Studies evaluating the possible evolution of malaria parasites in response to blood-stage vaccination

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Declaration

I, Victoria C. Barclay, verify that the work contained in this thesis is my own and of my own thought and origin. However, scientific research is of a collaborative nature and therefore I would like to recognize those involved in the experiments within each chapter.

Chapter 2: Derek Sim assisted with experimental sampling. The AMA-1 vaccine was produced in collaboration with Robin Anders, Vince Murphy and Rose Masciantonio (La Trobe University, Melbourne).

Chapter 3: Katrina Grech helped with experimental design. Derek Sim assisted with experimental sampling. The AMA-1 vaccine was produced in collaboration with Robin Anders, Vince Murphy and Rose Masciantonio, who provided me with the materials and taught me the relevant techniques. Karen Grocock helped with immunological assays. Cadhla Ramsden (The Pennsylvania State University) performed the phylogenetic analysis.

Chapter 4 and 7: Brian Chan assisted with experimental sampling. The AMA-1 vaccine was produced in collaboration with Robin Anders, Vince Murphy and Rose Masciantonio. Andy Bell designed the molecular assays. Lars Råberg helped with the repeated measures statistical analyses.

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So, when I started my PhD in Edinburgh I never thought I would finish it in America. I am excited to be continuing research here for another year, and looking forward to what my scientific future holds. I will not however, forget all the support that my friends and family back in Scotland gave me during these four years, I love you all!

Contents

Abstract

Drug resistance is one of the most medically relevant forms of pathogen evolution. To date, vaccines have not failed with the same depressing regularity as drugs. Does that then make vaccines evolution-proof? In the face of vaccination, pathogens are thought to evolve in two ways: by evolving epitope changes at the antigenic target of vaccination (epitope evolution); or by evolving changes at other antigenic loci, some of which may involve virulence (virulence evolution). The fundamental difference between these two forms of evolution is that virulence evolution could lead to disease outcomes in unvaccinated people that are more severe than would have been seen prior to evolution. One of the theoretical assumptions of virulence evolution is that more virulent parasites will have a selective advantage over less virulent parasites in an immunized host, and are thus more likely to be transmitted. The assumption is that more virulent parasites may be competitively more superior in mixed infections, or may be better able to evade/modulate the host immune response. Thus, the aim of this thesis was to experimentally test whether more virulent parasites have a within-host selective advantage in an immunized host or whether vaccine efficacy is more likely to depend on genetic differences at the targeted sites of vaccination.

I used clones (genotypes) of the rodent malaria *Plasmodium chabaudi* originally derived from wild-caught Thicket (*Thamnomys rutilans*) rats to infect laboratory mice and a rodent analogue of the candidate blood-stage malaria vaccine apical membrane antigen 1 (AMA-1). I found that within-host selection did not depend on parasite virulence, and that protective efficacy depended on genotype-specific differences at the vaccine target. Vaccine-induced protection was not enhanced by including a number of allelic variants. However, such genotype-specific responses were only observed when the vaccine was tested against genetically distinct *P. chabaudi* parasites. When one *P. chabaudi* genotype was serially passaged through naïve mice the derived line was more virulent and was subsequently less well

controlled by vaccine-induced immunity. In other experiments I found within host competition not to be immune-mediated. Thus my results suggest that vaccination has the potential to select for more virulent parasites but that the selective advantage is likely to be independent of competition. The selective advantage may be attributable to the enhanced immune evasion of more virulent parasites. However, without genetic markers of virulence, the mechanisms that mediate this selection remain unknown.

My thesis contributes towards a growing body of evidence that vaccines have the potential to differently alter the within-host parasite dynamics of particular pathogen genotypes and that the selection imposed is likely to be system specific, depending on the fine specificity of the vaccine-induced responses and the identity of infecting parasites. Although vaccine potency may not be enhanced by including more than one allelic variant of an antigen, multi-valent vaccines may be one of the best ways to avoid the inadvertent selection for more virulent malaria parasites.

1. General introduction

1.1. Evolution in biomedicine

Research on the evolutionary process has made notable impacts in many areas of biology. In contrast, evolutionary biologists have contributed rather little to medicine (Ewald 1994; Maynard Smith 1998; Stearns & Hoekstra 2005; Nesse et al. 2006). Indeed, medicine is a profession that offers practical help. Thus, in the first instance, evolutionary thinking may appear to be of little use, for example, to a surgeon who is performing an operation (Stearns et al. 2007). However, the importance in understanding evolutionary processes has become more apparent due to the emergence of medically relevant traits such as drug resistance (Ash 1996; Levy & Marshall 2004). In spite of resistance being a clear pathogen adaptation in response to drug intervention, it is surprising that the word 'evolution' is rarely used. For example, papers describing antimicrobial resistance often use 'emerge', 'arise', or 'spread' rather than 'evolve' (Antonovics et al. 2007). Thus there is evidently still a lack of acceptance or understanding of how evolution in biomedicine may assist in more effective methods for disease control.

To date, vaccines have not failed with the same depressing regularity as drugs. Does this then mean that vaccines are evolution proof? Unfortunately, as we will see with the disease examples given below, pathogen evolution in response to vaccination does occur. This is because host immunity imposes massive selection on pathogen populations and vaccines work to elicit those immune responses. In order not to repeat the same complacency which has lead to the widespread evolution of drug resistance it has become important that we consider how pathogens which are the focus of current vaccine development programmes may evolve in the face of vaccination (Read & Mackinnon 2008).

In contrast to the staggering successes of vaccines developed before the era of molecular biology, diseases of current vaccine development programmes, such as

HIV, seasonal flu, sleeping sickness, and malaria, persist by exhibiting large amounts of antigenic variation. To date, the biggest concern for vaccine developers has been that these diseases often induce protective responses which are strain (genotype)-specific, where immunity is often effective against genotypes that initiate the response, but less effective against variant genotypes. This can lead to the evolution of epitope variants in the population. Thus, vaccines not including distinct pathogen antigen variants may erode any potential benefits (Francois et al. 2001). In order to prevent selection of non-vaccine epitopes, vaccines against these highly diverse, chronic infections may have to be regularly updated to keep up with evolution (as is already the case with seasonal flu) or have to include several antigens and/or several allelic forms of one antigen to overcome antigenic diversity (McLean 1995; McLean 1998; Scherer & McLean 2002; van Boven et al. 2005).

However, selection of antigen-variants is not the only evolution that could occur in response to vaccination. A number of theoretical studies have shown that vaccination has the potential to select for (antigen) variants at loci other than those targeted by vaccination, and some of these changes may involve virulence (parasite-induced host damage) (Gandon et al. 2001; Gandon & Day 2003; van Boven et al. 2005; André & Gandon 2006; Restif & Grenfell 2006; Gandon & Day 2007; Mackinnon et al. 2008; Read & Mackinnon 2008; Williams & Day 2008). The thinking behind the idea of vaccine-driven virulence evolution draws heavily on the trade-off model of the evolution of parasite virulence (Anderson & May 1991; Bull 1994; Ewald 1994; Frank 1996). That model posits that parasites within a species vary in virulence, and that in order to maximize fitness, parasites have to balance the purported fitness costs of virulence (truncation of the infectious period by host death) against the purported fitness benefits of virulence (production of more transmission forms per unit time and/or longer time before immune clearance) (Anderson & May 1991; Ebert & Herre 1996; Frank 1996) (Fig. 1.1A).

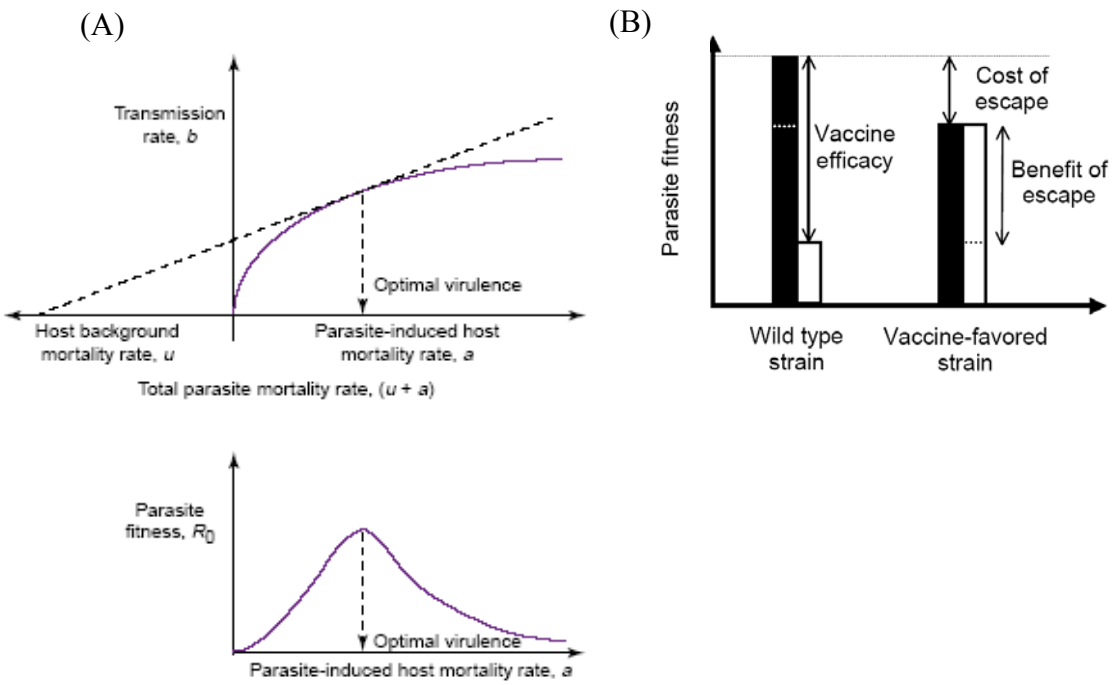

Figure 1.1. (A) The trade-off model of the evolution of virulence and (B) schematic representation of the comparison between a wild type strain and vaccine-favoured variant. (A) The model assumes a decelerating functional relationship between transmission rate, b and parasite-induced host mortality, a (virulence) (top panel). Because early host death curtails parasite transmission, the life time transmission success (R_0, the total number of secondary infections produced by a primary infection in a population of susceptible hosts) of a parasite is maximal at intermediate levels of virulence (bottom panel). Figures reproduced from Ebert & Bull (2003) and Ebert & Herne (1996). (B) The lifetime reproductive success (a relevant measure of fitness at endemic equilibrium) is plotted in naïve hosts (in black) and in vaccinated hosts (in white) for both strains. The efficacy of the vaccine is evaluated in naïve hosts while the benefit is evaluated in vaccinated hosts (in both cases, relative to the performance of the wild-type strain). Figure reproduced from Gandon and Day (2006).

The trade-off model of virulence evolution greatly clarifies how the damage a parasite causes to a host evolves in response to a *change* in some key factor of parasite biology (Frank & Schmid-Hempel 2008). In theory, vaccination has the potential to induce change in parasite biology by altering the costs and benefits of virulence. For instance, the cost of excessive virulence may be removed by vaccination, as the vaccine protects the host from death and in doing so keeps those

more virulent parasites alive. Thus, vaccination could induce higher levels of virulence (when measured in an unvaccinated host) because it drives such variants to higher frequency (Gandon et al. 2001; Gandon & Day 2003; André & Gandon 2006; Ganusov & Antia 2006) (Fig. 1.1B).

As mentioned above, one of the benefits of parasite virulence is assumed to be a greater total number of pathogen transmissible forms across the course of infection (Mackinnon & Read 1999a; Messenger et al. 1999; Mackinnon & Read 2004b). This could be achieved by a number of different ways. For instance, higher virulence may result in a greater number of transmissible forms per day of infection, an increase in the transmission period (duration of infection) due to reduced immune clearance/parasite immune evasion, or a combination of processes (Anderson & May 1982; Mackinnon & Read 2004a; Mackinnon & Read 2004b; Frank & Schmid-Hempel 2008; Schmid-Hempel 2008) (Fig. 1.2). Functional relationships between virulence and immune evasion were first described for the rabbit virus myxomatosis (Fenner & Ratcliffe 1965; Fenner & Myers 1978; Anderson & May 1982; Fenner 1983; Anderson & May 1991). The virus was introduced into wild rabbit populations in Australia as a biological control measure. That relationship indicated that the most virulent strains that killed their hosts were also those which were less rapidly cleared (Anderson & May 1991). All major parasite groups have been shown to deploy immune evasion mechanisms (Blaxter et al. 1992; Benedict et al. 2002; Hornef et al. 2002; Orange et al. 2002; Sacks & Sher 2002; Corley & Strand 2003). For example, some pathogens may secrete proteins that suppress the immune system by interfering with host signalling pathways and inflammatory processes (Hornef et al. 2002). Others may up-regulate the production of smokescreen molecules whose purpose is to distract the immune system from functionally important pathogen molecules. High levels of antigenic variation may allow some pathogens to stay ahead of the proliferating immune response (Brown & Brown 1965; Phillips et al. 1997; Nash 2002; Mackinnon & Read 2003; Mackinnon & Read 2004b; Dzikowski & Deitsch 2006; Dzikowski et al. 2006; Finlay & McFadden 2006; MacGregor & Matthews 2008). These are just a few examples of the sophisticated mechanisms of immune evasion. Enhanced immunity through vaccination may select for those

pathogens which deploy some of these mechanisms, as they are more likely to avoid vaccine-induced immunity.

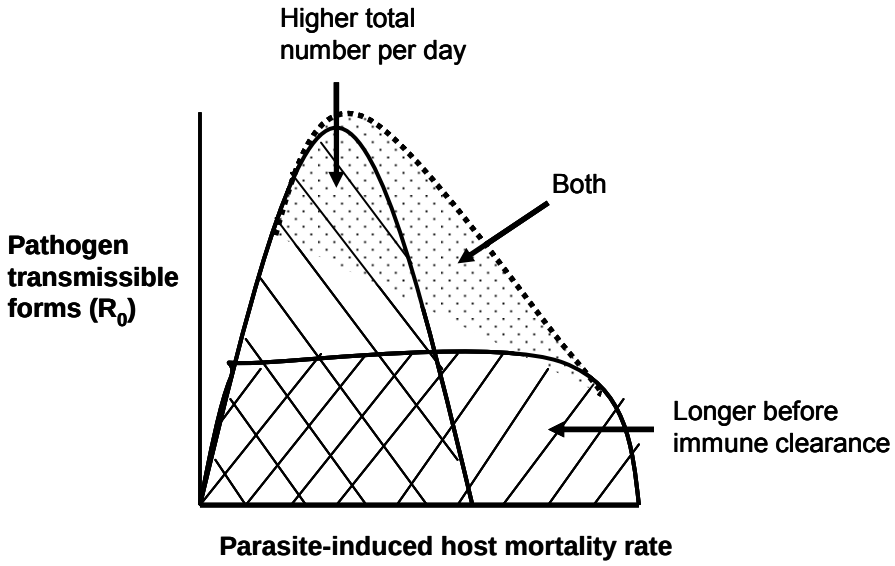

Figure 1.2. Possible fitness benefits of virulence hypothesised under the trade-off model of parasite virulence. More virulent strains might produce a greater number of transmission stages per day if they are less well controlled by host immunity or more aggressively exploit host resources, an increased period of transmission due to poor immune clearance, or a combination of both processes.

One of the other benefits associated with virulence can occur during within-species, between strains, parasite social interactions, where it is frequently argued that virulent strains will be competitively superior within hosts, so that within-host selection will favour increased virulence (Bremermann & Pickering 1983; van Baalan & Sabelis 1995; Frank 1996; Gandon et al. 2001; Alder & Losada 2002). This has been seen in the rodent malaria model *Plasmodium chabaudi* in laboratory mice, where more virulent genotypes suppress the densities and transmission of less virulent genotypes in mixed infections (de Roode et al. 2005b; Bell et al. 2006). Just as the relative fitness of drug-resistant strains, could be substantially enhanced when

co-infecting drug-sensitive competitors are removed by chemotherapy (Hastings 1997 ; Hastings & D'Alessandro 2000; Hastings 2003; Mackinnon 2005a; Hastings 2006; Wargo et al. 2007), vaccination may similarly affect the rate of evolution of epitope variants (Lipsitch & Samore 2002; Read & Mackinnon 2008).

The aim of this thesis was to experimentally investigate whether any fitness benefits of parasite virulence result in a disproportionate advantage in hosts immunized with a recombinant candidate malaria vaccine antigen. All else equal, it follows that a relative fitness advantage means that vaccination could lead to the evolution of increased virulence. The primary motivation for this work came from the 'Combination B' malaria vaccine, one of the first malaria vaccines to make it to field trials (Genton et al. 2002; Genton et al. 2003). The vaccine comprised three *P. falciparum* antigens which were known to be expressed during the pathogenic blood stage of infection: Merozoite Surface Protein 1 (MSP-1), MSP-2 and the Ring-Infected Erythrocyte Surface Antigen (RESA) (Saul et al. 1999). MSP-2 exists as two allelic families (variants) in *P. falciparum* known as 3D7 and FC27 (Smythe et al. 1990). The 3D7 variant was included in the vaccine formulation. In a double-blind, placebo controlled trial the vaccine was given to 120 children aged between 5 and 9 years old. In a follow up study, despite a reduction in the frequency of the 3D7 variant in the vaccinees, the prevalence of the FC27 variant, which was not included in the vaccine, was unaffected (Table 1.1). Interestingly, the FC27 variant itself had previously been associated with more morbid (virulent) cases (Engelbrecht et al. 1995). This was the first field evidence that a vaccine against malaria could exert selective pressure. However, what was not clear from those studies was whether the vaccine had exerted selection that was strain-specific (Fluck et al. 2004; Fluck et al. 2007) or whether more virulent variants had a selective advantage in immunized hosts.

	Treatment group	
	Placebo	Vaccine
3D7 allele	21	11 (.33)
FC27 allele	6	11 (.66)

Table 1.1. New infections following vaccination with Combination B. A new infection was considered to have occurred when an MSP2 strain that was not detected in the same child at week 0 or 4 was found at week 8, 10, 12, 14, 16 or 18. Twenty-one new 3D7-type infections were detected in placebo recipients, and only 11 were detected in vaccinees. In contrast, only 6 new FC27-type infections were found in placebo recipients, compared with 11 in vaccinees. The numbers in brackets indicate the frequency of each allele in those who received the Combination B vaccine. There was a significant difference between the allelic types in this efficacy ($\chi^2 = 4.1$; p=.042); the vaccine made no difference to the prevalence of the FC27-type parasites.

Over the past decade our laboratory has established one of the rodent malarias, *P. chabaudi,* as a model for studying the evolution of virulence (Mackinnon et al. 2002; Mackinnon & Read 2004a; de Roode et al. 2005a; de Roode et al. 2005b; Mackinnon et al. 2005b) (see section 1.4 below). The experiments I present in this thesis use a bank of genetically diverse *P. chabaudi* parasites that vary in virulence to infect laboratory mice immunized with the candidate malaria vaccine known as Apical Membrane Antigen 1 (AMA-1). I specifically asked: (i) whether parasite antigenic differences at the targeted vaccination locus and/or at other loci (which may be involved in virulence) influence vaccine-imposed selection; (ii) whether immunization with more than one antigen variant significantly improves host health and/or alters vaccine-imposed selection compared to when immunization is with one of the variants; (iii) whether vaccination has the potential to alter competitive interactions between genetically diverse malaria parasites.

