudi adami* DS antigens following vaccination. The data also show that protection against lethal *P. chabaudi adami* DS challenge is dependent on the type of DNA expression vector used, with survival correlating with in vitro cellular responses that were induced by vaccination with the VR1020 and MCP-3 libraries. The ELI vaccines promote significant survival, rather than reductions in parasitemia, in challenged mice, which is an important observation due to the high lethality associated with a *P. chabaudi adami* DS challenge (2, 21).

Vaccination of BALB/c mice with the VR1020/30K or MCP-

3/30K library induced a specific cellular immune response to native antigens shed by IRBCs which was characterized by a significant increase in splenocyte proliferation, as well as by detectable levels of IFN-γ and IL-4 secretion. The ability of the VR1020/30K and MCP-3/30K genomic libraries to promote proliferation of splenocytes in response to live *P. chabaudi adami*-IRBCs demonstrates that the genomic approach to vaccination is specific to epitopes or antigens produced by the parasite and not an artifact produced by nonspecific stimulation of the immune system by foreign DNA. The presence of cellular responses in vitro induced by *P. chabaudi adami* ELI correlates with the protection observed in vivo after lethal challenge with blood-stage parasites, as significant splenocyte proliferation was not observed after vaccination with the nonprotective CTLA4/30K library. The production of opsonizing antibodies to promote phagocytosis in vitro (regardless of the vector used) revealed another possible mechanism by which parasite clearance after ELI may occur.

The nature of the epitopes or antigens that are recognized by splenocytes from vaccinated mice is clearly of interest, as this may identify sequences in the library encoding protective antigens. Sequencing of a sample of 664 plasmids derived from the VR1020/30K pool showed that open reading frames, predicted to encode peptides of various sizes (with many homologous to *P. falciparum* sequences), are indeed present in the library (unpublished data). Recently it has been shown that peptides of 20 aa synthesized to span the length of MSP1 (38) could induce T-cell proliferation in mice. Multiple epitopes were recognized in two strains of mice, with two epitopes discovered that were able to induce effector T cells capable of delaying growth of lethal *P. yoelii* YM following adoptive transfer into immunodeficient mice without inducing detectable antibody responses (38). In addition, peptides were able to protect mice against *P. yoelii*, suggesting that the T-cell epitopes may be useful as a vaccine against *P. yoelii* (38) Given that the plasmids in the genomic library will encode a variety of in-frame peptides, a major contributory factor to protection observed with ELI is possibly due to T-cell help delivered by the library.

Although there was a response to IRBC stimulation in the VR1020/30K- and MCP-3/30K-vaccinated mice, significant levels of splenocyte proliferation did not occur when mice were vaccinated with the CTLA4/30K library. This result is curious as all three libraries were constructed by the same method with the same stock of *P. chabaudi adami* genomic DNA and show similar properties in terms of coding capacities. One possibility is that the nature of the CTLA4 vector itself may be contributing to the lack of proliferation. The CTLA4 vector expresses proteins as fusions with the CTLA4-human Ig moiety (approximately 60 kDa), and the fusion partner is thus considerably larger than the fused *P. chabaudi adami* sequences, based on a peptide of size 20 to 100 aa. It is possible that many of the epitopes contained within the CTLA4/30K pool may have been obscured upon folding of the large CTLA4 moiety and poorly processed or presented to the immune system. CTLA4 in vivo is a T-cell receptor involved in cell signaling and is known to be a negative regulator of T-cell activation (reviewed in reference 30); however, very high doses of CTLA4 (50 μg) are required to completely suppress the immune response in mice (23). DNA vaccines producing CTLA4 fusion proteins have previ-
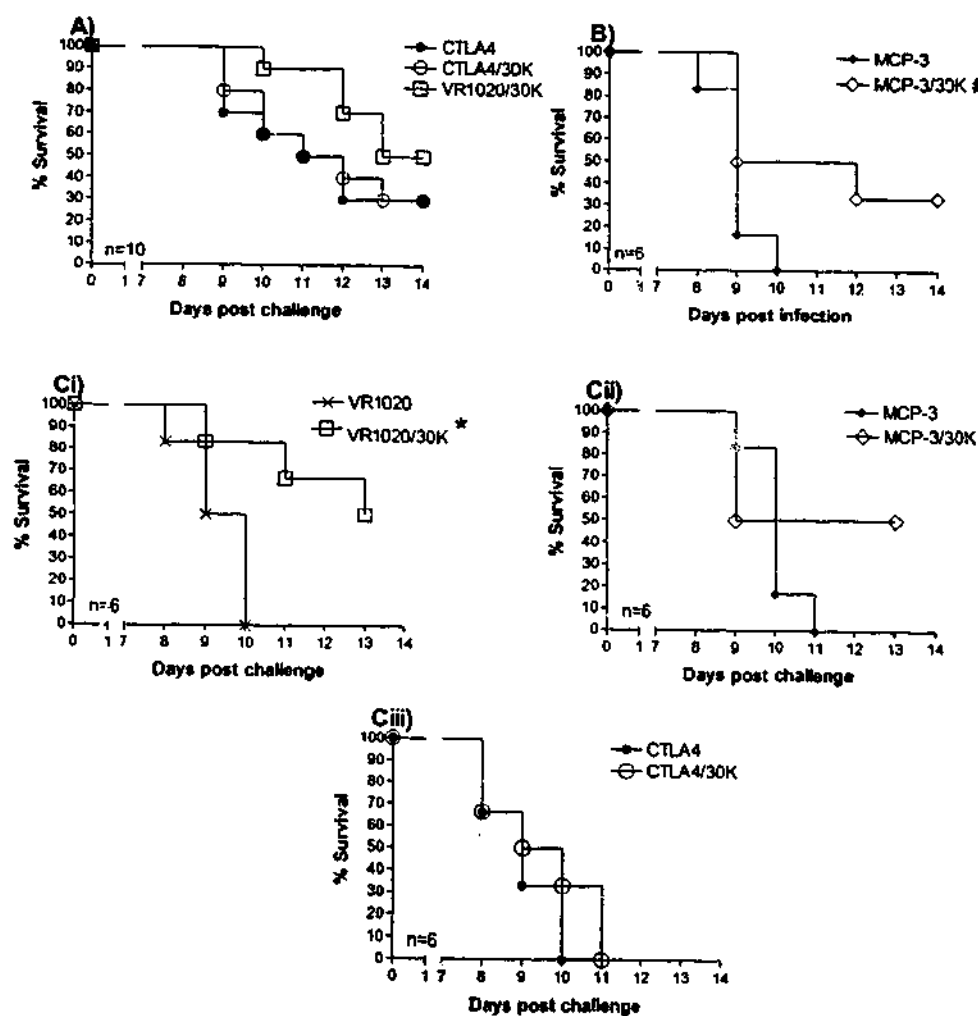
FIG. 4. Survival curves of mice vaccinated with VR1020/30K, MCP-3/30K, and CTLA4/30K genomic libraries and challenged with virulent *P. chabaudi adami*. Mice were vaccinated three times at 2-week intervals via the gene gun, followed by challenge with 100,000 *P. chabaudi adami*-IRBCs 2 weeks later. (A) Evaluation of efficacies of CTLA4/30K and VR1020/30K libraries. (B) Evaluation of efficacy of MCP-3/30K library. (C) Comparative evaluations of all three genomic libraries. *, $P = 0.05$; *, $P < 0.05$ compared to empty control vectors (Mantel-Haenszel test).