Although I base my thesis on malaria, I hope my findings will contribute towards an increased evolutionary understanding of how vaccines may impose selection on other diseases currently the focus of vaccine design programmes. In doing so I hope

provide information for the better design and use of vaccines for improved animal and human health in the future.

1.2. Evidence for vaccine-driven evolution

As mentioned above there are currently two separate views on how vaccines may impose selection upon pathogen populations: epitope evolution and the evolution of virulence (van Boven et al. 2005; Restif & Grenfell 2006). First, studies on epitope evolution focus on understanding how vaccines select for antigenic variants which are able to evade the protective effects of the vaccine (McLean 1995; McLean 1998; Wilson et al. 1998; Wilson et al. 1999; Francois et al. 2001; Scherer & McLean 2002; van Boven et al. 2005; Restif & Grenfell 2006). Second, studies on vaccine-induced evolution of virulence (parasite-induced host damage) are more concerned with how vaccines may alter the costs and benefits of parasite virulence (Gandon et al. 2001; Gandon & Day 2003; André & Gandon 2006; Gandon & Day 2007; Read & Mackinnon 2008; Williams & Day 2008).

The distinction between epitope and virulence evolution is heuristically useful and likely to be unambiguous in many cases. Therefore in the sections below I have separated the two processes for clarity. However, in some cases the distinction may be less clear as some epitopes will be virulence determinants (as may have been the case with the MSP2 FC27 variant in the Combination B malaria vaccine). However, the public health distinction remains: vaccine-driven virulence evolution will lead to disease outcomes in unvaccinated people more severe than would have been seen prior to vaccination; epitope change will not.

This thesis is concerned with investigating how a vaccine against the blood stage of malaria infection may impose selection on *Plasmodium* populations: will vaccine-imposed selection result in epitope evolution, virulence evolution, or perhaps a combination of both processes? Before I get into the specificities of the malaria model system I will use for addressing these questions, it appears appropriate that I should provide a number of disease examples where vaccine-driven evolution is

assumed to have occurred. However, first I would like to begin by describing some diseases where vaccines have been successful, with apparently no adverse evolutionary consequences.

Vaccination has spared millions of people the effects of devastating disease and should rightly be claimed a medical triumph. For example, vaccination has eradicated smallpox, is close to eradicating polio and controls a number of other diseases including *Haemophilus influenza* type B (Hib), measles, mumps, rubella, pertussis and diphtheria (Atkinson et al. 2000). However, the most striking common feature between these diseases is that they all cause acute childhood infections. Thus, the infection either kills the host or provides life-long sterilizing immunity. In the pre-vaccination era, there must have been intense selection for those diseases to over-come natural immunity but it is evident they did not. Some have argued that vaccines against these acute diseases were easy: natural immunity was already evolution-proof and all that was needed was for vaccines to mimic natural immunity (Read & Mackinnon 2008). Obviously, not all infectious diseases are alike, thus one should not assume that all vaccines are evolution-proof.

The diseases targeted by current vaccine development are markedly different to acute childhood infections. For instance, HIV, flu, sleeping sickness, and malaria are pathogens rich in diversity, and can result in chronic infections, presumably due to high levels of antigenic diversity and immunosuppressive properties. These diseases provide a challenge for vaccine developers as natural selection has provided a way of overcoming natural immunity. For example, comparing measles and influenza, due to continual antigenic evolution of influenza, vaccines must be continually updated to maintain their effectiveness, while with measles they do not (Boni 2008; Gog 2008). This makes vaccination a more effective control strategy for measles than for influenza, because influenza can, in effect, evolve to circumvent this control measure. The measles versus influenza situation demonstrates that an understanding of the evolutionary consequences of vaccination is crucial for designing successful vaccination programs.

1.2.1. Epitope evolution

Conventionally, epitope evolution may be regarded as a mutational change at the antigenic target of vaccination leading to the emergence of *de novo* mutants. However, vaccine-induced epitope evolution can also cause the emergence of pre-existing strains which are different at the target epitope(s) to the vaccinating strain. The following diseases provide examples where a change in gene frequency has occurred following vaccination and thus are likely to fall under the heading of epitope evolution.

First, there is strong evidence that hepatitis B virus (HBV) populations are evolving in response to widespread vaccination through changes at target epitopes. HBV is a major cause of liver disease including liver cirrhosis and hepatocellular carcinoma (Yokosuka & Arai 2006). The use of HBV vaccines started in the late 1980s in developed countries and dramatically reduced the incidence of disease (FitzSimons et al. 2005). The neutralizing (protective) antibodies induced by vaccination target epitopes located within the *a* determinant, a common immunodominant region on the outer protein coat (surface antigen). In 1990, the first mutation (a single amino acid substitution) in the S gene coding for the *a* determinant of the surface antigen was described (Carman et al. 1990). Since then several other mutants have been discovered (Ghany et al. 1998; Protzer-Knolle et al. 1998). Thus, in the presence of vaccination there appears to be a selective advantage to mutation in the target site of vaccination. What remains to be determined is whether these mutants will inflict significantly greater host disease in the unvaccinated population.

Second, whole-cell vaccines against *Bordetella pertussis* (the causative agent of whooping cough) resulted in a worldwide reduction in disease incidence. The current vaccine contains a number of proteins, including pertactin *(Prn)* and pertussis toxin *(Ptx)*. *Prn* facilitates attachment to host cells, and *Ptx* is involved in immune evasion and possibly resource extraction. Since the introduction of vaccines containing these proteins, changes in allele frequency have been recorded worldwide: non-vaccine alleles tend to be more frequent in vaccinated individuals than in unvaccinated individuals (Mooi et al. 1998; van Loo et al. 1999). For instance, in Finland and

Holland, the frequency of the vaccine-type pertactin allele *(Prn1)* went from essentially 100% in the pathogen population to less than 5% after the introduction of nationwide vaccination (van Loo et al. 1999; Elomaa et al. 2005). In addition, mutations in these two surface proteins have also been suggested as a reason for disease resurgence in the adult host population (WHO 2001; McIntyre et al. 2002; Ntezayabo et al. 2003). It is possible that these mutants have a selective advantage in vaccinated individuals as they are more able to evade vaccine-induced immunity.

Third, vaccination against infections with *Streptococcus pneumoniae* may provide an example where epitope evolution has arisen due to strain replacement: removal of one strain by vaccination evolves a niche for another strain to fill. *S. pneumoniae* is responsible for diseases such as meningitis, septicaemia, and pneumonia. The bacterium consists of 90 known serotypes with variable prevalence and virulence (Smith et al. 1993). Vaccines generally consist of between 7 and 11 of these serotypes (types of capsular polysaccharides). Since the widespread use of a 7-valent conjugate vaccine began in the United States in 2000, there has been a decrease in disease incidence caused by those strains targeted by the vaccine. Non-vaccine serotypes have increased in frequency in both disease cases and asymptomatic carriage (McEllistrem et al. 2003; Huang et al. 2005; Flannery et al. 2006). However, those non-vaccine serotypes are less virulent (induce less host damage/sickness). Thus, although there has been no net change in disease prevalence, vaccination has selected for those strains which are less nasty.

Fourth, immunization with the detoxified toxin ('toxoid' vaccine) against *Corynebacterium diphtheriae* has reduced the disease from a major child killer to one rarely seen at all. The disease is characterized by lesions, which are due to the production of a specific phage-encoded cytotoxin, found particularly in the tonsils, pharynx, larynx, and nose. Various authors have attributed decreased disease to be due to reductions in the frequency of the toxin-encoding phage in the bacterial population (Pappenheimer 1982; Ewald 1994; Ewald 1996; Ewald 2002; Soubeyrand & Plotkin 2002). The premise behind this is that in the presence of anti-toxin immunity there will be an evolutionary cost of toxin production to *C. diptheriae*.

The only data showing this evolution comes from Romania during the third quarter of last century (Pappenheimer 1982). However, given that the diphtheria toxoid vaccine is imperfect (Schneerson et al. 1996) the production of *more* toxin may be an alternative way to overcome the effect of the toxoid vaccine (Gandon et al. 2002)

Finally, the examples given so far of epitope evolution have come from uncontrolled field observations, therefore it is difficult to attribute the changes in gene frequency solely to selection imposed by vaccination. Disease resurgence for example, is usually due a number of complex factors. Major reasons can include low immunization coverage rates in adults and infants, waning immunity in the adult population and large movements of the population.

The current roll-out of vaccines against Human Papillomavirus (HPV), the primary etiologic factor in the development of cervical cancer (Bosch et al. 1995) may provide a disease where the effects of vaccination on the pathogen population can be measured over real-time. More than 35 distinct HPV types are know to infect the genital tract (Bernard et al. 1994). Certain types (types 16, 18) are designated high risk because of their association with cervical and other anogenital cancers (Trottier & Franco 2006). HPV infections can cause genital warts or lead to penile or anal cancer, although at rates well below the rates of cervical cancer in women (Partridge & Koutsky 2006). In 2006, a quadrivalent vaccine against types 6, 11, 16 and 18 was licensed by the Food and Drug Administration (FDA) for use in females who are 9 to 26 years of age (FDA 2006). The Advisory Committee on Immunization Practices (ACIP) has also voted to recommend routine HPV immunization of 11-to 12-year old females (CDC 2006). A theoretical framework has recently been used to predict epitope evolution in HPV (Poolman et al. 2008). That model demonstrated that depending on the degree of cross-immunity elicited by the vaccine, vaccination may either expand or contract the available niche of HPV. Monitoring the evolutionary consequences of HPV in the population will require collaborations between health workers, experimental biologists and bio-mathematicians. If these collaborations occur, there is great potential for HPV to be one of the first diseases where the evolutionary consequences of vaccination can be determined.

1.2.2. Virulence evolution

The classic example of virulence evolution in response to host immunity is the European rabbit-myxoma virus system. In the natural host (*Sylvilagus brasiliensis)*, myxoma virus causes a benign cutaneous fibroma. However, in European rabbits *(Oryctolagus cuniculus)* myxoma virus is lethal. The virus was deliberately introduced as a mechanism of biological control into wild European rabbit populations in Australia. Over the following years the virus first declined rapidly in virulence. Later virulence then increased again, as tests on non-coevolving control rabbits showed. At the same time the wild rabbits evolved to suffer less from the original virus (Fenner & Kerr 1994). This impressively documented example shows that virulence can evolve rapidly with large changes in the face of immunity, and that hosts evolve to reduce the costs of parasitism. Arguments for vaccine-driven virulence evolution imply that if hosts immunity can drive virulence upwards, vaccines which act to mimic or enhance natural immunity will similarly drive the evolution of virulence (Gandon et al. 2001; Gandon et al. 2003; André & Gandon 2006; Gandon & Day 2007; Mackinnon et al. 2008; Read & Mackinnon 2008; Williams & Day 2008).

Vaccine-driven virulence evolution is thought to have occurred in the avian cancer-causing herpes virus, Marek's Disease Virus (MDV). MDV causes substantial loss to the poultry industry each year. Vaccination started in the 1960s after the virus became economically important with the intensification of the chicken industry post World War II. Vaccination was with a live virus from a related non-oncogenic strain. This first generation vaccine initially yielded good control, but within a decade it was not providing adequate protection against virulent strains that appeared in the 1970s. One subsequent vaccine was introduced and similarly failed. Importantly, virulent strains have the ability to infect and exploit vaccinated and unvaccinated birds and are more virulent in these hosts (i.e. they induce more extreme symptoms) (Witter 1997; Read et al. 2004). Importantly, the failed vaccines were undermined by strains that were antigenically identical to the oncogenic strains of the pre-vaccine era. Thus, changes in viral aggression and immunosuppressive

capacity, not antigenic type, caused the vaccine failure (Witter 2001; Davidson & Nair 2004).

Similar to MDV, vaccine-driven evolution may provide an explanation as to why strains of a birnavirus, which causes the enteric and respiratory disease of chickens known as infectious bursal disease (IBD), have not been controlled by vaccination programmes. Since vaccinations begun in the 1980's, failure has been described in poultry operations around the world. In the United States, the re-emergence was due to the evolution of antigenically novel strains against which classical IBD vaccines were not sufficiently protective. In contrast, evolved European strains belong to the ancestral IBD serotype but are more aggressive. Like the newly evolved MDV strains, these very virulent European strains cause more severe disease in unvaccinated birds, with mortality rates of up to 60% (van den Berg et al. 2000; Rautenschlein & Haase 2005; Nouen et al. 2006).

The first controlled experimental evidence that vaccination may drive the evolution of virulence came from our laboratory using the rodent malaria *P. chabaudi* (Mackinnon & Read 2004a). In that study, one ancestral *P. chabaudi* genotype was serially passaged (i.e. the syringe transfer of parasite infected blood from one host to another) through whole-parasite immunized mice or naïve mice. Immune-derived lines evolved to be more virulent than lines that had evolved in naïve hosts, even after mosquito transmission, when virulence was measured during infection of non-immunized mice. An implication of that study was that more virulent genotypes had a selective advantage in an immunized host. That study by Mackinnon and Read (2004a) was a motivator for many of the experiments presented in this thesis.

1.2.3. Other possible vaccine-adapted phenotypes

Epitope and virulence evolution have been described as the main pathogen phenotypes that vaccination may alter. However, it is likely that a combination of different phenotypes may be favoured in vaccinated populations. A few of these include: enhanced immunosuppression, for instance by production of

immunomodulatory substances; the establishment of a 'smokescreen' effect that may have the advantage of distracting the immune system from functionally important molecules; changes in patterns of antigenic variation may occur, and/or antigenic repertoires and rates of change; changes in tissue tropism and immunologically privileged sites and/or increased sequestration; and activation of alternative host cell invasion pathways (Read & Mackinnon 2008). It is likely that these are just a few of the possible outcomes that could occur in response to vaccination and there are likely a number of other pathogen strategies for evading protective responses in addition to those mentioned.

1.3. Thesis aims

As I have described above, vaccination could impose selection on the parasite population by driving the evolution of epitope variants which are able to escape immunity or by altering one or more of the costs and benefits associated with parasite virulence. Here I use clonal genotypes of the rodent malarias *P. chabaudi* to experimentally investigate which parasite traits are most likely to influence selection during vaccination with the candidate malaria vaccine AMA-1. These results are relevant to the design and implementation of future malaria vaccines and will hopefully generate an increased recognition of the need to understand immediate epidemiological benefits and the longer-term evolutionary risks of interventions for a number of different diseases. The following specific questions form the basis of each chapter.

- For two antigenically distinct AMA-1 variants, are the protective effects of immunization due to strain (genotype) specific differences at the target epitopes, or is a more virulent *P. chabaudi* genotype better at evading vaccine-induced heterologous immunity? [Chapter 2]

- Across six *P. chabaudi* genotypes, how does genetic diversity at the AMA-1 locus and genotype virulence play out during immunization? When two of

the genotypes are identical at the AMA-1 locus, but differ in their virulence, does vaccination induce protective responses which are more effective against the genotype of lesser virulence? [Chapter 3]

- If strain-specific responses are such a big problem to vaccine developers, are multi-allele vaccines a solution to antigenic polymorphisms? What are the effects of single and multi-allele vaccines on within-host selection of variants, and does parasite virulence mediate the level of evasion from vaccine-induced immunity? [Chapter 4]

- Serial passage of a *P. chabaudi* genotype through whole-parasite immunized mice has previously been shown to drive the evolution of virulence more rapidly than those evolved through naïve mice. Does serial passage drive the evolution of a *P. chabaudi* genotype which is less well controlled by AMA-1 vaccination? [Chapter 5]

- Selection for more virulent genotypes is known to occur during mixed *P. chabaudi* infections. Could vaccination alter these competitive interactions? To investigate this I depleted mice of $CD4^+$ T cells and asked whether immune modulation alleviated or exacerbated competitive interactions between genetically diverse *P. chabaudi* parasites [Chapter 6].

- Following on from Chapter 6, does direct immunization with AMA-1 (known to induce strain-specific immune responses) alleviate or enhance the competitive interactions between two genotypes which differ in their virulence? [Chapter 7]

In Chapter 8, I discuss how the main findings of this thesis address the questions laid out above, and the implications of this for future vaccine development research on the evolution of virulence, and the relevance of using animal models.

1.4. Specificities of the malaria model

1.4.1. Malaria

Malaria is a disease caused by parasites of the genus *Plasmodium*. Classically, four species of *Plasmodium* were known to infect humans: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, of which *P. falciparum* is the most common and causes the most severe disease. However, during a recent epidemic in Malaysian Borneo a large number of cases were found to be due to *P. knowlesi*, a malaria parasite of Old World Monkeys (Garnham 1966; Cox-Singh et al. 2008). Thus, it is now appreciated that there are likely to be a number of other species that can naturally infect humans.

Malaria is endemic in more than 90 countries, principally in the developing world, with 300-500 million new infections each year and one million deaths, mainly in children and pregnant woman (Hentschel 2002). Malaria parasites undergo a complicated life cycle (Fig. 1.2). Infection is initiated by inoculation of sporozoites, an infectious form of the parasite, through mosquitoes. Sporozoites multiply in the liver, with a single sporozoite yielding 30,000-40,000 merozoites that are released into the blood stream. Each of these can invade a red blood cell (RBC). Inside a RBC, the merozoite transforms into a trophozoite. This divides asexually to generate up to 32 merozoites for *P. falciparum* and around 8 for the rodent malaria *P. chabaudi*. This process of asexual replication repeats itself every 48 (*P. falciparum*) and 24 (*P. chabaudi*) hours, and, in humans, results in the fevers characteristic of malaria. After invading a RBC, a small proportion of asexual parasites convert into male or female gametocytes, which are essential for transmitting the infection to other hosts through female anopheline mosquitoes (Hisaeda et al. 2005). In the mosquito, gametocytes turn into gametes which fertilize each other to form an ookinete which crosses the mosquito's mid gut wall where it encysts and forms an oocyst. Meiosis is followed by asexual replication resulting in new sporozoites which migrate to the salivary glands where, during the blood-meal of a mosquito, they are injected in to a new host. The various effector functions of the host immune

system are similarly complex with numerous components of the response operating at different stages of the parasite's life cycle (Fig. 1.3). Human disease severity in the field is remarkably variable ranging from asymptomatic infections to life-threatening cerebral malaria (Alles et al. 1998). Variation in severity of malaria may be attributed to a number of factors including geography, nutritional status, disease load, immunity and host genetics (Baird 1998; Phillips 2001; Mackinnon et al. 2002; Miller et al. 2002).