ously been reported to enhance antibody titers in mice and sheep (6, 11, 13, 22, 29). However, not all cases of the use of CTLA4 fusion vaccines have resulted in enhanced cellular responses (11), as was found in the present experiment. Overall, although the reason underlying the poor responsiveness induced by the CTLA4 library is not clear, the result shows that this vector is not useful for delivering malaria ELI vaccines in mice.

Gene gun vaccination with the genomic libraries did not produce humoral responses detectable upon ELISA analysis of sera, presumably due to the diverse repertoire of *P. chabaudi adami* sequences delivered in the libraries, as seen previously (31). Therefore, another method, opsonization, was used to establish the presence of antibody after vaccination and to further explore protective mechanisms mediated by genomic vaccination. Vaccination with all the genomic libraries constructed produced sera with significantly enhanced uptake of IRBCs by macrophages in vitro compared with that of IRBCs incubated with sera from empty vector-vaccinated control animals. In *P. chabaudi* infections, it has been shown that the antibody-mediated immune response is directed primarily to epitopes on the surface of IRBCs, with these antibodies en-

hancing phagocytosis and subsequent destruction of IRBCs in vitro (25, 26). In infected mice, IgG2a and IgG3 antibodies are dominant during ascending primary parasitemia and help to promote phagocytosis and opsonization of IRBCs (reviewed in reference 37). Opsonizing activity found in sera from immune individuals has also been associated with protection against *P. falciparum* (15). Our results show that ELI is able to induce opsonizing antibodies that may contribute to the efficacy of the VR1020/30K and MCP-3/30K vaccines.

The MCP-3 and CTLA4 vectors were used in an attempt to enhance the efficacy of genomic vaccination above the levels obtained by using the secretory VR1020 expression vector (31), since this enhancement would have made subsequent devolution of the libraries an easier task. Recently, we have shown that i.d. DNA vaccination with MSP4/5 fused to MCP-3 can significantly enhance survival in mice after challenge with blood-stage *P. chabaudi adami*, although there was no significant reduction in peak parasitemia compared to that in control animals (29). This construct also produced significant increases in antibody compared to a VR1020/MSP4/5 construct. Comparison of the efficacies of the VR1020/30K (secretory), MCP-3/30K (chemoattractant), and CTLA4/30K (lymph node-tar-

geting) libraries emphasized the need for cellular as well as humoral responses to protect against lethal *P. chabaudi adami* challenge. However, vaccination with the MCP-3/30K library did not significantly enhance any of the parameters measured (protection, splenocyte proliferation, opsonization, and IFN-γ and IL-4 production) compared to vaccination with the VR1020/30K library. The use of MCP-3 as a fusion partner was not detrimental to the library's protective efficacy, but this was not the case when mice were vaccinated with the CTLA4/30K library. Similarly, i.d. DNA vaccination with MSP4/5 fused to CTLA4 did not protect mice after challenge with blood-stage *P. chabaudi adami* (29). Clearly, the choice of fusion partner is important when utilizing targeting vectors in DNA vaccine studies. The results of the present study demonstrate that standard secretory vectors are sufficient to promote protective immune responses by using a malarial genomic library.

Priming of mice with the CTLA4/30K library did not promote significant splenocyte proliferation specific to *P. chabaudi adami*-IRBCs, which may have contributed to the lack of protection observed in the present study. Sera obtained after vaccination with CTLA4/30K did however promote phagocytosis of IRBCs in vitro. This suggests the possibility that CTLA4/30K vaccination primed the immune system with enough T- and B-cell epitopes to produce antibody and promote phagocytosis, but this response was not sufficient to protect mice from death.