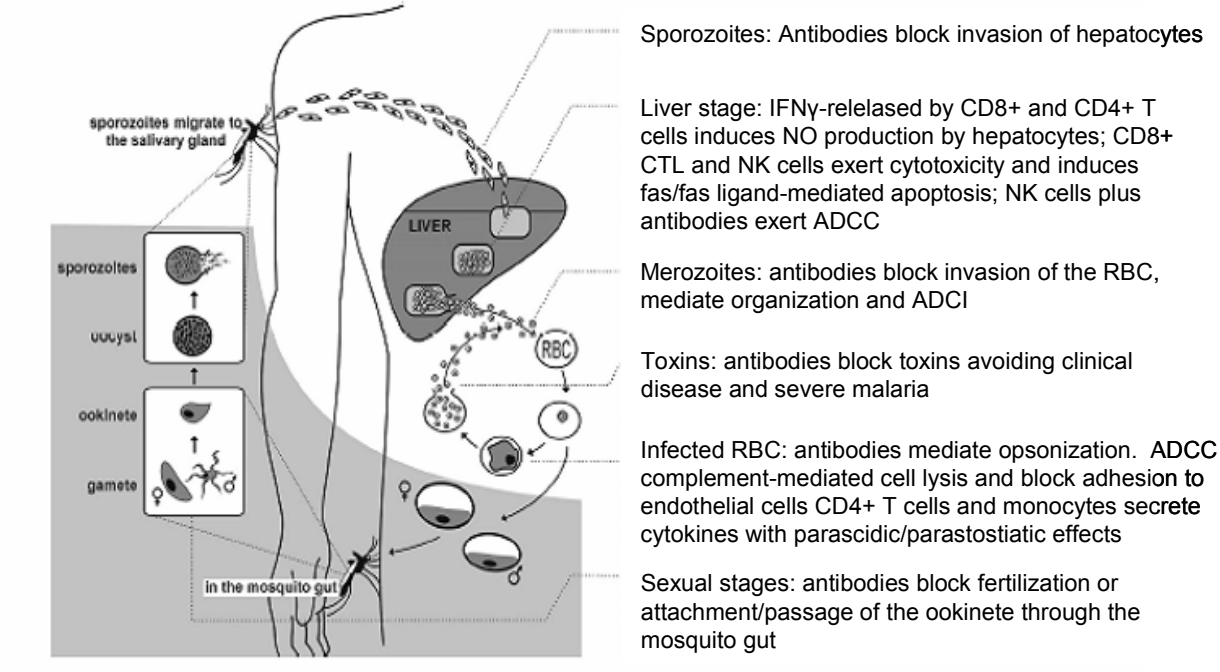

Figure 1.3. Life cycle of *Plasmodium* depicting the potential targets and mechanisms of protective immune responses. Figure reproduced from (Hoffmann & Miller 1996).

1.4.2. Malaria vaccines

Four stages of the malaria parasite life cycle have been the targets of vaccine development efforts (Fig. 1.4). The first two stages are referred to as the 'pre-erythrocytic stages' (i.e. before the parasite invades the human red blood cells); these

are the sporozoites inoculated by the mosquito into the human bloodstream, and the parasites developing inside human liver cells. The other two targets are the stages when the parasite is invading or growing in the RBCs (blood, merozoite, or erythrocytic stage); and the transmission stages, when the parasites emerge from RBCs and fuse to form a zygote inside the mosquito vector (gametocyte, gamete, or sexual stage). Despite continued efforts there are still currently no effective malaria vaccines. Many candidate malaria vaccines are directed at the disease-causing forms of the malaria life-cycle, the asexual blood-stage. Erythrocyte invasion is a complex process that requires contact, adherence and reorientation of the red blood cell by the merozoite. This is a rapid process involving a number of parasite proteins that are located on the surface of the merozoite and thus accessible to circulating antibodies. Eighteen clinical trials with asexual blood-stage vaccines have been registered in the main clinical trials registries (websites: http://www.clinicaltrials.gov or http://www.who.int/trialsearch/). The most advanced asexual blood-stage vaccines at present are based on recombinant forms of merozoite surface proteins (MSP) 1, 2 and 3, apical membrane antigen 1 (AMA-1) and the glutamate rich protein (GLURP) (Reed et al. 2006).

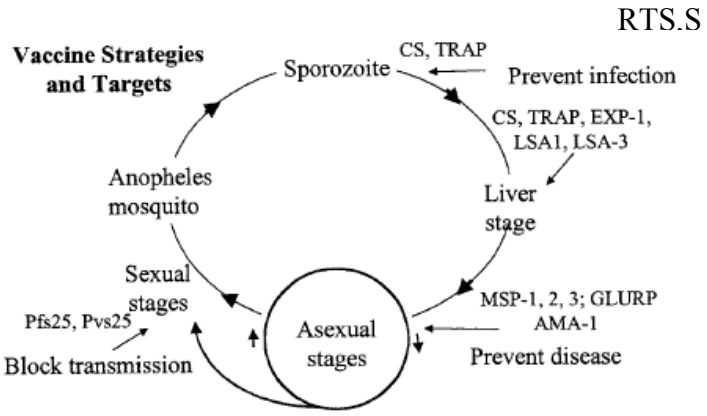

Figure 1.4. Examples of vaccine targets of the malaria life cycle. CS= circumsporozoite; RTS,S (sequences of the CS protein and the hepatitis B surface antigen (HBsAg); TRAP= thrombospondin-related adhesive protein; EXP-1 = exported antigen 1; LSA1 = liver stage antigen 1; MSP-1 = merozoite surface protein 1; GLURP = glutamate-rich protein; AMA-1 = apical membrane antigen 1; Pf = *P. falciparum*; Pv = *P. vivax*. Figure reproduced from (Ballou et al. 2004a).

1.4.3. Apical membrane antigen 1 (AMA-1)

AMA-1 is a promising vaccine candidate as it possesses fewer polymorphisms than other merozoite antigens (Anders et al. 1998; Hodder et al. 2001). AMA-1 is expressed in the sporozoite (Silvie et al. 2004), hepatic (Krzych et al. 1995; Bodescot et al. 2004) and erythrocytic (Triglia et al. 2000; Carvalho et al. 2002; Ballou et al. 2004b; Mitchell et al. 2004; Cortes et al. 2005; Greenwood 2005) stages of the *Plasmodium* life cycle. There is still much to be determined with the respect to the role of AMA-1 in erythrocyte invasion. However, failure to disrupt *ama-1* in *Plasmodium* (Triglia et al. 2000) and *Toxoplasma gondi* (Hehl et al. 2000) strongly suggests that AMA-1 has vital functions. It appears as though AMA-1 is originally trafficked as an 83-kDa protein to micronemes of the apical complex of the merozoite (Fig. 1.5A) (Triglia et al. 2000; Carvalho et al. 2002; Ballou et al. 2004b; Mitchell et al. 2004; Greenwood 2005). AMA-1 undergoes proteolytic processing into smaller fragments, and at the time of merozoite release, a 66kDa processed form is distributed around the merozoite surface (Peterson et al. 1989; Narum & Thomas 1994; Howell et al. 2005). The protein likely represents a key link between the weak initial contact involving MSPs and irreversible tight associations formed with microneme proteins (Cowman & Crabb 2006). The AMA-1 gene sequence has been determined for a number of different isolates of *P. falciparum* (Peterson et al. 1989; Thomas et al. 1990; Marshall et al. 1996; Oliveira et al. 1996) and for other *Plasmodium* species (Marshall et al. 1989; Peterson et al. 1990; Waters et al. 1990; Cheng & Saul 1994; Kappe & Adams 1996). The amino acid sequence is a classical type 1 membrane protein with a large NH-terminal putative ectodomain and a presumed transmembrane domain towards the COOH-terminus (Anders et al. 1998). The ecodomain of PfAMA-1 is largely composed of three domains: a relatively N-terminal domain 1, a central domain II, and a C-terminal domain III (Nair et al. 2002; Bai et al. 2005; Pizarro et al. 2005). Reduction and alkylation is required to stabilize the eight intramolecular disulphide bonds which are essential for inducing protective immune responses (Anders et al. 1998) (Fig. 1.5B). A number of different studies have demonstrated that each of these domains may be required to generate protective immunity against malaria. For instance, domain I appears to exhibit the most

polymorphisms, with clustering occurring at one end of a hydrophobic cleft, thought to be associated with a ligand binding site (Bai et al. 2005; Coley et al. 2007). X-ray crystallography and cell culture experiments have demonstrated a crucial role for domain II in the attachment/invasion of red blood cells (Fraser et al. 2001; Nair et al. 2002; Bai et al. 2005; Feng et al. 2005; Pizarro et al. 2005; Collins et al. 2007). Finally, genetic analyses have demonstrated domain III to be under balancing selection, and that some anti-domain III antibodies can block invasion (Nair et al. 2002; Mueller et al. 2003).

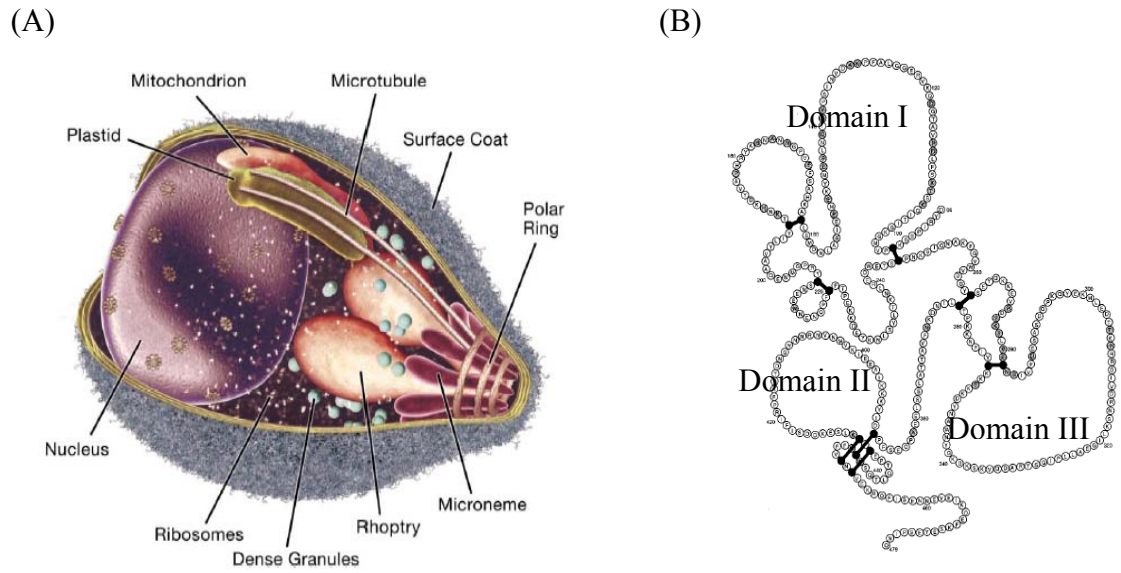

Figure 1.5. Schematic diagram of the parasite merozoite stage (A), and the structure of the three domains of AMA-1 and the disulphide bonds recognized by protective immune responses (B). (A) The major organelles and cellular structures of the merozoite are highlighted. AMA-1 is trafficked to the microneme where it is proteolytically cleaved before relocalization to the merozoite outer membrane (B) The AMA-1 ectodomain consists of three domains that are interlinked by disulphide bonds. Letters represent amino acids with darker shaded letters showing amino acid substitutions occurring among *P. chabaudi* strain DS and DK (556KA) used in this thesis. Figures reproduced from Hodder et al. (1996) and Cowman & Crabb (2006).

Because AMA-1 is relatively conserved in the genus it has been possible to use this protein from murine and primate *Plasmodium* species in vaccine efficacy trials in

monkeys, apes and mice (Marshall et al. 1989; Peterson et al. 1990; Anders et al. 1998). Murine *P. chabaudi* AMA-1 can be expressed and purified from *E. coli* constructs and trials comparing the full-length AMA-1 (AMA-1A) with the refolded ectodomain (AMA-1B) found highly significant protection with AMA-1B immunization of mice (Anders et al. 1998). Since then AMA-1 has been confirmed as a promising vaccine candidate. Naturally acquired antibody to *P. falciparum* AMA-1 (PfAMA-1) in endemic populations is associated with protection from the most virulent human malaria, *P. falciparum* (Thomas et al. 1994; Johnson et al. 2004; Polley et al. 2004; Cortes et al. 2005; Rodrigues et al. 2005). Immunization with AMA-1 induces antibodies that inhibit invasion, conferring protection against parasite challenge in a number of animal models (Deans et al. 1988; Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998; Narum et al. 2000; Stowers et al. 2001; Stowers et al. 2002; Healer et al. 2004). Humans as well as other species immunized with single allele AMA-1 vaccines have raised antibodies conferring protection from erythrocyte invasion in vitro (Hodder et al. 2001; Kocken et al. 2002). Moreover, there are currently at least six different vaccines based on the AMA-1 allele from the *P. falciparum* 3D7 strain currently in efficacy trials in humans (Polhemus et al. 2007; Maher 2008; Thera et al. 2008).

1.4.4. Plasmodium chabaudi model

Although malaria must ultimately be understood in humans, much of our knowledge on the pathogenesis of disease depends on studies in non-humans species (Miller et al. 2002; Lamb et al. 2006). This is particularly critical in the current context, where one of the key phenotypes (virulence) can only be assayed in a live host. Mouse malaria models offer an opportunity to study the mechanisms involved in malaria disease since there are some similarities between infection in humans and mice. For instance, both *P. falciparum* and *P. chabaudi* have a lack of preference for young red blood cells (reticulocytes) over older ones (normocytes) (Jarra & Brown 1989; Clough et al. 1998). Both species also display cytoadherence: the sticking of parasitized cells to other host cells (Rowe et al. 1995; Roberts et al. 2000) and undergo clonal variation (Biggs et al. 1991; Roberts et al. 1992). However, there are

also several potentially important differences. For instance, *P. chabaudi* infections frequently reach parasitaemias an order of magnitude higher than that found in human malaria infections (Collins & Jeffery 1999; Mackinnon & Read 1999a), and the relative importance of strain-transcending and strain-specific immunity may differ. A fuller evaluation of the strengths and weaknesses of the *P. chabaudi* model will be assessed in the General Discussion of my thesis (Chapter 8).

There are four species of *Plasmodium* that infect mice (*P. chabaudi*, *P. yoelli*, *P. berghei* and *P. vinckei*) and these were originally isolated from wild-caught thicket rats (*Thamnomys rutilans)* in Africa (Carter & Walliker 1976; Beale et al. 1978; Landau & Boulard 1978). To obtain clonal genotypes (lines of parasites derived from a single parasites) from these field samples, each isolate (each from a different host) were injected into groups of 14-30 mice with an inoculum expected to contain an average of one parasite (Beale et al. 1978). Successful infections were thus assumed to have been established by a single parasite which produced a clonal population. Clonal genotypes were shown to be distinct using electrophoretic enzyme analysis (Carter 1978). Some of the isolates from which clonal genotypes were derived comprised more than one species of parasite, the predominant one being *P. chabaudi*, with occasional *P. yoelli*. Therefore, after cloning, the species of each genotype was confirmed as *P. chabaudi* by morphology (Carter & Walliker 1975). Clonal genotypes of *P. chabaudi* are stored as frozen stabilites in liquid nitrogen with subscript codes used to identify their position in clonal history (Mackinnon & Read 1999b). These genotypes have significant genetic variation in virulence, measured as anaemia, weight loss and mortality during infection of laboratory mice (Mackinnon & Read 1999a). Experiments from our laboratory have utilized the availability of *P. chabaudi* genotypes to study parasite genetic variation upon which selection can act. Competition experiments between *P. chabaudi* genotypes which differ in their virulence have now strongly demonstrated that more virulent genotypes have a competitive advantage over less virulent genotypes; they produce more asexual and mosquito-infective stages (gametocytes) and are less rapidly cleared by the host (Mackinnon & Read 1999a; de Roode et al. 2003;

Ferguson et al. 2003; de Roode et al. 2004a; de Roode et al. 2004b; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006).

In nature, infections with malaria frequently consist of more than one *Plasmodium* genotype (Babiker et al. 1991; Conway et al. 1991; Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Tanner et al. 1999; Jafari et al. 2004). Mixed infections can arise due to the bite of a single mosquito inoculating more than one genotype or via successive bites from multiple mosquitoes infected with different genotypes (Grech et al. 2008). A substantial body of epidemiological evidence is consistent with competitive interactions observed in laboratory experiments of *P. chabaudi* (Daubersies et al. 1996; Arnot 1998; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006).

In theory, competitive interactions between different strains could be mediated by a number of different biological processes, including the host immune response (Read & Taylor 2001). One study from our laboratory indicated that competition could be immune-mediated (Råberg et al. 2006). In those experiments, competitive suppression of an avirulent *P. chabaudi* genotype was significantly reduced in nude mice, which cannot produce mature T cells, than in control mice. As mentioned above our laboratory has demonstrated competition to be a potent source of selection for virulence. Thus, the study by Råberg et al. (2006) inadvertently raised the possibility that current vaccination programmes, which are aimed at inducing T cell-dependent responses, might exacerbate within-host competition. In other words, vaccination may select for the competitive fitness benefit associated with virulence.

Consistent with competition experiments demonstrating that vaccines may enhance selection for virulence (Råberg et al. 2006), serial passage (i.e. the syringe transfer of malaria infected blood from one host to another) of a single *P. chabaudi* clone through whole-parasite immunized hosts, derived a parasite line which contained variant genotypes which induced more virulent infections in naïve mice compared to those lines which were derived by serial passage through immunologically naïve mice (Mackinnon & Read 2004a). An explanation for this was that immunity

selected for more virulent genotypes which were less well controlled. In another study, which did not involve serial passage, a more virulent *P. chabaudi* genotype was less well controlled during immunization of mice with the putative vaccine candidate AMA-1 than a less virulent genotype (Grech et al. 2008 *in prep*). Thus, all of the empirical experiments described so far implied that more virulent variants may have a selective advantage in an immunized host.

In contrast, however, competitive interactions between genetically diverse *P. chabaudi* strains were neither exacerbated nor alleviated during direct immunization experiments with AMA-1 or with live parasites (Grech et al. 2008). That study suggested that vaccination may not drive the evolution of virulence by exacerbating within host competition.

Thus, prior to this thesis, it was unclear which of the parasite fitness benefits associated with virulence (increased transmission, reduced immune clearance or competition) might allow more virulent strains to have a disproportionate advantage in immunized hosts. This thesis has aimed to expand on the previous studies from our laboratory in an attempt to clarify whether any of the parasite benefits associated with virulence influence selection imposed by vaccination with AMA-1. A strong grasp of these concepts could prove important in the design of future malaria vaccines.

1.5. Concepts and terminology

Throughout my PhD I attended and presented at various conferences and gave departmental seminars. I became increasingly aware of how sensitive people can be to the use of terminology to describe certain processes of my research project. I use the following definitions of these apparently controversial terms.

First, I will define the terms isolate, genotype and line in the context of *P. chabaudi*, the rodent malaria model I use in the experiments of this thesis. I define an isolate as a sample of parasites collected from wild-caught thicket rats (*Thamnomys rutilans*)

on a unique occasion. An isolate may contain more than one species of *Plasmodium*, and more than one genotype of a given *Plasmodium* species. I define a clonal genotype as an infection derived in the laboratory from the asexual blood form of a single haploid *P. chabaudi* parasite. I define a line as parasites which have undergone a particular treatment (for instance passage through immunized mice). Parasites in a line usually have certain characteristics in common, but are not necessarily genetically identical.

Second, I use the terms immunization and vaccination interchangeably. Essentially I am describing the process by which immunity is induced. This may be by directly administering an adjuvant together with an antigen that is derived from or similar to the infecting agent. The response following vaccination will be termed vaccine-induced immunity, which will have consequences for both host and infecting agent.

Third, immunization is administered with the aim of generating protective responses. In the literature, the definition of a positive protective response is very much system specific. For example, immunologists often equate a positive response to be the generation of antibodies to the immunizing antigen or successful passive transfer of antibodies from one individual to another (often measured in vitro using techniques such as ELISA), even though there is no evidence that hosts were protected against anything. Protection can be directed measured as alleviation of host disease symptoms *or* as a reduction in the number of infecting pathogens. To measure whether immunization had induced host responses I measured three parameters: antibody levels, protection from disease, and protection from *P. chabaudi* parasite density. Antibodies to the immunizing antigen were measured in serum by ELISA . I used protection from weight loss and/or red blood cell loss as indicators of protection from disease. Protection from parasite density was measured by quantifying the total number of parasites in a host using either real time qPCR or counting blood smears.