The combination of both specific cellular immune responses and opsonization of IRBCs by macrophages are two possible mechanisms which may be responsible for the protection observed with the VR1020/30K and MCP-3/30K libraries. The detection of IFN-γ and IL-4 in splenocyte cultures of mice vaccinated with these two libraries suggests priming of Th1 and Th2 cell subsets within the spleen. With *P. chabaudi* it has been shown that splenic CD4$^+$ T cells purified from immunologically intact mice during ascending parasitemia produce high levels of IFN-γ and IL-2 to limit infection, while IL-4 and IL-10 are produced by splenic CD4$^+$ T cells during descending primary parasitemia (33). B-cell-deficient mice, however, are not able to mount a Th2 response sufficient to completely resolve the primary parasitemia (33). Acute *P. chabaudi adami* infections in B-cell-deficient mice are suppressed at the same rate as those in normal mice, but complete depletion of CD4$^+$ T cells results in the inability to control parasite growth, emphasizing the importance of CD4$^+$-T-cell activation during blood-stage infection (reviewed in reference 37).

With these results, a mechanism by which ELI might induce a protective response has been established. The results suggest the possibility that the genomic libraries encode previously undiscovered combinations of malarial epitopes or whole antigens which are protective. We have now begun exploiting the synergistic properties of the VR1020/30K library, in combination with known antigens, by codelivery in bicistronic vectors. This has resulted in a significant reduction in peak parasitemia in challenged mice covaccinated with the 30K vaccine and known antigens, and this reduction is greater than that observed with the VR1020/30K library alone. Thus, the 30K vaccine may allow us to identify new unknown antigens that synergize with known antigens. The ELI vaccine may thus represent a method to mimic the multivalent nature of the acquired immune response seen in resistant malaria-exposed humans. Mimicking this response has been proposed to be the best approach to produce an effective malaria vaccine (12). Our laboratory is now in the process of dividing a subpool of the original 30,000 plasmids contained within the VR1020/30K library into groups based on predicted functions (epitope types and homology with known malaria parasite sequences) and sizes, and these pools are being evaluated in vaccination experiments in an attempt to further define the protective epitopes in the vaccine.

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# Appendix III

**Expression library immunization protects mice against a challenge with virulent rodent malaria.**

**Smooker, P. M., Y. Y. Setiady, A. Rainczuk, and T. W. Spithill**

# Expression library immunization protects mice against a challenge with virulent rodent malaria

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## Abstract

Although several candidate vaccine antigens have been developed for malaria, there is as yet no effective single vaccine available. There is a growing consensus that the ultimate malaria vaccine will be multivalent, requiring the identification of a suitable cocktail of antigens. However, evaluation of the multitude of potential malaria vaccine antigens in suitable combinations is a daunting task. Here we describe the validation of expression library immunization (ELI) as a tool for the discovery of sequences protective against malaria infection. A genomic *Plasmodium chabaudi* expression library was constructed comprising ten separate pools of 3000 plasmids. Over three vaccine trials using biolistic delivery of pools composed of 616 to 30,000 plasmids we report up to 63% protection of mice from a challenge with *P. chabaudi adami DS*, a highly virulent strain. Overall, ELI protected 36% of vaccinated mice against virulent challenge compared with only 3.2% survival of control mice. These results demonstrate that ELI is a suitable approach for screening the malaria genome to identify the components of multivalent vaccines. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Expression library immunization; DNA vaccines; Murine malaria; *Plasmodium chabaudi*

## 1. Introduction

Each year between 1.5–2.7 million people die from malaria (usually *P. falciparum*) infection, and a further 300–500 million people contract the disease [1]. Over a number of years, significant advances in the development of vaccines against malaria have been made in laboratory animals, non-human primates and in human volunteers [2,3]. Several candidate antigens are undergoing clinical testing in humans although, as yet, there is no effective, generally applicable vaccine.

The advent of nucleic acid vaccination has delivered another tool for the development of malaria vaccines [4]. For example, studies with malaria vaccine candi-

dates in Hoffman's laboratory showed that vaccination of mice with a DNA vaccine encoding CSP1 from *P. yoelii* protects against subsequent sporozoite infection [5]. Furthermore, vaccination of mice with DNA encoding two liver stage antigens (CSP1 and HEP17) can enhance this protection above that achieved with either antigen alone, and also enables mouse strains of a wide range of haplotypes to be protected, thereby overcoming genetic restriction [4,6]. Further progress is being made using multivalent vaccines based on known antigens. It has been shown that delivery of four *P. falciparum* antigens (as DNA vaccines) into rhesus monkeys can induce CTL responses against each of the encoded proteins [7], and that the vaccination of mice with four DNA plasmids encoding *P. falciparum* proteins resulted in the induction of antibodies against each protein [8]. Shi et al. [9] showed that a multicomponent, multistage recombinant protein can induce antibodies that inhibit both sporozoite

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invasion of hepatoma cells and growth of blood-stage parasites. These results demonstrate that a multivalent DNA vaccine is possible, and that it may be necessary in order to overcome genetic restriction in the human population and to generate immunity against multiple stages of the parasite life-cycle. Although the existing candidate antigens may ultimately prove to be sufficient for inducing protection in humans, the development of the optimal multivalent vaccine may require the testing of as yet unidentified gene products in novel combinations. Each gene identified in the *P. falciparum* genome sequencing project can be tested individually or in concert with a number of other genes [10]. However, this will be laborious since, for routine testing of protective efficacy in murine models the homologous genes from rodent malaria strains must first be isolated. As an approach to vaccine identification we have elected to utilise a novel method, ELI, which will enable the rapid screening of the malarial genome without prior knowledge of the antigens encoded by the genomic DNA.

ELI was first applied to protect mice against *Mycoplasma pulmonis* by vaccination with cloned genomic DNA in pools of 3000–27,000 plasmids [11,12]; a cumulative dose of less than 1 ng of each plasmid was sufficient to induce protective responses. Here we describe the evaluation of ELI as an approach to malaria vaccine discovery. Validation of this technology requires a demonstration that vaccination with an expression library can protect against subsequent malaria infection. We have chosen to use the *P. chabaudi adami DS*-BALB/c mouse model, which yields an extremely stringent test (survival) to the applicability of ELI. Libraries were constructed using genomic DNA, which has the disadvantage compared to cDNA of including non-coding DNA but the marked advantage of including all antigens from all stages of the *Plasmodium* life-cycle. Thus, it will be possible to not only test for efficacy against blood-stage infection (as described here) but every stage of the parasite life-cycle.

## 2. Materials and methods

### 2.1. Creation of plasmid pools

*P. chabaudi adami DS* genomic DNA was isolated from Ficoll-purified erythrocytes of infected BALB/c mice (parasitemia 20–30%). The purified DNA was partially digested with *Tsp*509I and the digested fraction between 1 and 3 kbp isolated by gel elution. VICAL vector VR1020 [13] was prepared by digestion with *Bam*HI and *Bgl*II and the insertion of an *Eco*RI linker constructed from the oligonucleotides 5'GATCCGGGAATTCAA and 5'GATCTT-

GAATTCCCG. After creation, the new vector was digested with *Eco*RI, treated with alkaline phosphatase and ligated with *Tsp*509I digested *P. chabaudi* genomic DNA. Ligation mixes were transformed into *E. coli* DH5α and selected on solid media containing 50 μg/ml kanamycin. After overnight growth *E. coli* colonies were combined into pools. A total of 10 pools (termed 3KA-3KJ) each comprising approximately 3000 individual clones were constructed and stored at −80°C as glycerol stocks. At a later stage, one of these pools (3KA) was replated and 3080 individual colonies picked into microtitre wells. Pools of 616 of these clones were used for vaccination in Trial 3.

### 2.2. Isolation of plasmid DNA and construction of vaccination cartridges

Library pools stored as glycerol stocks were grown to confluence on solid media prior to inoculation into liquid media. 5–10 confluent plates were used to inoculate one litre and grown with shaking at 37°C for 6 h prior to harvest. Plasmid preparation and endotoxin removal was performed as described by Boyle et al. (1998) [14]. Briefly, DNA was purified from a cleared lysate by PEG8000 precipitation and endotoxin was removed by three extractions with Triton-X114. Purified DNA was precipitated onto gold microcarriers and these attached to plastic supports as per manufacturer's recommendations (Bio-Rad Laboratories, USA). DNA was combined with carriers at a ratio of 100 μg DNA/50 mg carriers (except for Trial 2, group 8, where 250 μg DNA was used). Each projectile contains approximately 1 μg DNA (2.5 μg Trial 2, group 8). It was noted that the use of excess DNA resulted in significant clumping of the gold microcarriers, which may have reduced the efficiency of subsequent vaccination.

### 2.3. Mice and vaccinations

All mice were BALB/c, female, and 5–6 weeks of age at the time of first vaccination. For vaccination the abdominal region was shaven and particles delivered by the Helios gene gun (Bio-Rad Laboratories, USA) with a pulse of helium gas at 400 psi.

### 2.4. Infection of mice, blood sampling and parasitemia measurements

Blood from an infected mouse with a known parasitemia (1–10%) was taken and immediately diluted in PBS to give the required dosage ($1 \times 10^5$ infected RBC/dose). Mice were infected by intraperitoneal infection at day 0, and parasitemia assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa

staining of tail smears. Mean peak parasitemia levels and day to peak parasitemia were compared using the Mann–Whitney non-parametric $t$-test.

## 3. Results

### 3.1. Characterisation of library clones

To construct the sibling libraries (pools of plasmids, abbreviated as "pools" in this report), *P. chabaudi* genomic DNA was isolated, digested and cloned into a modified VR1020 vector containing an *Eco*RI restriction site. Peptides resulting from the in-frame insertion of *P. chabaudi* DNA are secreted from host cells by virtue of the TPA signal peptide encoded by VR1020 sequences, as we have previously shown for *Fasciola* glutathione *S*-transferase [15]. Ten pools (A–J) of 3000 clones each were constructed, and these plasmid pools were evaluated by vaccination trials in this study. One protective pool (3KA) was re-plated and clones picked and stored individually. Several clones from this pool were examined in a number of ways, including the sequence analysis of 24 randomly selected clones (Table 1). The majority of in-frame peptides encoded were of a size less than 20 amino acids. It is expected that a substantial number of these peptides would be due to the fortuitous encoding of nonsense amino acids, which soon terminate at a stop codon (usually UAA) due to the high $A+T$ content of *P. chabaudi* DNA. However, 13% of clones encode in-frame peptides greater than 50 amino acids. As it is expected that no more than 1 in 6 (16%) of all clones will be inserted in-frame, many of these longer reading frames should encode authentic *P. chabaudi* peptides. We are currently undertaking a large scale sequence analysis project comprising all plasmids in pool 3KA-4, and these results will be reported elsewhere.

### 3.2. Trial 1

In the first trial pools were tested in combinations as follows: 3000 members (3 K, pool A); 9000 members (9 K, pools B,C,D); 30,000 members (30 K, pools A–J). Plasmid DNA was isolated from each of these combined pools, endotoxin removed and the DNA used to vaccinate BALB/c mice using the Helios gene gun, as previously described [15]. A total of six vaccinations were performed at weeks 0, 3, 6, 9, 12 and 17. Humoral responses to *P. chabaudi* late trophozoite-stage antigen were assessed after the second vaccination, and again after each subsequent vaccination. Detectable humoral responses did not develop, and therefore the mice were challenged after the sixth vaccination. Two control groups were also included (Table 2): VR1020 (expression vector with no insert) and VR1020GST (an irrelevant protein) [15]. The VR1020GST group was included to ensure that a protective response was not due simply to the expression of any encoded protein. These mice developed antibody responses to *F. hepatica* GST (data not shown) as previously described [15].