Fourth, throughout my thesis I will often refer to protective responses against malaria infection being strain/strain-specific. A strain-specific response is one which is

effective in protecting individuals against malarial infection with parasites of the same (homologous) strain that induced the response, but is often less effective against challenge infection with parasites of a different (heterologous) strain (Mendis et al. 1991; Anders et al. 1998; Martinelli et al. 2005; Cheesman et al. 2006).

Fifth, this thesis is concerned with how vaccination may drive evolutionary change in a pathogen population. One of these changes may involve pathogen virulence. Virulence is a term that can be used to describe the ability of any agent of infection to produce disease (defined in turn as harm to host). In this thesis, virulence is measured by the number of asexual *P. chabaudi* parasites, and by the degree of weight and red blood cell loss in mice during an infection.

Sixth, there have been a number of studies which have begun to look at the selective forces within a host which could lead to the evolution of virulence. For instance, where mixed infections are common, natural selection is expected to favour higher levels of parasite virulence if virulent strains have a competitive advantage, as they do in *P. chabaudi* (de Roode et al. 2003; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006). In my thesis I discuss within-host selection in the context of changes in strain (strain) frequency following immune modulation. Competition is one source of within host selection that could cause changes in frequency. Changes in frequency could also occur even if co-infecting strains are not competing i.e. are not affected by the presence of another strain. For instance changes in frequency will occur if one strain performs better in an immunized host.

Finally, the choice of terminology to describe variants that are favoured in vaccinated hosts has caused the most complaint at meetings I have attended. For example, the terms 'vaccine-escape' or 'vaccine-resistant' variants capture the essential concept, but seem to mean different things to different people. 'Escape' and resistance' are used by many in the biomedical community to mean the special case of mutations at protective epitopes. For example, vaccine escape of HBV mutants has been found to be due to mutations in the hepatitis B surface antigen (HBsAg) (Zanetti et al. 1988; Lee et al. 1991; Torresi 2002; Coleman 2006; Gibb et al. 2007). As I have described

above, epitope alterations are only one form of vaccine adaptation. Throughout this thesis I have tried to use the term which captures the form of adaptation that has occurred for a particular pathogen. Essentially all that matters is that these epitope or virulence variants are selectively favoured by vaccination. This may mean they are better able to invade/infect/penetrate the defences of a vaccinated host, or that once inside the immunized host, they have a higher per day transmission rate, or that they are cleared less quickly by the immune system than are wild type strains (Read & Mackinnon 2008).

1.6. Thesis arrangement

Each chapter in my thesis has been written up as a free-standing paper, therefore each has their own abstract, introduction and discussion. This inevitably means there is some repetition, particularly of methods and rationale. However, with this style I hope that any repetitiveness is off-set by the conceptual clarity that comes from papers. It also reflects the modern reality that science has to be reported in papers. All references are complied as one at the end of the thesis.

2. Differences at the target locus and not virulence predict protection induced by recombinant AMA-1 in the rodent malaria *Plasmodium chabaudi*

In prep for publication in *Proceedings of the Royal Society London Biological Sciences*.

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2.1. Abstract

Apical membrane antigen 1 (AMA-1) is a promising malaria vaccine candidate. Polymorphisms in this antigen are of concern to vaccine developers as these are thought to play a major role in vaccine-induced strain (genotype)-specific immunity. However, protection against malaria may not just depend on polymorphisms in the target antigen of vaccination, but on other genetically-encoded parasite traits, some of which may involve virulence. In this study AMA-1 derived from two separate *Plasmodium chabaudi* genotypes was used to immunize laboratory mice, and reciprocal parasite challenge trials were performed. These two genotypes had previously been shown to differ in AMA-1 sequence and in virulence. Thus, the aim of the study was to determine whether immunization induced protection that was strain-specific, and/or whether the more virulent genotype was less well controlled. I found vaccination with AMA-1 reduced parasites in a strain-specific manner, but that the affect was asymmetrical, with the virulent genotype being better controlled. In addition, we found that immunization did not protect against anaemia with the less virulence genotype, but immunization with either antigen reduced anaemia induced

by the virulent genotype. My results provide no support for the hypothesis that virulent genotypes may be harder to control by vaccination. The most appropriate method to reduce inadvertent selection via strain-specific immunity may be to include more than one antigen allele in vaccine preparations. I further suggest that measurements of disease as well as the parasitological status of the host should be used during vaccine efficacy studies to increase the validity of implementing certain vaccines.

2.2. Introduction

The genetic diversity of the *Plasmodium* genome appears to be a major reason that those people living in areas where malaria is endemic often require repeated exposure to generate an effective immune response (Ferreira et al. 2004). A number of animal and human studies suggest that immunity against malaria is strain (genotype)-specific: effective immunity to one strain of a given malaria parasite does not imply immunity to other distantly related strains (Jeffery 1966; Cardigan & Chaicumpa 1969; Powell et al. 1972; Jarra & Brown 1989; Crewther et al. 1996; Jones et al. 2000; Martinelli et al. 2005; Cheesman et al. 2006). Strain-specific immunity is of particular concern to malaria vaccine developers as most vaccine-candidate antigens are highly polymorphic surface proteins (Tanabe et al. 1987; Mendis et al. 1991; Miller et al. 1993; Thomas et al. 1994; Marshall et al. 1996). Thus, a vaccine which does not match all or most of the local variants at a testing site may be ineffective (Richie & Saul 2002; Saul 2007).

However, malaria parasites have other genetically-encoded traits upon which selection may act, and some of these may involve virulence (parasite-induced host harm) (Frank 1996; Mackinnon & Read 1999a; Mackinnon & Read 1999b). Classical models of parasite virulence describe that in order to maximize fitness, a parasite should optimize the costs and benefits of virulence (Frank 1996; Dieckman et al. 2002). A considerable body of theory now predicts that natural immunity, or mechanisms which act to enhance it such as vaccination, are likely to alter the costs and benefits of parasite virulence (Gandon et al. 2001; Gandon & Day 2003; van

Boven et al. 2005; André & Gandon 2006; Restif & Grenfell 2006; Gandon & Day 2007; Mackinnon et al. 2008; Read & Mackinnon 2008; Williams & Day 2008). Experimental evidence has started to appear in support of vaccine-driven evolution. First, under controlled laboratory conditions, a clonal genotype of the rodent malaria *P. chabaudi* evolved virulence more rapidly when serially-passaged through whole-parasite immunized mice than when passed through non-immunized mice (Mackinnon & Read 2004a). An implication of that study was that more virulent genotypes in the derived lines had a selective advantage in an immunized host. Second, in another study which did not involve serial passage, a more virulent *P. chabaudi* genotype was less well controlled than those genotypes of lesser virulence during infection of mice immunized with a rodent analogue of the malaria vaccine candidate Apical Membrane Antigen 1 (AMA-1) (Grech et al. 2008 *in prep*). More virulent genotypes may have a selective advantage if they can replicate more rapidly or can sustain higher densities for longer, making them relatively less vulnerable to any given concentration of immune effectors (Mackinnon & Read 1999; Chotivanich et al. 2000; Deans et al. 2006).

Thus vaccine-imposed selection could arise because of strain-specific immunity, because of virulence differences, or a combination of both process, if for instance, virulence determinants are also targets of strain-specific responses. In one field trial of a malaria vaccine known as "Combination B" in Papua New Guinea, it was unclear whether vaccination had imposed selection that was strain-specific, or whether the vaccine was less able to control more virulent strains (Genton et al. 2002; Genton et al. 2003; Fluck et al. 2004; Fluck et al. 2007). The vaccine consisted of three recombinant blood-stage proteins, merozoite surface protein 1 (MSP-1), MSP-2, and ring-infected erythrocyte surface antigen (RESA). The MSP-2 antigen is known to be highly polymorphic and the different *msp-2* alleles can be grouped into two families, FC27 and 3D7 (Smythe et al. 1990). Despite the FC27 type previously being known to cause more virulent infections (Engelbrecht et al. 1995), the 3D7 type was included in the vaccine formulation. Among parasites subsequently acquired by vaccinees, 3D7-type alleles were rarer than in people given a placebo whereas the prevalence of the FC27 form was unaltered (Genton et al.

2003). Thus, vaccination selected against the variant contained in the vaccine. This could have been because of strain-specific immunity, or because the virulent form, FC27, was less successfully controlled by vaccine-induced immunity.

In this study I aimed to investigate whether vaccination with AMA-1, is more likely to select for parasites in a strain-specific manner, or whether other parasite traits, which may involve virulence determinants at other antigenic loci, allow those more virulent strains to have a selective advantage in an immunized host. AMA-1 is a merozoite protein and is thought to be essential for erythrocyte invasion (Triglia et al. 2000; Carvalho et al. 2002; Ballou et al. 2004; Mitchell et al. 2004; Cortes et al. 2005; Greenwood 2005). Immunization with AMA-1 confers protection against parasite challenge in a number of animal models (Deans et al. 1988; Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998; Narum et al. 2000; Stowers et al. 2002; Healer et al. 2004). Antibodies from the immune sera of animals and humans immunized with AMA-1, as well as sera from those people who live in areas where malaria is endemic, can prevent erythrocyte invasion in vitro (Thomas et al. 1994; Hodder et al. 2001; Kocken et al. 2002; Johnson et al. 2004; Polley et al. 2004; Cortes et al. 2005; Rodrigues et al. 2005). In laboratory mice, the availability of genotypically distinct *P. chabaudi* genotypes has previously provided an experimental system for examining the impact of genetic diversity on the efficacy of AMA-1 vaccination (Marshall et al. 1989; Crewther et al. 1996; Anders et al. 1998). The nucleotide sequence of *P. chabaudi* AMA-1 (PcAMA-1) genotype DS differs from genotype DK (556KA) by 79 amino acids (Marshall et al. 1989; Crewther et al. 1996). Immunization of mice with the refolded ectodomain of DS AMA-1 or with rabbit antibodies raised against the same antigen have been shown to induce responses which are ineffectual against challenge with the heterologous DK genotype (Crewther et al. 1996). Interestingly, these genotypes are also known to differ in virulence; genotype DS achieves a higher number of total parasites across an infection compared to DK. Because reciprocal trials to test whether the more virulent DS strain is less affected by DK AMA-1 immunization have not been performed, it is not possible to say whether DK vaccine breakthrough is due to antigenic differences or to virulence differences, or both.

Here experimental mice were immunized with AMA-1 derived from either *P. chabaudi* strain DS or DK and then infected with either one of the strains. The aim of the study was to test whether AMA-1 induced selection that was strain-specific, or whether the more virulent DS genotype had a selective advantage and was thus less affected by heterologous immunization compared to the less virulent DK genotype.

2.3. Material and Methods

2.3.1 Parasites and hosts

Isolates containing *P. chabaudi adami* were originally collected from wild-caught thicket rats (*Thamnomys rutilans*) in the Congo (Brazaville) (Carter and Walliker 1976). Isolates were cloned, genotyped and stored in liquid nitrogen with subscript codes used to locate their position in clonal history. In this experiment we used *P. c. adami* genotype DS_{218} and DK_{112} originally genotyped from isolates 408XZ and 556KA respectively. Hosts were inbred female C57BL/6J mice aged six to eight weeks fed on 41B maintenance diet (Harlan, UK). Their drinking water was supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964) and they were kept in a 12L:12D cycle.

2.3.2. Immunizations, parasite challenge and sampling

Two experiments were performed (Table 2.1). Immunization protocols followed those of Anders et al. (1998) and Crewther et al. (1996). In the first, immunization was with the highly immunogenic ectodomain of the full AMA-1 protein derived from either parasite genotype DS or DK. The AMA-1B ectodomain was expressed and purified from *E. coli* constructs as described elsewhere (Anders et al. 1998). In the second experiment, immunization was via the passive transfer passive of

hyperimmune sera from rabbits which had previously been immunized with AMA-1B derived from parasite genotype DS or DK.

Clone	DK infection			DS infection		
Experiment	Control	DK AMA-1	DS AMA-1	Control	DK AMA-1	DS AMA-1
Experiment one	5	5	5	5	6	6
Experiment two	4	4	4	4[1]	4	4

Table 2.1. Experimental design. In experiment one, mice were immunized with recombinant AMA-1 antigen emulsified in Montanide ISA 720 (10 µg/100 µl) or with a control emulsion of PBS and Montainide (100 µl). Boost immunizations occurred 4 weeks after the primary immunization and two weeks prior to parasite challenge with either genotype (clone) DS or DK. In experiment two, mice were immunized with three milligrams of purified anti-DK AMA-1 or anti-DS AMA-1 IgG from the sera of immune rabbits one day before parasite infection and again 3 hours before parasite infection. Control mice were injected with a sham inoculation of rabbit serum of the same isotype. Figures show the number of mice in each treatment group. In experiment two one mouse died as indicated by the number in brackets.

In experiment one, mice were randomized into three treatment groups. Injections were performed intra-peritoneally with 10 µg of either DS AMA-1 or DK AMA-1 antigen emulsified in 100 µl of the adjuvant Montanide ISA 720 (Seppic, Paris, France). Control mice were injected with a sham inoculation which was an emulsion of 100µl PBS in the same concentration of Montanide ISA 720. Boost immunisations were conducted 4 weeks after the primary immunisation. Two weeks after the boost immunization, mice within each of the three treatment groups were randomized into two groups and infected intra-peritoneally with 10^5 parasites of genotype DS or DK.

In experiment two, rabbits were immunized with refolded AMA-1B derived from parasite genotype DS or DK emulsified in Montanide ISA 720. For the primary immunizations, rabbits received a total of 100-200 µg of emulsified antigen between two intra-muscular sites. Rabbits were boosted at 4-8 week intervals with the same

amount of antigen injected intra-muscularly and subcutaneously. Immunoglobulin G (IgG) was isolated from the sera of rabbits by affinity chromatography on protein A-Sepharose columns (Pharmacia/Amrad). Prior to immunization, experimental mice were randomized into three treatment groups. For passive transfer to mice, three milligrams of purified anti-DK AMA-1 or anti-DS AMA-1 IgG was administered intra-peritoneally one day before parasite infection and again 3 hours before parasite infection. Control mice were injected with a sham inoculation of rabbit antibody of the same isotype (IgG Sigma I5006). Before parasite challenge, mice within each of the three treatment groups were randomized into two groups and infected intra-peritoneally with 10^5 parasites of genotype DS or DK.

I followed the infection dynamics for 29 days. As of the first day of parasite infection (day 0), I daily measured body weights and took blood samples from the tail of experimental mice to estimate RBC density (by flow cytometry; Beckman Coulter). As of day 4 post-parasite infection onwards, I took daily blood samples from the tail to make Giemsa-stained blood smears for estimating daily parasite densities. I chose day 4 as this is around the first day that parasites can be detected in the blood using microscopy. Since *P. chabaudi* has a 24 hour replication cycle, the total number of parasites in any period can be estimated by summing the daily parasite counts.

2.3.3. Isotype ELISA

In the first experiment, three days prior to parasite challenge, ELISA assays were used to check that antigen immunizations had generated an antibody response. All mice were assayed for both anti-DS AMA-1 and anti-DK AMA-1 total IgG and IgG2b antibodies. I used IgG2b as previous work in my laboratory showed that C57BL/6 produce this allotype in response to *P. chabaudi* infection (K. Grocock, A. Graham unpublished). Protection induced by immunisation with recombinant AMA-1 in C57 mice is isotype independent (Burns et al. 2004). Serum fractions were separated by centrifugation from 20 µl of blood samples taken from the tail and stored at -80^{o}C. High binding 96 well ELISA maxisorb immunoplates (Nunc) were

coated with either DS AMA-1 or DK AMA-1 at a concentration of 1µg/ml in 0.06M carbonate buffer (0.04M $NaHCO_3$, 0.02M $NaCO_3$, pH 9.6) in a final volume of 50 µl per well. Plates were stored at 4°C overnight to allow the antigen to bind. Non-specific binding was blocked by incubating wells with 5% BSA: carbonate buffer (200 µl/well) for 2 hours at 37°C. Wells were then washed three times in Tris-buffered saline with 0.01% Tween (TBST). Serum samples were detected in a serial dilution 1/100-1/204800 using TBST as a diluent, in a final volume of 50 µl per well and incubated for 2 hours at 37°C. Wells were washed three times in TBST. HRP conjugated goat anti-mouse IgG2b detection antibody (Southern Biotech 1100-05) was diluted 1/4000 in TBST to a final volume of 50 ul per well. Plates were incubated for 1 hour at 37°C. Wells were washed three times in TBST followed by a final wash in distilled water. ABTS peroxide substrate (Insight biotechnology) was added at 100 µl per well and allowed to develop at room temperature for 20 minutes. Optical density was read at 405 nm using a spectrophotometer. IgG2b isotype antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D.) was greater than the mean (plus 2 standard deviations) O.D values observed for naïve mouse sera assayed against both DS and DK AMA-1 at 1/100 dilutions.

2.3.4. Trait definition and statistical analyses

In the first experiment, prior to parasite challenge, anti-DS AMA-1 and anti-DK AMA-1 total IgG and IgG2b antibodies were measured in mouse sera from all 32 mice giving a total of 64 measurements. Since *P. chabaudi* has a 24 hour replication cycle, the total number of parasites in any period can be estimated by summing the daily parasite counts. Mice infected with genotype DK actually gained weight so weight loss was not a suitable measure of virulence. All other data were analysed using General Linear Models (GLMs) in MINITAB (Minitab, version 14). To meet normality and homogeneity of variance assumptions, data on antibodies and red blood cell density were log transformed while all parasite densities were square route transformed. GLMs were used to test whether the magnitude of protection differed between the antigen immunizations (DK AMA-1 or DS AMA-1); that is whether

there was a statistical interaction between infecting genotype and immunizing treatments. Maximal models (response variable = infecting genotype + immunizing treatment + infecting genotype x immunization treatment) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. In experiment two one mouse died; this mouse was included in the calculation of daily densities until death, since death occurred as initial parasiteamias were declining.

2.4. Results

Table 1 gives details of the two experiments, including sample sizes.

2.4.1. Pre- parasite infection antibody titres

In experiment one, anti-DS AMA-1 and anti-DK AMA-1 IgG and IgG2b antibodies were measured in mouse sera to ensure our antigen immunization successfully generated antibody responses. Anti-DK and anti-DS AMA-1 total IgG and IgG2b antibody titres were higher in antigen-immunized mice than in sham-immunized controls (Fig. 2.1A; total IgG sham-immunized versus antigen immunized: $F_{1,62}$=68.72, p=<0.001; Fig 2.1B; IgG2b sham-immunized versus antigen immunized: $F_{1,62}$=8.92, p=0.004). Among antigen immunised mice only, immunization with DS AMA-1 induced higher total IgG titres than immunization with DK AMA-1 on both DS and DK AMA-1 coated ELISA plates (Fig. 2.1C; immunization: $F_{1,41}$=23.03, p=<0.001; immunizing treatment x ELISA antigen: $F_{1,40}$=1.18, p=0.28). Conversely, IgG2b antibodies were specific for the antigen they had been exposed to during immunization (Fig. 2.1D; immunizing treatment x ELISA antigen: $F_{1,40=}$9.99, p=0.003). For example, anti-DS AMA-1 IgG2b antibody tires were higher when assayed against the homologous DS antigen than the heterologous DK antigen and vice versa. Among antigen-immunized mice, antibody titres (total IgG or IgG2b)

were not predictive of protection from either parasite density or disease (data not shown correlation p >0.2 in all cases).