Five weeks after the final vaccination, mice were challenged with $1 \times 10^5$ *P. chabaudi*-infected erythrocytes, delivered by intraperitoneal injection. Parasitemias were monitored by Giemsa staining of blood smears from day 6 post-infection. As shown in Fig. 1a, all control animals (VR1020 and VR1020GST) succumbed to the infection by day 13. However, in each of the three groups vaccinated with *P. chabaudi* plasmid pools there were surviving animals (Fig. 1B). A total of 6 animals of the 28 that were vaccinated with *P. chabaudi* pools survived (21%), with the highest level of survival observed in groups of mice vaccinated with 30,000 plasmids. Table 2 shows that the peak parasitemia and day of peak parasitemia values were not significantly different between control groups and those vaccinated with *P. chabaudi* DNA. However, when the mean peak parasitemia of surviving animals (48%) is compared to non-survivors (57%), there is a

Table 1
Features of *P. chabaudi* library clones

| | |
|---|---|
| Plasmid pools created | 10 × 3000 clones (3KA–3 KJ) |
| Plasmid clones examined by restriction digestion | 58[a] |
| Number with inserts | 58 (100%) |
| Plasmid clones examined by sequencing | 24 |
| Number with reading frame expressing peptide[b] | 18 (75%) |
| Size range of encoded peptides[c] | 1–115 amino acids |
| Number greater than 20 aa | 10 (42%) |
| Number greater than 50 aa | 3 (13%) |
| Average size of encoded peptide | 25 amino acids |

[a] All clones examined were from pool 3KA.

[b] In-frame with the TPA signal sequence.

[c] Not including the TPA signal sequence.

significant difference, with survivors having on average a 9% reduction in peak parasitemia ($p < 0.01$). This suggests that limiting the degree of parasitemia by vaccination enhances the survival rate of mice.

### 3.3. Trial 2

In order to extend and confirm the observations for Trial 1 a second trial was conducted in which the pools of 3000 and 30,000 clones were re-tested. Several additional control groups were included, the most critical being the pool of 3000 clones in which the insert DNA had been excised from the plasmid by restriction enzyme digestion prior to loading the DNA onto gold particles (Table 2). This group was designed to eliminate the presence of *P. chabaudi* DNA itself as a factor in any protective response, as the DNA will be bound to gold particles and delivered to the tissues but no peptides can be expressed. One further control group was the co-delivery of the 30,000 clone pool at a higher dose (2.5 µg), to determine if there was a dose-response effect. Finally, in an attempt to stimulate the switch to a Th2 response (proposed to be involved in the resolution of parasitemia [16]) a plasmid encoding IL4 was co-delivered with some *P. chabaudi* pools [17].

In order to simplify the vaccination protocol, mice were given three vaccinations at three-weekly intervals and challenged 3 weeks after the final vaccination.

Table 2
Parasitemia measurements from mice challenged with *P. chabaudi adami* DS. Parasitemias were determined daily and the mean peak parasitemia determined for each group. *p*-values were determined using the Mann–Whitney non-parametric *t*-test

| Group | No. in group | Peak parasitemia | Day of peak | Survivors (%) |
|---|---|---|---|---|
| **Trial 1** | | | | |
| 1. VR1020 | 5 | $52 \pm 11$ | $8.8 \pm 0.84$ | 0 |
| 2. VR1020GST | 4 | $58 \pm 10$ | $8.8 \pm 0.79$ | 0 |
| 3. 3 K | 10 | $56 \pm 8.2$ | $9.3 \pm 1.3$ | 2 (20%) |
| 4. 9 K | 8 | $57 \pm 4.7$ | $9.0 \pm 0.99$ | 1 (13%) |
| 5. 30 K | 10 | $56 \pm 9.9$ | $9.9 \pm 1.6$ | 3 (30%) |
| *Summary* | | | | |
| Non-survivors | 31 | $57 \pm 8.3$ | $9.0 \pm 1.2$ | |
| Survivors | 6 | $48 \pm 5.3^a$ | $9.8 \pm 0.84$ | |
| **Trial 2** | | | | |
| 1. VR1020 | 15 | $61 \pm 9.5$ | $9.1 \pm 0.35$ | 0 |
| 2. 3KA Digested | 5 | $57 \pm 5.3$ | $9.6 \pm 0.89$ | 0 |
| 3. VR1020/GST | 5 | $54 \pm 19.2$ | $9.2 \pm 0.45$ | 0 |
| 4. IL4 | 5 | $51 \pm 12.5$ | $9.6 \pm 0.89$ | 0 |
| 5. 3KA | 9 | $52 \pm 7.5$ | $9.6 \pm 0.53$ | 3 (33%) |
| 6. 3KA + IL4 | 10 | $58 \pm 8.8$ | $9.1 \pm 0.32$ | 3 (30%) |
| 7. 30 K (1 µg) | 10 | $45 \pm 7.7^b$ | $9.3 \pm 0.48$ | 5 (50%) |
| 8. 30 K (2.5 µg) | 10 | $40 \pm 9.0^b$ | $9.7 \pm 0.95$ | 4 (40%) |
| 9. 30 K + IL4 | 10 | $46 \pm 6.9^c$ | $10.3 \pm 1.1$ | 3 (30%) |
| *Summary* | | | | |
| Non-survivors | 61 | $55 \pm 10.3$ | $9.4 \pm 0.68$ | |
| Survivors | 18 | $42 \pm 8.1^d$ | $9.8 \pm 0.88$ | |
| **Trial 3** | | | | |
| 1. VR1020 | 8 | $58 \pm 9.4$ | $8.3 \pm 1.0$ | 2 (25%) |
| 2. 3KA Digested | 8 | $59 \pm 6.0$ | $8.1 \pm 1.1$ | 0 |
| 3. GM-CSF | 8 | $58 \pm 7.9$ | $7.8 \pm 0.46$ | 0 |
| 4. 3KA + GM-CSF | 8 | $54 \pm 5.2$ | $9.3 \pm 1.0^e$ | 3 (38%) |
| 5. 3KA-1 | 8 | $59 \pm 6.3$ | $8.6 \pm 0.52$ | 4 (50%) |
| 6. 3KA-2 | 8 | $56 \pm 9.3$ | $8.1 \pm 0.64$ | 3 (38%) |
| 7. 3KA-3 | 8 | $64 \pm 6.1$ | $8.4 \pm 0.74$ | 3 (38%) |
| 8. 3KA-4 | 7 | $57 \pm 6.7$ | $9.1 \pm 1.3$ | 4 (63%) |
| 9. 3KA-5 | 8 | $59 \pm 7.4$ | $9.0 \pm 0.76$ | 4 (50%) |
| *Summary* | | | | |
| Non-survivors | 48 | $60 \pm 6.7$ | $8.1 \pm 0.82$ | |
| Survivors | 23 | $54 \pm 7.1^f$ | $9.3 \pm 0.81^d$ | |

[a] $p < 0.01$ (compared to non-survivors).
[b] $p < 0.001$ (compared to groups 1,2,3 combined).
[c] $p < 0.005$ (compared to groups 1,2,3 combined).
[d] $p < 0.0001$ (compared to non-survivors).
[e] $p < 0.005$ (compared to group 3).
[f] $p < 0.005$ (compared to non-survivors).

with $1 \times 10^5$ P. chabaudi infected erythrocytes. All control animals succumbed to the infection by day 12 (Fig. 2A). These included mice vaccinated with the vector alone, the digested pool, the cytokine alone and the irrelevant GST construct, a total of 30 animals. A significant number of mice (39%) vaccinated with P. chabaudi plasmid pools survived the infection. The survival rate in different groups ranged from 30 to 50% (Fig. 2B,C, Table 2). The survival rate was again highest in the groups which had received 30,000 plasmids (Fig. 2B).

The average peak parasitemia and day to peak parasitemia of animals in Trial 2 is shown in Table 2. A significant reduction ($p < 0.005$) in peak parasitemia was observed in those groups vaccinated with the 30,000 plasmid pool, either alone (1 or 2.5 µg) or with IL4, when compared to that in control animals. Codelivery of the IL4 expressing plasmid had no positive protective effect in this system (comparing Group 5 with 6, and Group 7 with 9), and there was no increase in efficacy induced by the vaccine containing an increased amount of DNA (comparing Group 7 with 8). However, in a result similar to that observed in Trial 1, the peak parasitemia of all surviving mice was significantly lower ($p < 0.0001$) than that seen in non-survivors (Table 2).

### 3.4. Trial 3

In order to further characterise protective plasmids present in the pool of 3000 plasmids (3KA), a third trial was conducted with groups of 616 plasmids selected from this pool. Five pools comprising 616 clones each (3KA-1 to 3KA-5) were constructed and tested (Table 2). In addition, GM-CSF was evaluated as a co-stimulatory sequence as it had recently been
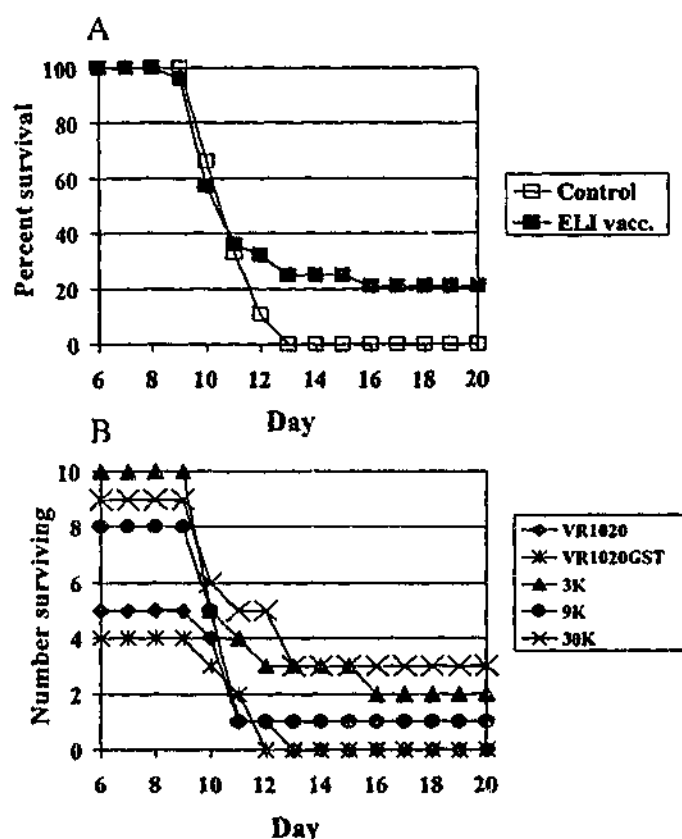