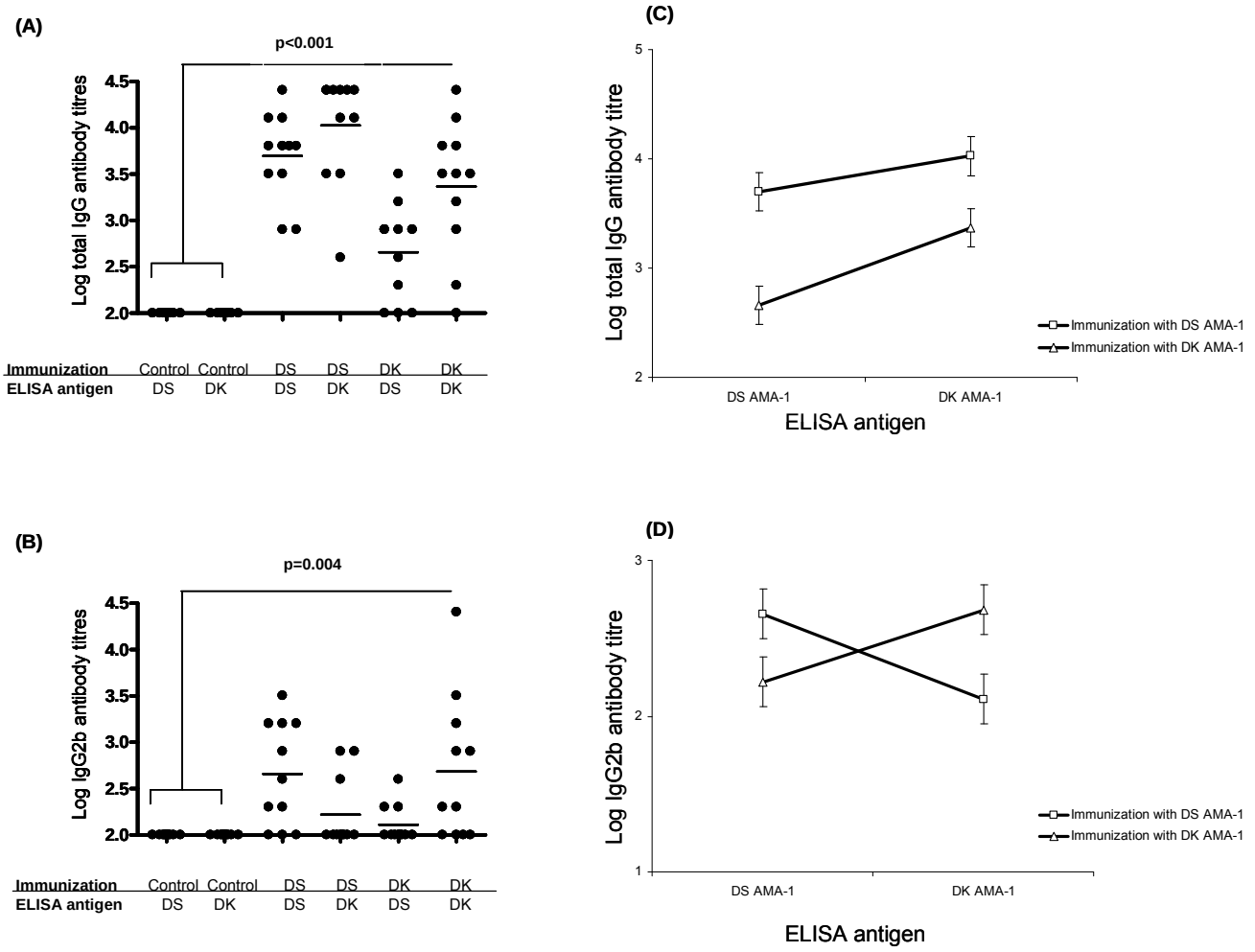

Figure 2.1. Total IgG or IgG2b antibody levels from the serum of experiment one mice. In A and B, each of the treatments used to immunize mice (sham-inoculated control, DS AMA-1 or DK AMA-1) and the AMA-1 test antigen used to coat ELISA plates are shown on the x axis. Each dot represents the antibody titre against a particular immunizing antigen. Horizontal lines indicate the mean antibody levels. Total IgG and IgG2b antibody levels in antigen immunized groups of mice were higher than in sham inoculated controls (p=<0.001, p=0.004 respectively). In C and D, each line represents the least means square of the immunizing antigen x ELISA antigen interaction with the associated standard error. Open squares represent immunization with DS AMA-1 and open triangles represent immunization with DK AMA-1

2.4.2. Genotype differences

Considering sham-immunized control infections only, genotype DS achieved higher total parasite densities than did DK (Fig. 2.2; $F_{1,14}$=25.40, p=<0.001). Similarly, in sham-immunised mice, infection with DS induced more anaemia than did genotype DK (Fig. 2.3; $F_{1,15}$=14.80, p=0.002). These genotype differences in performance and virulence did not differ between the two experiments (infecting genotype x experiment: $F_{1,14}$=0.02, p=0.88 and $F_{1,14}$=0.67, p=0.43 respectively). Thus, as reported previously (Crewther et al. 1996), DS was indeed the more virulent of the two genotypes.

2.4.3. Protective effects of vaccination on parasite density

The level of anti-parasite protection depended on the infecting genotype and the type of immunization (Fig. 2.2; immunizing treatment x infecting genotype: $F_{2,46}$=7.02, p=0.002), but in a similar way for antigen immunization and passive transfer of hyperimmune sera (immunizing treatment x infecting genotype x experiment: $F_{2,44}$=1.13, p=0.33). Visual inspection of Fig. 2.2 shows two things: (i) DK, the less virulent genotype, is less well controlled by immunization, having achieved similar parasite densities in the face of heterologous immunization as it did in the absence of immunization. In contrast DS, the more virulent genotype, is reduced by both types of immunization. (ii) Immunization with DS AMA-1 only generated homologous protection, whereas DK AMA-1 induced homologous and heterologous protection. Together, these results show that protection was asymmetrically strain-specific, and that the less virulent genotype was less well controlled by heterologous immunization.

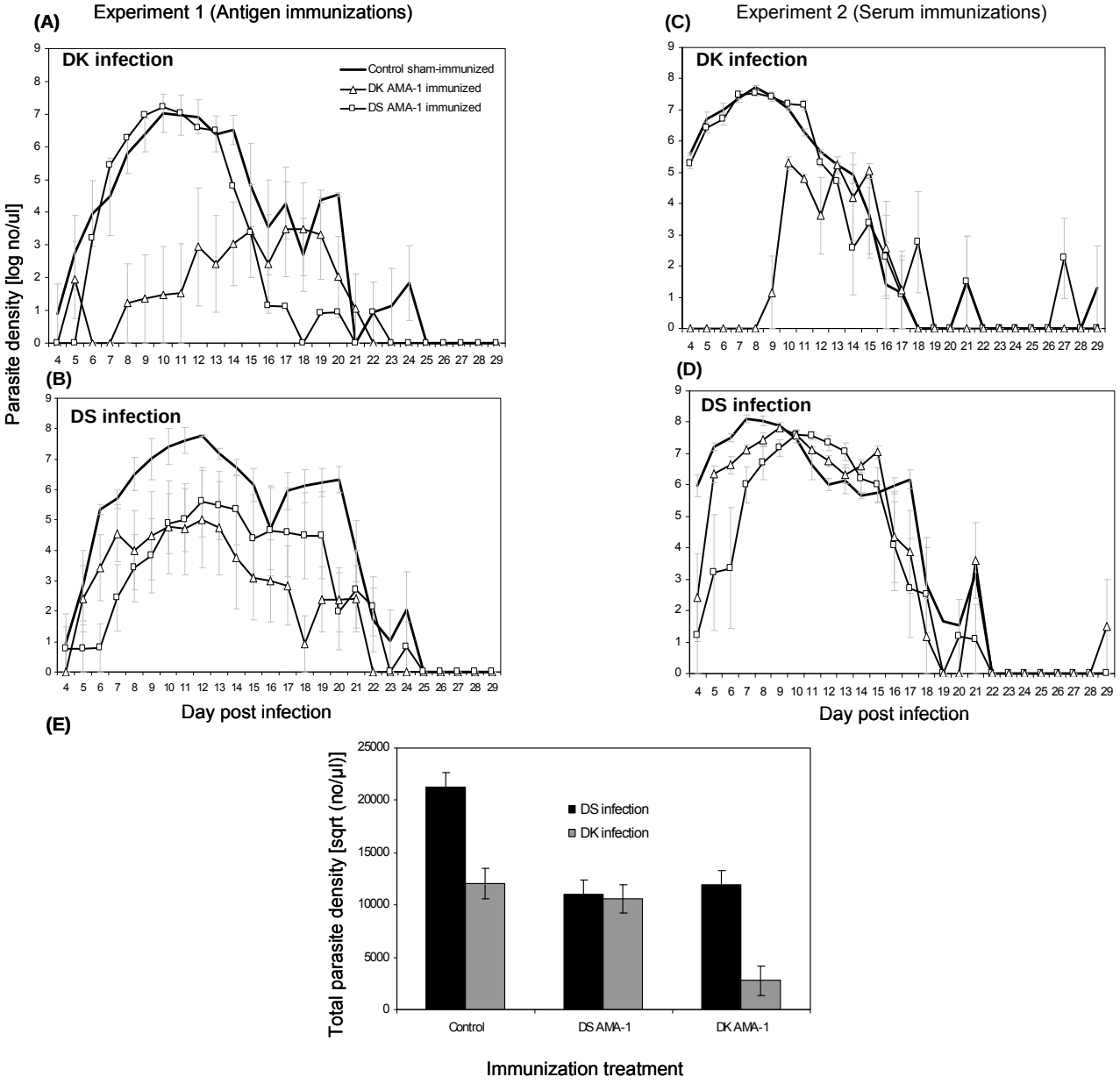

Figure 2.2. Parasite genotype density kinetics for experiment one (left panels) and experiment two (right panels). Each line represents the mean of up to 6 mice (+/- 1 s.e.m.) during infection with genotype DK (A,C) or genotype DS (B,D) under either sham inoculated control (solid black line), DK AMA-1 (open triangles) or DS AMA-1 (open squares). Figure 2E illustrates the total parasite density interaction when the data from experiment one and two were combined. Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m) during infection with genotype DK (grey bars) or genotype DS (black bars).

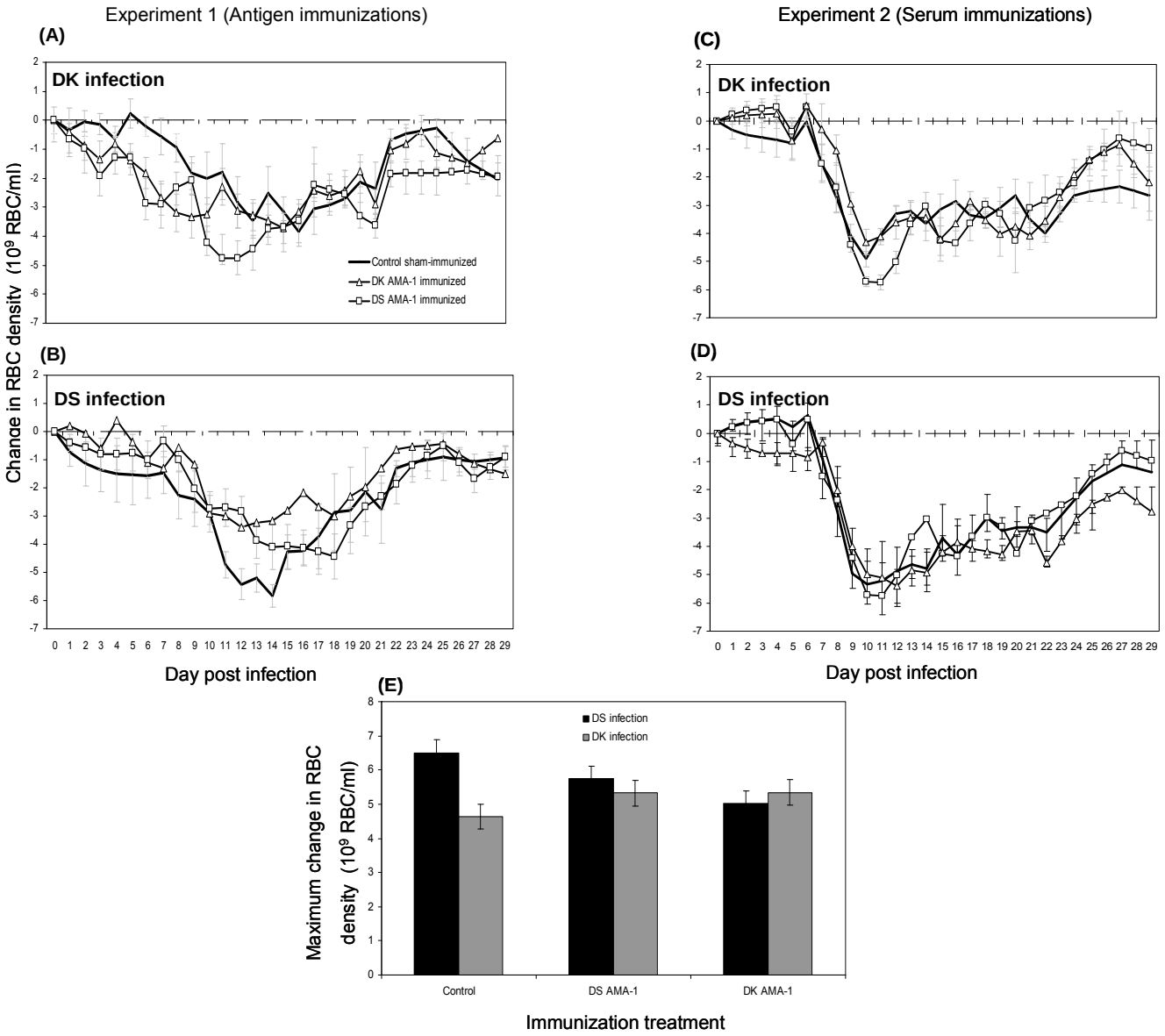

Figure 2.3. Effect of *Plasmodium chabaudi* infecting genotype and immunization treatment on red blood cell densities for experiment one (left panels) and experiment two (right panels). Each line represents the mean of up to 6 mice (+/- 1 s.e.m.) during infection with genotype DK (A,C) or genotype DS (B,D) under either sham inoculated control (solid black line), DK AMA-1 (open triangles) or DS AMA-1 (open squares). Figure 3E illustrates the maximum change in red blood cell loss interaction when the data from experiment one and two were combined. Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m) during infection with genotype DK (grey bars) or genotype DS (black bars).

2.4.4. Protective effects of vaccination on virulence

Protection from anaemia depended on the immunization and the infecting genotype (Fig 2.3; immunization x infecting genotype: $F_{2,46}$=4.70, p=0.014), and similarly for antigen immunization and passive transfer of hyperimmune sera (immunizing treatment x infecting genotype x experimental block: $F_{2,44}$=1.72, p=0.19). Visual inspection of Fig. 2.3 reveals no significant difference in red blood cell losses between control and immunized mice infected with DK (Fig. 2.3A,C,E; Immunization: $F_{2,24}$=1.43, p=0.26). Immunization protected against anaemia during infection with genotype DS (Fig. 2.3B,D,E; Immunized versus sham: $F_{1,27}$=5.08 p=0.003) with the magnitude of protection being similar under DS or DK AMA-1 (DS AMA-1 versus DK AMA-1: $F_{1,18}$=1.79 p=0.19). Thus, as with the parasite data, protection from anaemia due to infection with genotype DS was similarly generated by either antigen immunization.

2.5. Discussion

The aim of this study was to determine in vivo whether a more virulent *P. chabaudi* genotype (DS) was less well controlled by heterologous immunization than a less virulent genotype (DK). Thus, I was testing whether more virulent parasites have a selective advantage in an immunized host or whether vaccine efficacy depended on strain-specific differences at the target locus. I found that anti-parasitic protection was asymmetrically strain-specific, but it was the less virulent genotype that was less well controlled by heterologous immunization (Fig. 2.2). In terms of protection from anaemia, both antigens were equally protective during infection with the virulent genotype (Fig. 2.3). My results were comparable for direct antigen immunization or

via the passive transfer of hyperimmune rabbit serum, and consistent with previous studies which have shown that immunization with DS AMA-1 failed to protect against heterologous challenge (Crewther et al. 1996). Thus, vaccine efficacy may depend on differences at a target locus, but a single antigen immunization need not always induce protection that is strain-specific. The potency of a vaccine may be increased by surveillance for the most common or most cross-reactive allelic variants at a vaccine testing site.

Do my results here go any way to explaining the selection observed in the field trial of the 'Combination B' malaria vaccine (Genton et al. 2002; Genton et al. 2003)? In that trial it was implied that strain-specific selection had resulted as representatives from both MSP-2 allelic families had not been included in the formulation (Fluck et al. 2004; Fluck et al. 2007). However, since the FC27 allelic family, which was not included in the vaccine and was subsequently unaffected, had previously been associated with more morbid infections (Engelbrecht et al. 1995), it was also possible that the vaccine was just less effective at protecting against more virulent variants (Mackinnon & Read 2004a; Mackinnon et al. 2008; Read & Mackinnon 2008). My results go against those latter arguments that a more virulent variant may be less well controlled, and that strain-specificity may result from not including a number of allelic variants, or from choosing a variant which induces less cross-strain protection.

The results from my study apparently contradict those experiments by Mackinnon et al. (2004a), which suggested virulent variants may have a selective advantage in an immunized host. An explanation for the discrepancy between the two studies could be that that accelerated evolution of virulence as previously reported may be a feature only seen during serial passage of a single *P. chabaudi* genotype through whole-parasite immunized hosts. In the results presented here, which did not involve serial passage, we saw no evidence that a virulent genotype is more likely to be selected. In order to resolve these discrepancies in future experiments I will evolve *P. chabaudi* through AMA-1 immunized hosts (See Chapter 8.4 Future directions).

It is clear from my results that the two different antigens were not equally protective.

DK AMA-1 induced anti-parasitic protection that was asymmetrical, and was equally protective as DS AMA-1 at protecting against anaemia during DS infection. On the other hand, DS AMA-1 only generated protection against homologous parasite infection. The cross-strain protection I observed here could be explained by the ability of some inhibitory antibodies raised under DK AMA-1 vaccination able to recognize conserved epitopes in the heterologous genotype, or by cross-reacting with other *P. chabaudi* antigens, or AMA-1 like sequences, in natural protein complexes (Hodder et al. 2001). In this experiment I also measured antibodies in vitro purely as an indicator that a response had been generated against the immunizing antigen (Fig. 2.1). However, I did note that total IgG was cross-genotype reactive, while IgG2b was predominantly genotype-specific, recognizing the antigen that it had been exposed to during immunization. However, since the precise antibody isotypes induced by AMA-1 immunization that are required for parasite clearance are still unclear (Burns et al. 2004), we do not draw anything conclusive from these results.