Fig. 1. Survival curves of control and ELI vaccinated mice in Trial 1. Panel (A) shows the overall survival curves for the trial, collating results for all control animals and all P. chabaudi vaccinated animals. Panel (B) shows the survival curves of the individual groups.
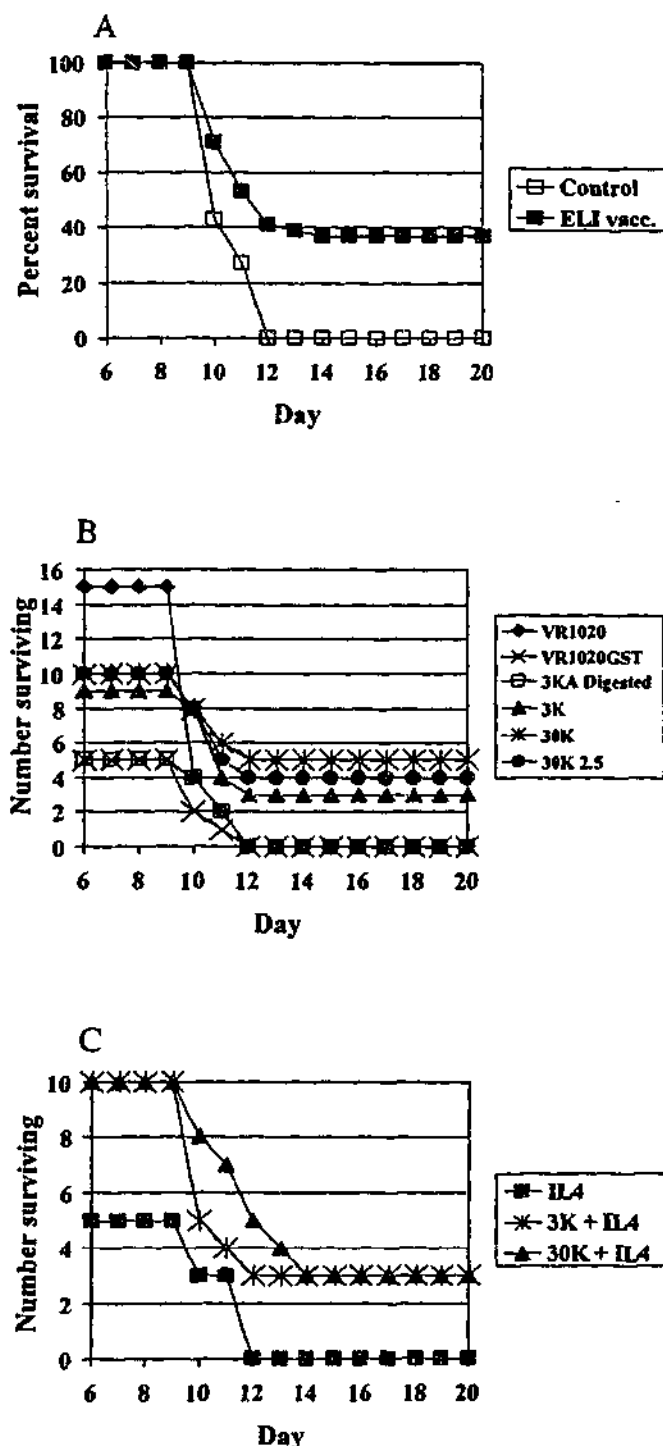


Fig. 2. Survival curves for Trial 2. Panels (A) and (B), as for Fig. 1. Panel (C) shows the survival curve for animals vaccinated with cytokine sequences and the corresponding control groups.

shown that this cytokine can enhance the effectiveness of a malaria antigen delivered as a DNA vaccine [18]. The immunization and challenge regime was identical to the previous trial. The majority of mice vaccinated with control sequences (Groups 1, 2 and 3) succumbed to the infection by day 14 (survival 2 of 24, 8%) whereas 21 of the total of 47 mice (45%) vaccinated with *P. chabaudi* sequences survived (Fig. 3). 18 of 39 (46%) of the mice vaccinated with different pools of 616 plasmids survived, whereas 38% of mice co-vaccinated with 3000 plasmids and GM-CSF survived. There was no significant reduction in the levels of peak parasitemia between control groups and those

vaccinated with *P. chabaudi* sequences (Table 2). Overall, however, surviving mice (as a group) had significantly reduced peak parasitemia levels ($p < 0.005$) compared to non-survivors, as seen in Trials 1 and 2; in addition, the time to peak parasitemia was significantly delayed ($p < 0.0001$) in surviving animals (Table 2).

## 4. Discussion

The results presented clearly show that ELI with a *P. chabaudi* genomic expression library is able to significantly protect mice against infection with a lethal challenge of *P. chabaudi*. Over the three trials which have been performed, only 2 of 63 control animals have survived infection (3.2%), compared with 45 of 124 animals vaccinated with *P. chabaudi* sequences (36%). The mechanism by which ELI vaccination might induce a protective response in this model is not yet known. Prior to infection, sera were analysed for humoral responses to vaccination: none were observed in an ELISA against antigens from *P. chabaudi* (late trophozoite-stage). DNA vaccination has recently been shown to primarily induce a T-cell memory response rather than an overt humoral response [19,20]. Vaccine-induced immunity to malaria in mice has been shown to involve the induction of cytophilic antibodies (IgG2a) or complement fixing isotypes [reviewed in 21]. It has also been recently demonstrated that murine macrophages, in the presence of sera from an acute stage infection, can opsonise and internalise parasitised red-blood cells [22]. In this context it is plausible that vaccination of mice with pools of *P. chabaudi* plasmids encoding B-cell and T-helper cell epitopes primes the immune system by inducing immunological memory. After infection, the anamnestic response results in a faster appearance of specific antibodies that can effect clearance of parasitised cells, significantly reducing peak parasitemias in vaccinated mice and promoting survival. The pool of 30,000 plasmids, tested in Trial 2, was the only pool to induce a reduction in average peak parasitemia compared to control groups: this decrease may be due to the induction of antibodies against a wider array of target antigens compared to vaccination with 616 or 3000 plasmids, thus increasing the efficiency of clearance of parasitised cells. Given that a substantial proportion of the encoded in-frame peptides are short (less that 20 amino acids) it is possible that a major contributory factor in the protective response is the supply of T-cell help. This is analogous to the experiment conducted by Amante and Good [23], who vaccinated mice with a lysate of blood-stage *P. yoelii* parasites and were able to show that CD4 + T-cells isolated from vaccinated mice and cul-
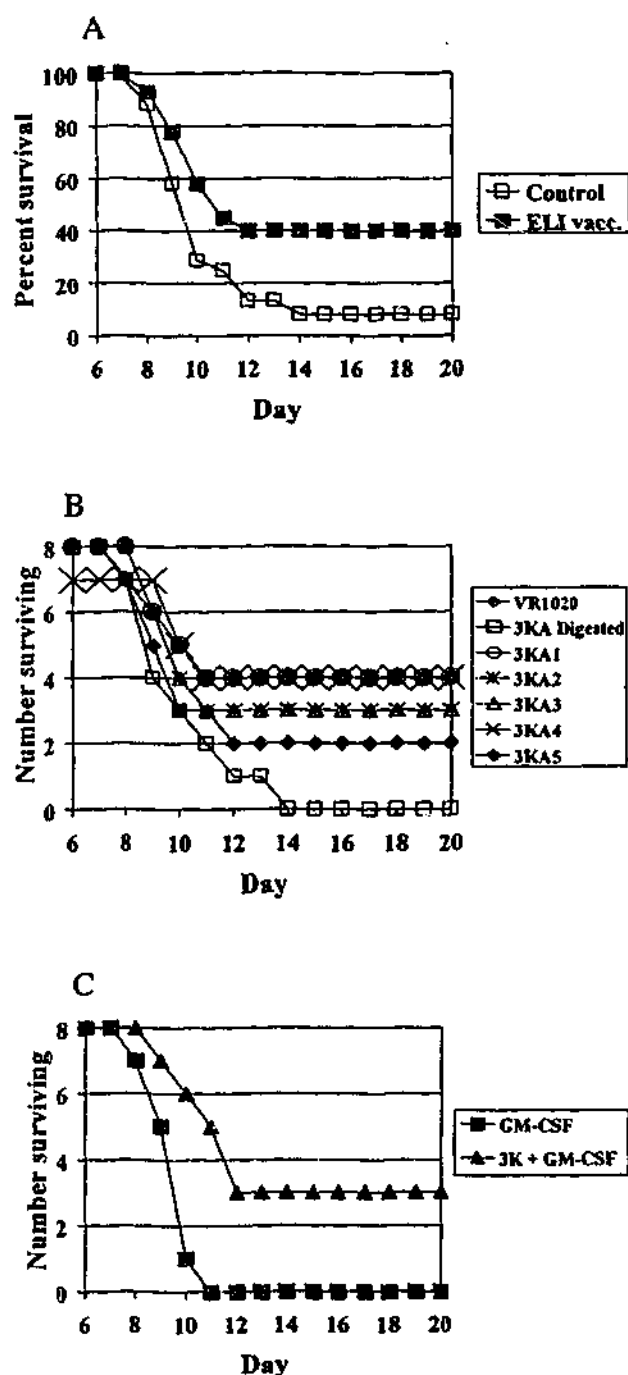


Fig. 3. Survival curves for Trial 3. Legend as for Fig. 2

tured in vitro were able to mediate protection in reconstituted nude mice.

A further observation is the maintenance of protection in each of the groups of 616 plasmids derived from pool 3KA. Although there will be some overlap between these pools (as the library was amplified by re-plating) this suggests that there may be a large number of potential protective molecules encoded by *P. chabaudi* DNA distributed among these pools. This result may not be surprising: of the limited number of *Plasmodium* proteins which have been tested as vaccines there are several which have given some degree of a protective response against blood-stage challenge. These include MSP1, MSP2, RESA, AMA1, RAP1 and SERA, which have been shown to give protective efficacy in mice, non-human primates or humans [24–29; reviewed in 2,3,21,30]. This suggests that there may be a number of potential blood-stage vaccine molecules expressed by the malaria parasite. Our preliminary sequence data from the plasmid pools suggests that approximately 13% may express a bona-fide *P. chabaudi* peptide greater than 50 amino acids in length (Table 1). Hence from a pool of 616 clones there will be approximately 80 such peptides produced and it is possible that this is a sufficient number to induce an appropriate immune response that can limit a subsequent malaria infection. Additionally, as mentioned above, it may be that a substantial contribution to the protective effect is from clones that encode shorter in-frame peptides, which serve as T-helper cell epitopes. It is plausible that it is the multivalent nature of these DNA vaccines which is critical in inducing a multi-immune protective response that exceeds the threshold required for protection, as proposed by Doolan and Hoffman [4]. This conclusion is consistent with the analysis of human malaria infections which suggest that, after repeated exposure, humans acquire resistance to malaria by specific immunity to a range of erythrocyte-stage antigens [31–33]. We are currently analysing the sequence of all plasmids from one pool of 616 clones to identify all expressed peptides, for further refinement of the vaccine.

This report has demonstrated the feasibility of using ELI to protect a mammalian host against lethal infection with a complex protozoan parasite. Using a genomic expression library of *Leishmania major*, the ELI approach has been shown to reduce lesion formation in challenged mice [34], confirming the utility of this approach in a non-lethal model. Subdivision of these *Leishmania* plasmid pools resulted in the loss of protection with certain pools, indicating that there may be fewer potential protective epitopes encoded by these *Leishmania* libraries than in the malaria libraries described here. Alternatively, it is possible that the observed loss of protection against *L. major* is an artefact due to loss of some plasmids from the pools following overnight amplification of the bacterial cultures.

Several features of the approach we have used merit further comment. Firstly, the decision to utilise genomic DNA rather than cDNA will allow these libraries to be tested against all malaria life-cycle stages. We will shortly test the libraries described here for efficacy against sporozoite challenge, to search for protective liver-stage antigens. As the ultimate malaria vaccine will most likely be multivalent, and comprise antigens from more than one life cycle stage, this is an important feature that obviates the need to make multiple cDNA libraries corresponding to each stage. A second advantage of using a genomic DNA library for ELI is that all genes will be represented according to their gene dosage, rather than their expression level. This may be important in allowing the representation in the vaccine of relatively poorly expressed, but critical, cell surface proteins that are essential for parasite survival, and which can be targeted by the acquired immune response).

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