As observed in this study and as attributed previously by others (Crewther et al. 1996; Healer et al. 2004), immunization with DS AMA-1 induced protection that was strain-specific. To the best of my knowledge, there are no reports that variants of AMA-1 have different intrinsic virulence, so that any strain-specific immunity induced by including just one antigen, should not directly alter virulence. However, caution should be taken when vaccines contain antigens involved in pathogenesis e.g. red cell invasion, as these could be inadvertently linked to virulence. Population-wide surveillance for the most common allelic variants at a vaccine testing site may reduce the chance of selection. Another approach has been to design vaccines which combine more than one allele of an antigen (Hoffmann & Miller 1996; Bolad & Berzins 2000; Richie & Saul 2002; Polley et al. 2007). For example, one group immunised rhesus monkeys with a mixture of two allelic forms of PfAMA-1 designated AMA1-C1, or the component alleles and measured responses in vitro using growth inhibition assays (GIA) and ELISA (Kennedy et al. 2002; Malkin et al. 2005; Miura et al. 2007a). However, antibodies were similarly effective regardless of whether immunization was with a single variant or AMA1-C1. Thus, questions

had been raised to the necessity of using multi-allele vaccines [Chapter 4, Appendix 1 (Barclay et al. 2008b)].

Here I found DK AMA-1 was the most effective vaccine at inducing both cross-strain protection from parasites and disease, and reducing homologous parasite densities. In spite of this it was ineffective at alleviating host disease during homologous challenge. Thus, my results highlight the importance of considering measurements of disease as well as the parasitological status of the host during vaccine efficacy studies.

In conclusion, I found that vaccination with AMA-1 reduced parasites in a strain-specific manner, but that the effect was asymmetrical, with the virulent genotype being better controlled. Thus, my results provide no support for the hypothesis that virulent variants may be harder to control, and more strongly support the hypothesis that vaccine efficacy may depend on strain-specific differences at a target locus. However, since a number of blood-stage vaccine antigens are involved in pathogenesis, one of the safest ways to avoid inadvertent selection may be to include all known variants of a locus in vaccine formulations. So far, in vitro studies by others have indicated that the inclusion of more than one allelic form of an antigen may not increase vaccine potency (Kennedy et al. 2002; Malkin et al. 2005; Miura et al. 2007b; Lalitha et al. 2008). As the results presented here highlight the importance of measuring host disease as well as parasitological status of the host, in future experiments I plan to investigate in vivo any benefits of including more than one antigen allele in vaccine formulations [Chapter 4, Appendix 1 (Barclay et al. 2008b)].

3. Virulence or sequence diversity: what mediates within-host selection during immunization with Apical Membrane Antigen-1?

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3.1. Abstract

Vaccines against malaria have the potential to impose selection in a number of ways. Selection for non-vaccine strains is expected to occur if vaccination induces protective immune responses that are strain-specific. However, malaria parasites also differ in virulence (parasite-induced host damage). Where virulence is (for instance) associated with immunosuppression, or immunoevasion, more virulent genotypes may have a selective advantage over less virulent genotypes in an immunized host. In this study I tested whether more virulent *P. chabaudi* parasites had a selective advantage during immunization with the malaria vaccine candidate Apical Membrane Antigen 1 (AMA-1). I found no selective advantage to virulence: vaccination reduced those parasites which were most similar to the vaccine target to a greater extent than those that were different. My results apparently contradict previous studies which have suggested that there is a within-host advantage to

virulence. Selection imposed by vaccination is likely to depend on the fine specificity of the immune response induced by alternative immunizing antigens.

3.2. Introduction

Much of our current knowledge on *Plasmodium* genetic variability comes from molecular studies of proteins which are putative malaria vaccine candidates. The genes for merozoite stage vaccine antigens are known to be highly polymorphic (Escalante et al. 1998; Mahajan et al. 2005) such that antibodies raised to one form of the protein bind less efficiently to heterologous forms present in other genotypes (strain-specific immunity) (Richie & Saul 2002; McKenzie et al. 2008). Polymorphisms are of particular concern to vaccine developers as the success of a particular vaccine may depend on the genetic relatedness to the vaccine strain at individual target sites (Richie & Saul 2002).

However, malaria parasites also differ in virulence (here defined as parasite-induced host harm) (Frank 1996; Mackinnon & Read 1999a). Studies in *P. chabaudi* have demonstrated that the variation in virulence observed during infection of laboratory mice are likely to be due to differences in a number of genetically-encoded parasite traits (Taylor & Read 1998; Mackinnon & Read 1999a; Mackinnon & Read 1999b; de Roode et al. 2005b; Bell et al. 2006). In the field, different 'strains' of the human malaria *P. falciparum* are thought to be responsible for differences in disease severity (Deans et al. 2006). In theory, these genetically-encoded traits may allow more virulent strains to have a selective advantage in an immunized host, when virulence is for instance associated with immunosuppression, or immunoevasion, (Gandon et al. 2001; Mackinnon & Read 2004a; Mackinnon & Read 2004b; Mackinnon et al. 2008).

The trial of the malaria vaccine known as 'Combination B' in Papua New Guinea provided the first field evidence that a vaccine against this disease could exert selective pressure (Genton et al. 2002). This vaccine contained a single antigen from each of three polymorphic loci of *P. falciparum*. One of these loci, Merozoite

Surface Protein 2 (MSP-2), is dimorphic, with each parasite having an allele from one of two allelic families (labelled 3D7 and FC27). The MSP-2 allele in the Combination B vaccine came from the 3D7 family. Among parasites subsequently acquired by vaccinees, 3D7-type alleles were rarer than in people given a placebo, thus, vaccination selected against the variant contained in the vaccine. Interestingly, the FC27 allelic family is associated with more virulent infections (Engelbrecht et al. 1995). Thus, what was not clear from this field trial was whether the vaccine imposed selection that was strain-specific (Fluck et al. 2004; Fluck et al. 2007) or whether more virulent parasites were less well controlled because they had a selective advantage.

More virulent genotypes could have a selective advantage in an immunized host by utilizing a number of advantages associated with virulence. For instance, during infection of naïve hosts, more virulent *P. chabaudi* genotypes transmit more successfully, are less rapidly cleared by the host, and have an advantage in competition over less virulent genotypes (Mackinnon & Read 1999a; de Roode et al. 2005b; Bell et al. 2006; Mackinnon et al. 2008; Read & Mackinnon 2008). In theory, one or more of those virulence benefits may also accrue or even be enhanced by vaccination. For instance, if more virulent genotypes persist for longer as immunity develops in naïve mice it is likely that these strains will be less rapidly cleared by vaccine-induced immunity.

Consistent with this, *P. chabaudi* serially passaged (by syringe transfer of parasites in blood from one host to another) through whole-parasite immunized mice became more virulent than parasites passaged through naïve mice, even after mosquito transmission (Mackinnon & Read 2004a). In another study, which did not involve serial passage, more virulent *P. chabaudi* genotypes were less well controlled by immunization with the putative vaccine candidate Apical membrane Antigen-1 (AMA-1) than were less virulent genotypes (Grech et al. 2008 *in prep*).

However, competition experiments failed to find a within host advantage to virulence in mice vaccinated AMA-1 (Grech et al. 2008). Furthermore, reciprocal

immunization and parasite challenge trials with AMA-1 derived from two *P. chabaudi* genotypes which differed in virulence appeared to induce protective responses that were asymmetrically strain-specific (Chapter 2). In that experiment the more virulent DS genotype was better controlled by heterologous immunization than the less virulent genotype.

In light of this mixed evidence, I extended the earlier experiments of Grech et al. (2008 *in prep*) to ask whether vaccination with AMA-1 is more likely to impose selection in a strain-specific manner or whether virulent genotypes will be less well controlled. The aim of this study was to determine whether vaccination with a different AMA-1 antigen to the study by Grech et al. (2008 *in prep*) was also less able to control more virulent genotypes. These results should inform current malaria vaccine programmes, many of which are focused on reducing strain-specificity by including a number of different antigens or variants of a particular antigen in vaccine formulations.

AMA-1 is a promising vaccine candidate for human malaria as it possesses fewer polymorphisms that other candidate merozoite antigens (Anders & Smythe 1989; Hodder et al. 2001). There are currently at least six different vaccines based on the AMA-1 allele from the *P. falciparum* 3D7 strain currently in efficacy trials in humans (Polhemus et al. 2007; Maher 2008; Thera et al. 2008). However, polymorphic sites in the AMA-1 protein have been shown to be of immunological importance (Peterson et al. 1989; Marshall et al. 1996; Polley & Conway 2001; Polley et al. 2003; Dutta et al. 2007). For instance, anti-AMA-1 antibodies from animals and human field sera inhibit parasite growth in a strain-specific manner (Crewther et al. 1996; Anders et al. 1998; Hodder et al. 2001; Polley et al. 2003; Cortes et al. 2005; Polhemus et al. 2007). Allelic replacement experiments have directly implicated sequence polymorphisms in antigenic escape (Healer et al. 2004), and cross strain inhibition assays suggest that the extent of escape correlates with sequence difference between the vaccine and the target strain (Kennedy et al. 2002). AMA-1 polymorphism has not to my knowledge ever been associated with parasite virulence.

3.3. Materials and Methods

3.3.1. Parasites and hosts

Blood isolates were originally collected from wild-caught thicket rats (*Thamnomys rutilans*) from different geographical areas of the African continent (Carter & Walliker 1976; Beale et al. 1978). *P. c. chabaudi* were isolated from the blood of rats of the Central African Republic, while *P. c. adami* were isolated from the blood of rats of the Congo (Brazzaville). Laboratory rodents were infected with sub-inoculations of blood and the parasites were cloned and genotyped. Clonal genotypes were stored as frozen stabilites in liquid nitrogen with subscript codes used to identify their position in clonal history (Beale et al. 1978; Mackinnon & Read 1999a)

In this experiment I used *P. c. adami* genotypes DS_{444} and DK_{217} (originally 408XZ and 556KA respectively) and *P. c. chabaudi* CB_{1268}, BC_{237}, AQ_{224}, and AS_{12193}. Herein these genotypes will be referred to as DS, DK, CB, BC, AQ and AS. These genotypes were selected to look at the role of genetic diversity on vaccine efficacy. Parasite genotypes AQ, AS and BC have been shown to differ in their sequence at the AMA-1 locus but are known to be of similar virulence. Parasite genotypes AS and CB are known to be identical at their AMA-1 locus but CB is known to be more virulent than AS. Hosts were inbred female C57BL/6JolaHsd mice aged 6-8 weeks (Harlan England) were maintained as described previously (de Roode et al. 2004b).

3.3.2. Sequence analyses of the ama-1 alleles of P. chabaudi

The following ama-1 alleles were originally sequenced by Grech et al. (2008 *in prep*) and are stored in GenBank with the associated accession numbers: Genotype DK (EU219729), AS (EU1052842), CB (EU219733), AQ (EU219730) and BC (EU219732). Primers were designed to amplify the ama-1 gene as two fragments.

Primer sequences for fragment 1 were: forward 5' CTTGGGTAATTGTTCCGA 3' and reverse 5' GGTTTCCCAATCTTCACG 3'. Primer sequences for fragment 2 were: forward 5' GGGTCCAAGATATGTAG 3' and reverse 5' TGGTGTTGTGTGTGATGC 3'. Grech et al. (2008 *in prep*) derived the AS virulent (AS_{vir}) line by serial passage of the ancestral AS genotype through immuologically naïve mice (See Chapter 5) and sequenced parasites in the line using the above primers. AMA-1 from genotype DS was amplified by PCR as two fragments.
Primer sequences for fragment 1 were: forward 5' CTTGGGTAATTGTTCCGA 3' and reverse 5' GGTTTCCCAATCTTCA CG 3'.
Primer sequences for fragment 2 were: 5' TGAGAATCAACCATTTTGTTTTA 3' and reverse 5' CATCTTTTCTGCTTTGGGCT 3'.
PCR was performed using Platinum® Taq DNA polymerase (Invitrogen), with the thermocycle profile ; 95^{o}C for 7mins, then 93 ^{o}C for 1 min, 52^{o}C for 1 min and 72^{o}C for 1 min (x 30 cycles) ending at 72^{o}C for 10mins. Sequencing was performed by the University of Edinburgh sequencing service.

For phylogenetic analyses, two *P. yoelii* sequences (GenBank accession numbers U4597, U45971) and a single *P. berghei* sequence (GenBank accession number U45969) were chosen as outgroup taxa. Maximum likelihood (ML) phylogenetic trees were inferred using PAUP* (Swofford 2003) using the best-fit transversion model of nucleotide substitution (TVM+I) as identified by Modeltest (Posada & Crandall 1998) and tree bisection-reconnection branch swapping. The robustness of each node in the resultant tree was determined using a ML bootstrap re-sampling process incorporating the best-fit ML model with 1,000 replications.

To further investigate the relationship between DS, DK and AQ, recombination detection analysis was conducted using the complete sequence data set. Potential recombination break-points were identified using the GARD algorithm (Pond et al. 2006) available as part of the datamonkey package (www.datamonkey.org). An identified breakpoint was verified by manually breaking the sequence alignments up into two regions, and running ML phylogenetic trees followed by a bootstrap analysis (as above) on each region of the alignment separately.

3.3.3. Immunizations and isotype ELISA

I used an immunization protocol adapted from Anders et al. (1998). Prior to immunization, mice were randomized into two groups (Table 3.1). Immunization was with the highly immunogenic ectodomian of the full AMA-1 protein derived from parasite genotype DS. Mice were injected intraperitoneally with 10 µg of protein emulsified in 100 µl of the adjuvant ISA 720 (Seppic, France). Control mice were injected with 100 µl emulsion of PBS in ISA 720. Boost immunisations were conducted 4 weeks after the primary immunisation.

	Immunization	
Parasite clone		
	Control sham-immunized	DS AMA-1
DS	5	6
CB	5[1]	6[2]
BC	5[1]	6[1]
AQ	5[1]	6
AS	5	5
DK	5	5
Experimental total		**64**

Table 3.1. Experimental design. Immunization was either a sham-immunization or with DS AMA-1. Groups of mice were immunized with one of the four treatments before being separated into groups of 5 or 6. Infection was either with parasite genotype (clone) DS, CB, BC, AQ, AS or DK. During the experiment 6 mice were found dead as indicated by the numbers in brackets.

To ensure my antigen immunization successfully generated antibody responses, IgG2b antibodies in mice sera were measured 3 days prior to parasite challenge by ELISA using DS AMA-1 coated wells. I measured the quantity of antigen-specific IgG2b antibodies since protection induced by immunisation with recombinant AMA-1 in C57 mice is isotype independent (Burns et al. 2004) and previous work in my laboratory showed that C57BL/6 produce IgG2b in response to *P. chabaudi* infection

(K. Grocock, A. Graham unpublished). Sera fractions were separated by centrifugation from 20 µl of blood taken from a tail snip and were stored at -80^{o}C. High binding 96 well ELISA Maxisorb immunoplates (Nunc) were coated with DS AMA-1 at a concentration of 1µg /ml in 0.06M carbonate buffer (0.04M $NaHCO_3$, 0.02 M $NaCO_3$, pH 9.6) in a final volume of 50 µl per well. Plates were stored at 4^{o}C overnight to allow the antigen to bind. Non-specific binding was blocked by incubating wells with 5% BSA: carbonate buffer (200 µl/well) for 2 hours at 37^{o}C. Wells were then washed three times in Tris buffered saline with 0.01% Tween 20 (TBST). We used end-point dilution methods to detect IgG2b titres: serum samples were detected in a serial dilution 1/100-1/204800 using TBST as a diluent, in a final volume of 50 µl per well and incubated for 2 hours at 37^{o}C. Wells were washed three times in TBST. HRP conjugated goat anti-mouse IgG2b detection antibody (Southern Biotech 1100-05) was diluted 1/4000 in TBST to a final volume of 50 µl per well. Plates were incubated for 1 hour at 37^{o}C. Wells were washed three times in TBST followed by a final wash in distilled water. ABTS peroxide substrate (Insight Biotechnology) was added at 100 µl per well and allowed to develop at room temperature for 20 minutes. Optical density was read at 405nm using a spectrophotometer. IgG2b isotype antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D.) was greater than the mean (plus 2 standard deviations) O.D values observed for naïve mouse sera assayed against both DS and DK AMA-1 at 1/100.

3.3.4. Parasite challenge and monitoring of within host dynamics

Groups of treated mice (control sham-immunized or DS AMA-1 immunized) were injected intra-peritoneally with 10^5 parasitized red blood cells (RBCs) of either genotype DS, CB, BC, AQ, AS or DK (Table 1). I followed the infection dynamics for 29 days. As of the first day of parasite infection (day 0), we daily measured weight and took blood samples from the tail of experimental mice to estimate RBC density (by flow cytometry; Beckman Coulter). From day 4 post parasite infection onwards I took blood samples from the tail to make Giemsa-stained blood smears for estimating daily parasite densities. We chose day 4 as this is around the first day that

parasites can be detected in the blood using microscopy. Since *P. chabaudi* has a 24 hour replication cycle, the total number of parasites in any period can be estimated by summing the daily parasite counts.

3.3.5. Trait definition and statistical analyses

As in Chapter 2, mice infected with genotype DK actually gained weight and therefore weight loss was not a suitable measure of virulence. All data were analysed using General Linear Models (GLMs) in MINITAB (Minitab, version 14). To meet normality and homogeneity of variance assumptions, data on antibodies and red blood cell density were log transformed while all parasite densities were square route transformed. To capture any vaccine-induced protection from anaemia, for each genotype, we analyzed the minimum red blood cell density and the mean red blood cell density across the course of infection. GLMs were used to test whether the magnitude of protection induced by DS AMA-1 vaccination differed between the six genotypes (DS, DK, AS, CB, AQ, BC); that is whether there was a statistical interaction between infecting genotype and immunizing treatment. Maximal models (response variable = infecting genotype + immunizing treatment + infecting genotype x immunization treatment) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model.

3.4. Results

Table 3.1 gives details of the immunization treatments, infecting genotype and sample size of the experiment. Some mice died; these were included in the calculation of daily densities until death, and in the analyses of peak parasite densities since death always occurred as initial parasitaemias were declining.

3.4.1. Sequence diversity within the AMA-1 region of P. chabaudi genotypes

Table 3.2 and Fig. 3.1A illustrate the *ama-1* nucleotide sequence similarity between genotype DS, from which the vaccine was derived, and the other genotypes

previously sequenced by Grech et al. (2008 *in prep*). We found genotype AQ to be the most similar to the vaccine genotype, sharing 95.8% *ama-1* nucleotide sequence similarity. Genotype AS was 94.3% while CB was 94.2% identical to the vaccine antigen. Genotype AS and CB were chosen to look at the role of virulence during vaccine-efficacy and differed from each other by one synonymous mutation. Genotype BC shared 94.2% nucleotide sequence identity to the vaccine antigen and differed from AS and CB by one non-synonymous mutation. Genotype DK was the most diverged differing from the vaccine antigen by 93.3% (Table 3.2).

To further investigate phylogenetic relationships, recombination detection analysis was conducted using the complete sequence data set. This analysis supported the presence of a recombination event occurring between DS, DK and AQ at approximately the 765th nucleotide position (Fig. 3.1B and C). Before the breakpoint, the *ama-1* sequence of genotype AQ was more similar to sequence of the vaccine antigen DS, and after the breakpoint was more similar to genotype DK

	DS	AQ	AS	CB	BC	DK
DS		50	67	69	69	79
AQ	29		85	84	86	63
AS	34	49		1	1	85
CB	34	49	0		2	36
BC	34	49	1	1		36
DK	35	35	21	21	21	

Table 3.2. Relatedness of parasite genotypes across the ectodomain of AMA-1. The numbers above the black diagonal line indicate the number of nucleotide differences between each of the parasite genotypes. The numbers below the black diagonal indicate the number of amino acid differences between each of the parasite genotypes. DS is the genotype from which the AMA-1 vaccine was derived.

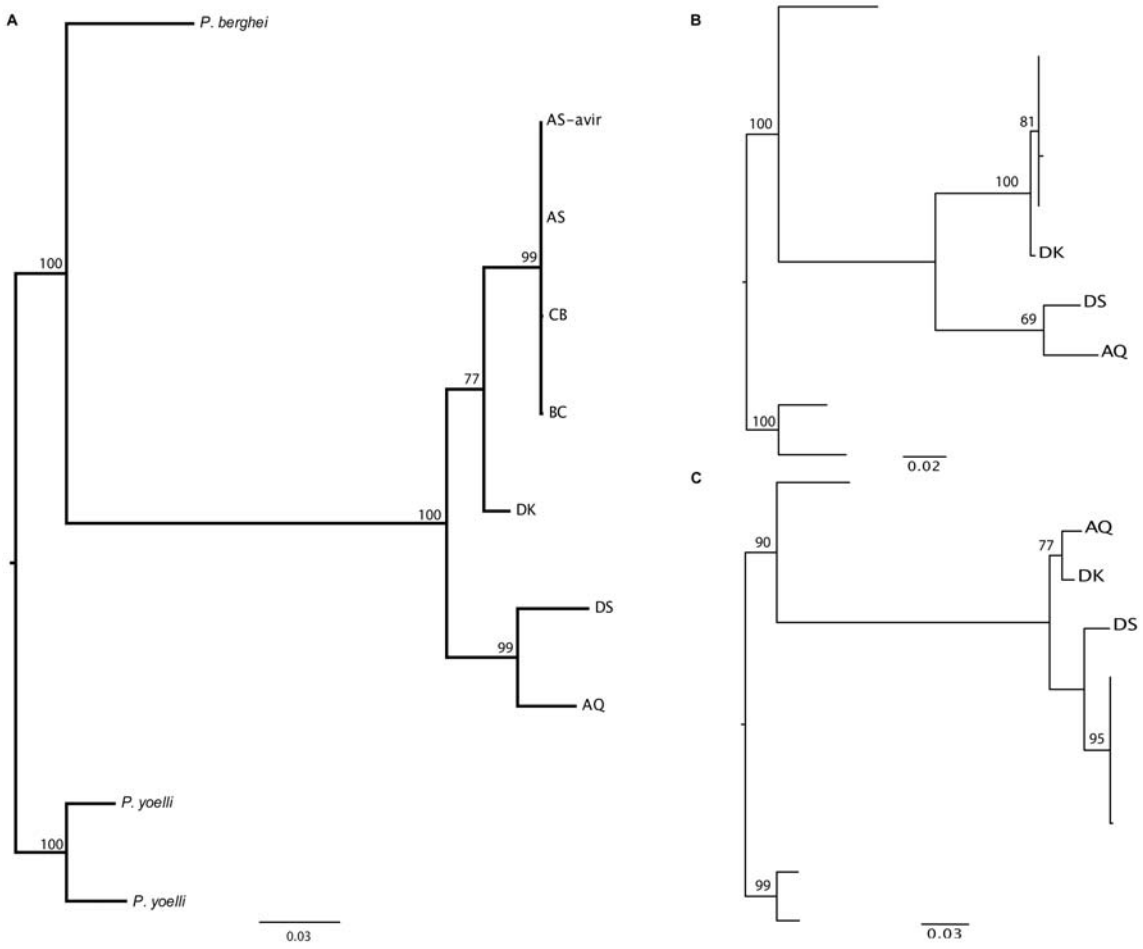

Figure 3.1. Maximum likelihood (ML) phylogenies of the AMA-1 genes of *P. chabaudi* (genotypes AQ, AS, AS-avir, BC, CB, DK, DS), *P. berghei and P. yoelii* (outgroup). Horizontal branch lengths represent the number of nucleotide substitutions per site. (A) ML phylogeny of the complete gene with ML bootstrap values shown at each node based on 1,000 replications. (B,C) ML phylogenies showing the recombination event between AQ, DK and DS with a break-point at nucleotide position 765 (the tree represented in b was derived from nt 1-765, and c from 765-1512).

3.4.2. Pre- parasite challenge anti- AMA-1 IgG and IgG2b antibody titres

Fig. 3.2 illustrates the total IgG and IgG2b antibody titres from control sham-immunized and DS AMA-1 immunized mice. Immunization with DS AMA-1

induced antibody titres that were higher than sham-immunizations (Fig 3.2A, total IgG, sham immunized v antigen immunized: $F_{1,62}$=452.04, p=0.001; Fig. 3.2B, IgG2b, sham immunized v antigen immunized: $F_{1,62}$=31.88, p<0.001). There were some non-responders to the antigen immunization. However, antibody titres prior to challenge did not predict subsequent parasite intensities (Fig. 3.3, all correlations p >0.2), so that mice with low antibody titres were not necessarily less well protected from parasite infection than those with high titres.

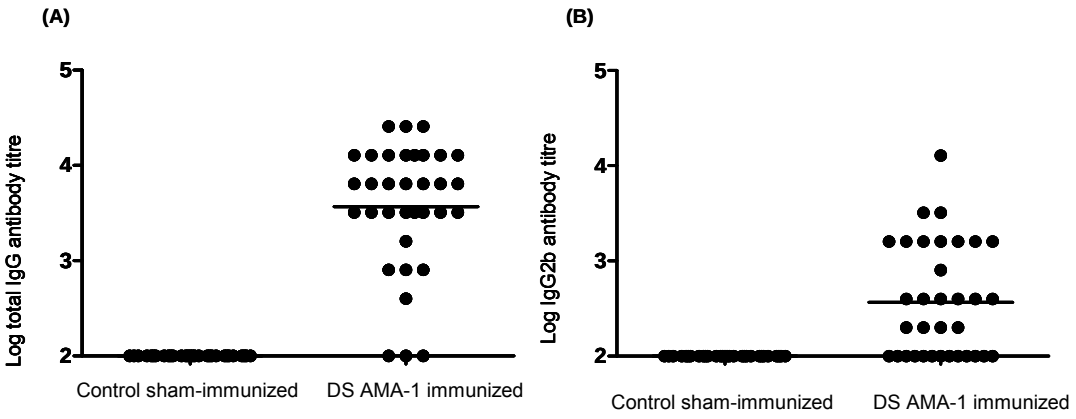

Figure 3.2. Total IgG and IGg2b antibody titres from the serum of mice assayed on DS AMA-1 coated ELISA plates. Mice were either sham-immunized or immunised with DS AMA-1. Total IgG (A) and IgG2b (B) antibodies titres were measured. The dots in each panel represent the antibody titre for individual mice. Horizontal lines indicate mean antibody levels. Total IgG (p<0.001) and IgG2b (p<0.001) antibody titres were higher in antigen immunized groups of mice than in sham-immunized controls.

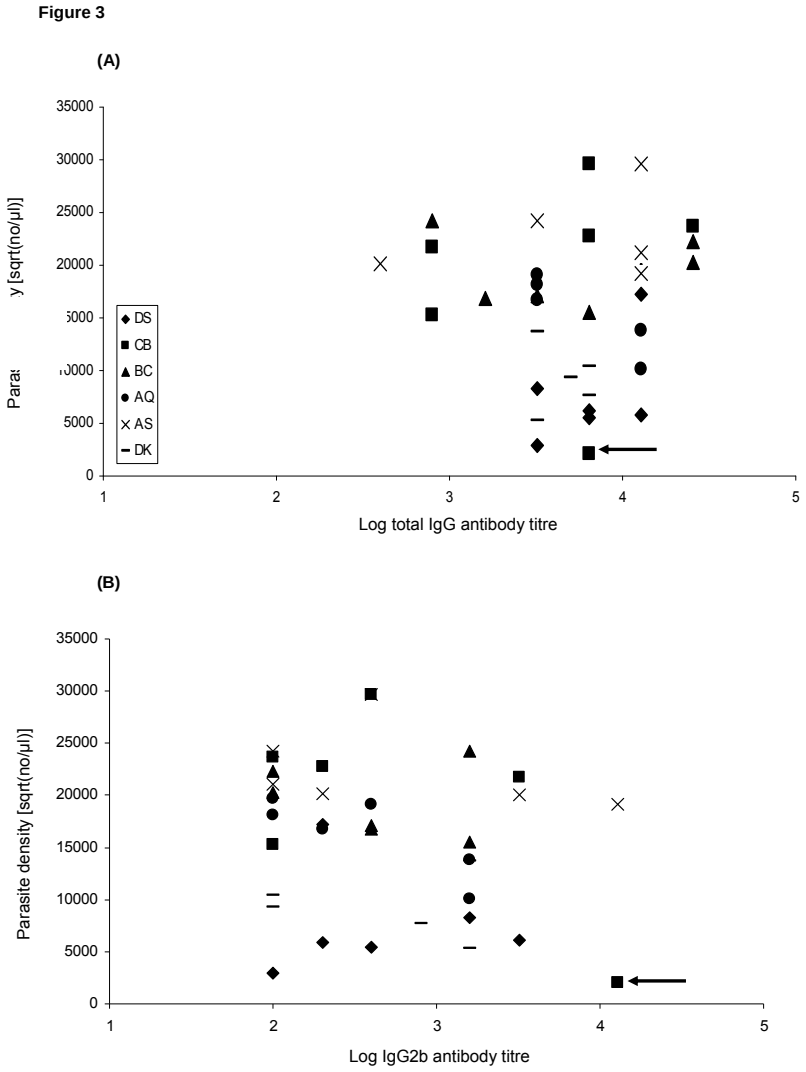

Figure 3.3. The relationship between pre-parasite infection total IgG and IgG2b antibodies, and the number of parasites subsequently present in DS AMA-1 immunized mice. Total IgG (A) and IgG2b (B) antibodies were measured. The symbols in each panel represent individual mice infected with either genotype DS (diamonds), CB (squares), BC (triangles), AQ (circles) AS (cross) or DK (single dash). There was no correlation between pre-parasite challenge antibodies and the total parasite density for any of the genotypes ($p>0.2$). One CB-challenged mouse had very high antibody titres and very low parasite densities (indicated by the arrow).

3.4.3. Genotype differences in virulence and parasite densities

The kinetics of parasite density and red blood cell density of the six parasite genotypes in DS AMA-1 immunized and in sham-immunized mice are illustrated in Figures 3.4 and 3.5. As found previously (Mackinnon & Read 1999a; de Roode et al. 2005a; Bell et al. 2006; Grech et al. 2008 in prep), in sham-immunized animals the total parasite densities and anaemia induced by the different genotypes varied significantly (Fig. 3.4; $F_{5,24}$=7.24, p=<0.001, and Fig. 3.5; $F_{5,\ 23}$=6.98, p=<0.001 respectively). Specifically comparing parasite genotypes AS and CB which had identical amino acid sequences at the target region of AMA-1, sham-immunized hosts infected with parasite genotype CB had higher parasite densities and were more anaemic than sham-immunized hosts infected with AS (parasites: $F_{1,8}$=13.88, p=0.006; anaemia: $F_{1,7}$=9.39, p=0.018). Thus, CB was the more virulent of these two genotypes. The other parasite genotypes ranked in the following descending order of virulence: BC, AQ then DK.

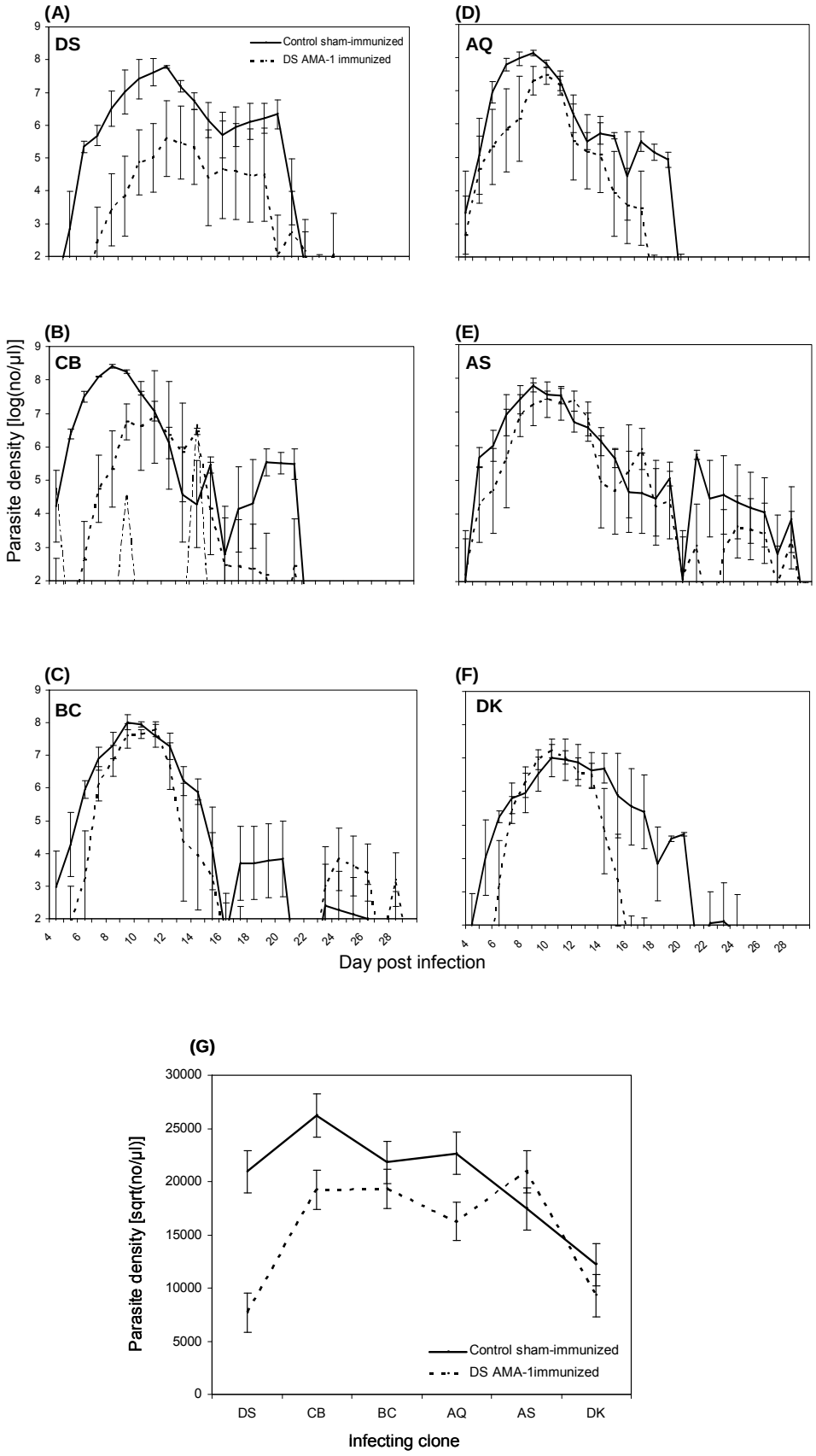

Figure 3.4. Legend on next page

Figure 3.4. Effect of *Plasmodium chabaudi* genotype (clone) and immunization on parasite density kinetics. (A-F) The infecting genotype is indicated in the top left corner of each graph. (B) One antigen immunized mouse infected with CB had lower parasite densities than the rest of the mice in that group indicated by the thin dashed line. Each thick line represents the mean change in parasite density over time per treatment group (+/- 1 s.e.m). (G) Each data point represents the least square means and associated standard error for each parasite genotype. Solid lines represent the dynamics in control sham-immunized hosts while the dashed lines represent the dynamics in vaccinated hosts. Significant protection was conferred against homologous parasite DS and heterologous AQ but not against any of the other genotypes.

3.4.4. Protective affects of immunization on parasite density

Across the course of infection, the anti-parasite effect of vaccination differed among parasite genotypes (Fig. 3.4A-G; infecting genotype x immunization: $F_{5,52=}4.14$, p=0.003).

Vaccination conferred protection against hosts infected with the homologous parasite DS (DS vaccinated versus DS control; $F_{1,9}=20.36$, p=0.001) and the heterologous parasite with the greatest sequence similarity AQ (AQ vaccinated versus AQ control $F_{1,9}=9.06$, p=0.015). No protection was conferred against hosts infected with the more virulent genotype BC (BC vaccinated versus BC control; $F_{1,9}=2.27$, p=0.16) or the less virulent genotypes AS (AS vaccinated versus AS control; $F_{1,8}=2.01$, p=0.19) or DK (DK vaccinated versus DK control $F_{1,8}=0.98$, p=0.35).

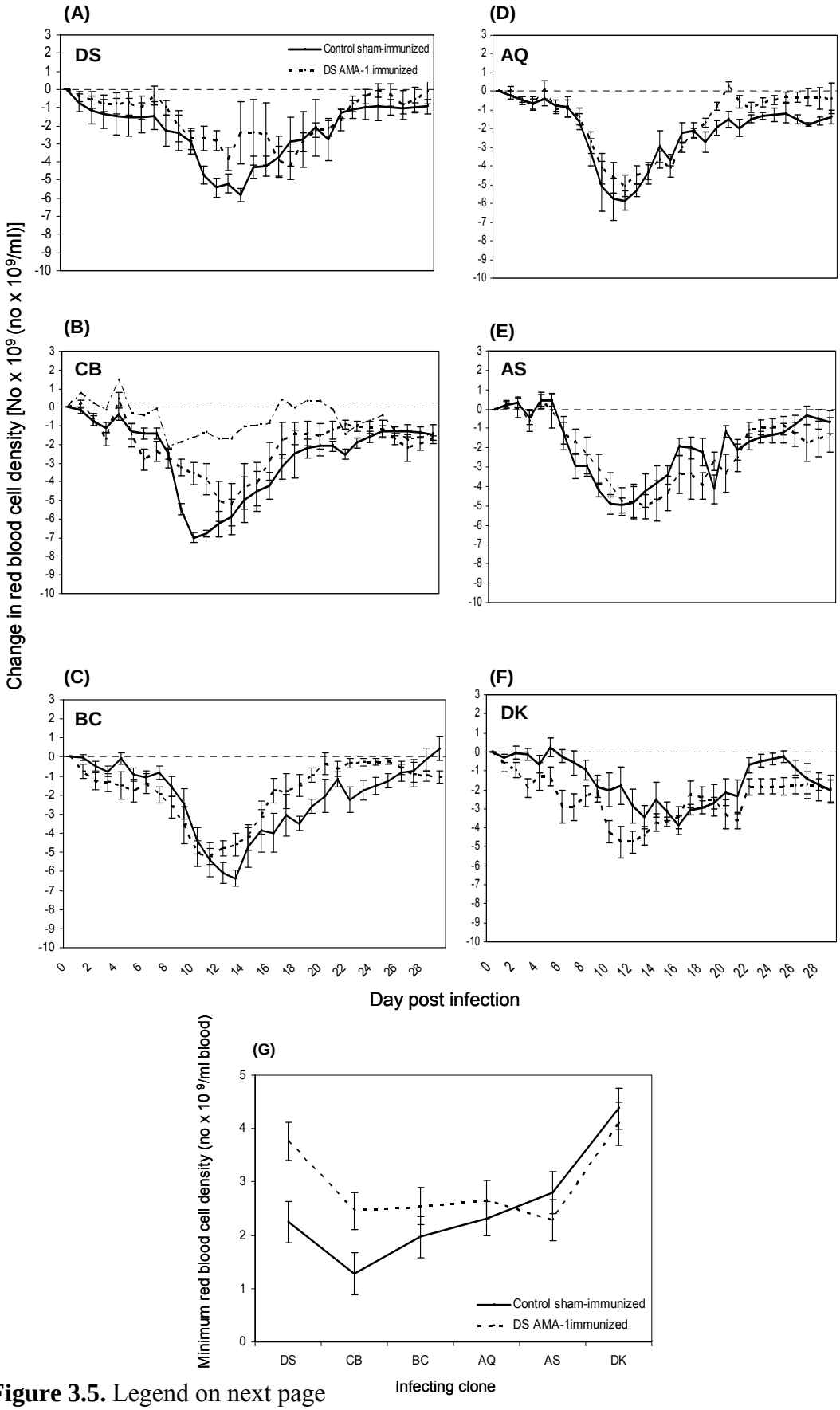

Figure 3.5. Legend on next page

Figure 3.5. Effect of *Plasmodium chabaudi* genotype (clone) and immunization on red blood cell density kinetics. (A-F) The infecting genotype is indicated in the top left corner of each graph. (B) One antigen immunized mouse infected with CB had higher red blood cell densities than the rest of the mice in that group indicated by the thin dashed line. Each thick line represents the mean change in parasite density over time per treatment group (+/- 1 s.e.m). (G) Each data point represents the least square means and associated standard error for each parasite genotype. Solid lines represent the dynamics in control sham-immunized hosts while the dashed lines represent the dynamics in vaccinated hosts. Significant protection was conferred against homologous parasite DS and the heterologous genotype CB but not against any of the other genotypes.

Visual inspection of Fig. 3.4B and G may suggest that vaccination provided protection against the heterologous genotype CB. However, pairwise comparison found vaccination did not afford protection (CB vaccinated versus CB control; $F_{1,9}$ =2.57, p=0.14). An explanation for the statistical and visual differences can be explained by the fact that the least square means error plotted in Fig. 3.4G does not show the error contributed by the one mouse vaccinated with DS AMA-1 and infected with genotype CB, which had higher IgG2b antibody levels, lower parasite densities and did not suffer from anaemia compared to the other mice in that treatment group (see Fig 3.3, 3.4 and 3.5). That mouse was an outlier in all statistical analyses. However the statistical output did not change whether that mouse was included or not.

Specifically comparing parasite genotypes AS and CB which were chosen to look at the role of virulence in vaccine efficacy, we found that vaccination conferred better protection against infections with genotype CB than AS, and this was true whether the analysis included (infecting genotype x immunization: $F_{1,17=}$4.71, p=0.004) or excluded (infecting genotype x immunization: $F_{1,16=}$6.02, p=0.026) the outlier mouse mentioned above. Thus, there was no evidence that vaccination was less effective against the more virulent genotype CB. If anything it was more protective against this genotype than the genotype of lesser virulence AS.

Together these results suggest that vaccination generated the most effective anti-parasitic protection against those strains which were most similar to the vaccine antigen. Vaccination reduced densities of the homologous genotype DS and the heterologous genotype AQ, which out of all the heterologous genotypes was most similar at the *ama-1* sequence to the immunizing antigen (Table 3.2, Fig. 3.1). There was no evidence that virulence played a role in vaccine efficacy.

3.4.5. Protective affects of vaccination on virulence

Vaccination protected against anaemia (Fig.3.5; immunization: $F_{1,56}$=6.76, p=0.012), but whether the extent of the protection differed among parasite genotypes was ambiguous. Formally there was no evidence that it did (immunization x infecting genotype: $F_{5,51,}$=2.19, p=0.069), but this result was marginal, and pairwise comparisons indicated protection was afforded during infection with the homologous parasite genotype DS (DS vaccinated versus DS control: $F_{1,8}$=8.57, p=0.019) and against the heterologous genotype CB (CB vaccinated versus CB control: $F_{1,8}$=47.24, p<0.001). Vaccination protected against anaemia due to infection with genotype CB whether the outlier mouse in the statistical analyses mentioned above was included in the analysis or not. No protection was conferred against hosts infected with genotype AQ (AQ vaccinated versus AQ control $F_{1,8}$=0.08, p=0.77), BC (BC vaccinated versus BC control; $F_{1,8}$=2.66, p=0.14) AS (AS vaccinated versus AS control; $F_{1,7}$ =1,34, p=0.29) or DK (DK vaccinated versus DK control $F_{1,7}$=0.34, p=0.58).

These results show that vaccination protected against anaemia during infection with the homologous genotype DS. However, unlike the pattern observed with the parasite data, vaccination did not protect against infection with genotype AQ which was most similar at the *ama-1* sequence to the immunizing antigen. Out of the heterologous genotypes, vaccination afforded most protection from anaemia during infection with genotype CB.

3.5. Discussion

The motivation for the experiments presented here came from the trial of the Combination B malaria vaccine (Genton et al. 2002; Genton et al. 2003). In that trial it was unclear whether vaccination had imposed selection that was strain-specific (Fluck et al. 2004; Fluck et al. 2007) or whether more virulent parasites had a selective advantage in vaccinated hosts. Consistent with my previous studies (Chapter 2), the data we present here go against the latter argument. I found that immunization with AMA-1 derived from *P. chabaudi* genotype DS induced protection that was strain-specific: vaccination reduced those parasites which were most similar to the vaccine target to a greater extent than those that were different. There was no evidence that vaccine-induced immunity was less effective against virulent genotypes. For example, when we compared two genotypes which had identical AMA-1 protein sequences but differed in virulence, there was no evidence that more virulent genotype (CB) was harder to control. If anything vaccination was less effective against the less virulent genotype (Fig. 3.4 and 3.5). In contrast, neither *ama-1* sequence diversity nor virulence appeared to more strongly influence protection from anaemia.

Phylogenetic analyses revealed that the *ama-1* sequence of genotype AQ most closely resembled genotype DS from which the vaccine was derived (Fig. 3.1, Table 3.1). Vaccination was most effective at reducing parasite densities during infection with that genotype (Fig. 3.4). However, a recombination detection analysis identified a breakpoint nucleotide at position 765 for genotypes DS, DK and AQ supporting the presence of a recombination event.

Intragenic recombination has been proposed as a mechanism of generation of diversity in AMA-1 (Eisen et al. 1999), as well as other merozoite proteins such as MSP-1 (Tanabe et al. 1987; Miller et al. 1993; Tanabe et al. 2007) and MSP-2 (Marshall et al. 1991; Snewin et al. 1991). Sequence analysis of a Papua New Guinea isolate (KF1916) revealed that the AMA-1 allele appeared to have arisen by a recombination event from alleles classified as members of two different allelic

families. The KF1916 AMA-1 was identical to the FC27 AMA-1 allele (family I) from nucleotide positions 1-558, after which 25 nucleotide substitutions were observed. The FCR3 AMA-1 allele (family II) was identical to the KF1916 allele from nucleotide position 558 to the 3' end (Eisen et al. 1999).

In my study I found that the AQ AMA-1 allele more commonly resembled the vaccine genotype DS between nucleotide positions 1-765, and resembled genotype DK for the remainder of the sequence. This allelic diversity in the *ama-1* gene of genotype AQ presumably occurred during meiotic recombination in the *Anopheles* mosquito, when gametocytes belonging to either genotype DS or DK were also present.

The breakpoint nucleotide position is of interest given the recent data investigating the regions of the AMA-1 ectodomain which may generate protective immunity. For instance, domain I appears to exhibit the most polymorphisms, with clustering occurring at one end of the hydrophobic cleft, thought to be associated with a ligand binding site (Bai et al. 2005; Coley et al. 2007). X-ray crystallography and cell culture experiments have demonstrated a crucial role for domain II in the attachment/invasion of red blood cells (Fraser et al. 2001; Nair et al. 2002; Bai et al. 2005; Feng et al. 2005; Pizarro et al. 2005; Collins et al. 2007). Finally, genetic analyses have demonstrated domain III to be under balancing selection, and that some anti-domain III antibodies can block invasion (Nair et al. 2002; Mueller et al. 2003). In my analyses, the recombination breakpoint occurred close to where domain II started, so that genotype AQ was actually more similar to DK for domain II. Thus, my finding that anti-DS antibodies were more effective against AQ implies that the conserved target epitope lies upstream of the breakpoint, perhaps in domain 1.

My results strongly imply that the anti-parasite affects of AMA-1 vaccination correlate with sequence similarity between the infecting strain and the vaccine antigen, and that vaccine efficacy is unaffected by the virulence of challenge parasites This data apparently contradicts a previous study which, using the same

genotypes as we used here, found evidence that both sequence similarity and parasite virulence affected vaccine efficacy (Grech et al. 2008 in prep). Most notably, protection was significantly better against AS than against CB, the two genotypes which differed in virulence but have identical amino acid sequences. The only obvious difference between the two studies was that Grech et al. (2008 *in prep*) immunized with DK-derived AMA-1 antigen whereas I used DS AMA-1. The most likely explanation for the contrast in results is therefore that different immunizing antigens can impose selection in different ways. For example, some antigens may generate protective antibodies that have higher affinity to certain AMA-1 residues, or other AMA-1 like protein sequences, in some parasites more than others (Hodder et al. 2001). Serial passage of a *P. chabaudi* through whole-parasite immunized mice evolved virulence more rapidly than did serial passage through immunologically naïve hosts (Mackinnon & Read 2004a). In this study I saw no parasite advantage to virulence. An explanation for the contrast in results could be explained by differences in the immune response induced by single antigen immunizations in comparison to whole-parasite immunization. Furthermore, perhaps selection for virulence is especially obvious when antigenically identical parasites are evolved, or when immunization is with many highly diverse antigens, as must occur with live parasite immunization. In future experiments I will evolve single *P. chabaudi* genotypes through AMA-1 immunized mice to measure whether this vaccine has the potential to drive the evolution of similar rapid virulence.

My observations suggest that single AMA-1 vaccine antigens may not be sufficient to protect against *Plasmodium* populations in the field due to allelic heterogeneity. Perhaps one of the best solutions to avoid inadvertent selection for more virulent strains, as apparently happened with the Combination B vaccine, may be to include all known variants of a particular antigen in a vaccine formulation. One group investigated whether the potency of an AMA-1 vaccine increased by including more than one allele (Kennedy et al. 2002; Malkin et al. 2005; Miura et al. 2007). The resulting antibodies were similarly effective during parasite invasion assays regardless of whether immunization was with a single allele or a mixture of two. In Chapter 4 of this thesis we have extended on those studies to test in vivo whether a

bi-allelic AMA-1 immunization affords the host greater protection from parasites and disease, and how this alters within host selection.

In this study I found that heterologous anti-parasitic protection did not correlate with anti-disease protection, and vice versa. For example, immunization reduced the parasite densities of genotype AQ but did not provide significant protection against anaemia caused during infection with that genotype. The fact that there are two different types of malarial immunity - immunity against parasites and immunity against disease - is poorly understood on a molecular basis although widely appreciated (Schofield & Grau 2005). An explanation for the two different responses observed here could be that the specificity of the anti-AMA-1 antibody response lies with the generation of inhibitory antibodies which may target a hypervariable region located around a conserved hydrophobic pocket on domain I common between the vaccine antigen and genotype AQ (Coley et al. 2007). These inhibitory antibodies may be faster at inhibiting parasite invasion of red blood cells than the mechanisms of red blood cell replacement (erythropoiesis) (Chang & Stevenson 2004). These results imply that for vaccine studies to be executed most effectively, disease symptoms, as well as the parasitological status of the host, should be measured.

Vaccines which can control disease symptoms without preventing parasite transmission may have important evolutionary consequences (Gandon et al. 2001). For instance, vaccines against *Bordetella pertussis*, the causative agent of whooping cough, have controlled disease symptoms but not bacterial colonization. Thus, vaccination has been attributed as the reason for persistence and re-emergence in the adult host population (WHO 2001; McIntyre et al. 2002; Ntezayabo et al. 2003). If selection imposed by vaccination heavily depends on the fine specificity of the immune response, it will become increasingly important that we understand how each of six AMA-1 based vaccines currently in human trails may alter *Plasmodium* populations. Such studies will provide important information on the evolutionary consequences of AMA-1 vaccination.

4. Mixed allele malaria vaccines: host protection and within-host selection

This chapter, with a few minor editorial changes, has been published as Victoria C. Barclay, Brian H.K. Chan, Robin F. Anders, Andrew F. Read. 2008. Mixed allele malaria vaccines: Host protection and within-host selection. *Vaccine in press* (See Thesis Appendix 1).

4.1. Abstract

Malaria parasites are frequently polymorphic at the antigenic targets of many candidate vaccines, presumably as a consequence of selection pressure from protective immune responses. Conventional wisdom is therefore that vaccines directed against a single variant could select for non-target variants, rendering the vaccine useless. Many people have argued that a solution is to develop vaccines containing the products of more than one variant of the target. However, we are unaware of any evidence that multi-allele vaccines better protect hosts against parasites or morbidity. Moreover, selection of antigen variants is not the only evolution that could occur in response to vaccination. Increased virulence could also be favored if more aggressive strains are less well controlled by vaccine-induced immunity. Virulence and antigenic identity have been confounded in all studies so far, and so we do not know formally from any animal or human studies whether vaccine failure has been due to evasion of protective responses by variants at target epitopes, or whether vaccines are just less good at protecting against more aggressive strains.

Using the rodent malaria model *Plasmodium chabaudi* and recombinant apical membrane antigen 1 (AMA-1), I tested whether a bi-allelic vaccine afforded greater protection from parasite infection and morbidity than did vaccination with the component alleles alone. I also tested the effect of mono- and bi-allelic vaccination on within-host selection of mixed *P. chabaudi* infections, and whether parasite virulence measured in unvaccinated hosts predicts pathogen titres in immunized

hosts. I found that vaccination with the bi-allelic AMA-1 formulation did not afford the host greater protection from parasite infection or morbidity than did mono-allelic AMA-1 immunisation. Mono-allelic immunization increased the frequency of heterologous genotypes in mixed genotype infections. There was no evidence that any type of immunization regime favoured virulence. A single AMA-1 variant is a component of candidate malaria vaccines current in human trials; my results suggest that adding extra AMA-1 alleles to these vaccines would not confer clinical benefits, but that that mono-allelic vaccines could alter AMA-1 allele frequencies in natural populations.

4.2. Introduction

Malaria parasite antigens which are the targets of protective immune responses are frequently polymorphic, with antigen-coding genes having multiple allelic forms (Richie & Saul 2002). Polymorphisms likely arise as a consequence of immune-mediated selection because host responses can be more effective against parasites of the immunising genotype than against different genotypes (strain-specific immunity) (Jeffery 1966; Mendis et al. 1991; Anders et al. 1998; Martinelli et al. 2005; Cheesman et al. 2006). Sequence polymorphisms have been directly implicated in antigenic escape (Kennedy et al. 2002; Healer et al. 2004; Saul 2007), and in malaria endemic areas, immunity is acquired slowly, probably because repeated exposure is required to generate an effective response against a repertoire of genotypes (McGregor et al. 1956; McGregor 1974; Day & Marsh 1991). The existence of antigenic polymorphism is therefore of considerable concern to malaria vaccine developers because it implies that single antigen vaccines will have trouble inducing protective immunity against polymorphic targets (Hodder et al. 2001; Genton et al. 2003; Fluck et al. 2004; Genton & Reed 2007).

One approach to minimizing vaccine-induced strain specificity has been to design vaccines which combine more than one allele of an antigen (Hoffmann & Miller 1996; Bolad & Berzins 2000; Richie & Saul 2002; Polley et al. 2007). However, the inclusion of more than one allelic form of an antigen may not be sufficient to

overcome substantial polymorphisms (Saul 2007), and there is little experimental evidence that multi-allele vaccines actually afford the host more protection from morbidity than do single antigen vaccines.

Furthermore, selection of antigen-variants is not the only evolution that could occur in response to widespread vaccination. Theoretically, vaccination has the potential to cause evolutionary change in parasite virulence (parasite-induced host damage) by altering the way natural selection acts on parasite populations (Gandon et al. 2001; Gandon & Day 2003; André & Gandon 2006; Restif & Grenfell 2006; Gandon & Day 2007; Mackinnon et al. 2008; Read & Mackinnon 2008; Williams & Day 2008). In experimental evolution experiments, the rodent malaria *Plasmodium chabaudi* became virulent more rapidly if serially passaged through mice previously immunized with live parasites (Mackinnon & Read 2004a). The most likely explanation for this is that more aggressive variants are less well controlled by immunity.

To date, we still do not fully understand how vaccines will alter gene frequencies in malaria parasite populations. Evidence for selection in the field comes from a small phase 1-2b trial of the "Combination B" blood-stage malaria vaccine (Genton et al. 2002). This vaccine contained a single antigen from each of three polymorphic loci of *P. falciparum*. One of these loci, Merozoite Surface Protein 2 (MSP-2), is dimorphic, with each parasite having an allele from one of two allelic families (labelled 3D7 and FC27). The MSP-2 allele in the Combination B vaccine came from the 3D7 family. Among parasites subsequently acquired by vaccinees, 3D7-type alleles were rarer than in people given a placebo. Vaccination thus selected against the variant contained in the vaccine. Interestingly, the FC27 allelic family is associated with more virulent infections (Engelbrecht et al. 1995). Therefore, it is not clear whether the vaccine-imposed selection was due to immune specificity (Fluck et al. 2004; Fluck et al. 2007) or whether the vaccine was less good at controlling more virulent infections.

Many candidate vaccines against malaria are directed against the asexual blood stage, with the principal target being the merozoite. Apical Merozoite Antigen-1 (AMA-1) is a promising vaccine candidate as it possesses fewer polymorphisms than other merozoite antigens (Anders et al. 1998; Hodder et al. 2001). AMA-1 is thought to play a major role during erythrocyte re-modelling and invasion (Cowman & Crabb 2006). Immunization with AMA-1 confers protection against parasite challenge in a number of animal models, probably by inducing antibodies which inhibit invasion (Deans et al. 1988; Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998; Narum et al. 2000; Stowers et al. 2002; Healer et al. 2004). Furthermore, humans and other species immunized with single-allele AMA-1 vaccines raise antibodies which inhibit erythrocyte invasion *in vitro* (Hodder et al. 2001; Kocken et al. 2002). In endemic populations, naturally acquired antibody to *P. falciparum* AMA-1 (PfAMA-1) is associated with protection from falciparum malaria (Thomas et al. 1994; Johnson et al. 2004; Polley et al. 2004; Cortes et al. 2005; Rodrigues et al. 2005). At least six different vaccines based on the AMA-1 allele from the *P. falciparum* 3D7 strain are currently in efficacy trials in humans (Polhemus et al. 2007; Maher 2008; Thera et al. 2008).

However, there are more than 60 polymorphic sites in the *P. falciparum* AMA-1 protein, and most of these are non-randomly dispersed point mutations on domain I (Peterson et al. 1989; Marshall et al. 1996; Polley & Conway 2001; Polley et al. 2003; Dutta et al. 2007). These point mutations may be of immunological importance. Protection in mice is strain-specific, and growth and invasion inhibition assays (GIA) and ELISA show that antibodies from animals and human field sera inhibit growth in a strain-specific manner (Crewther et al. 1996; Anders et al. 1998; Hodder et al. 2001; Polley et al. 2003; Cortes et al. 2005; Polhemus et al. 2007). Allelic replacement experiments have directly implicated sequence polymorphism in antigenic escape (Healer et al. 2004), and cross strain inhibition assays suggest that the extent of escape correlates with sequence distance between the vaccine and the target strain (Kennedy et al. 2002). In an attempt to overcome strain-specificity, vaccine researchers are beginning to combine allelic variants of AMA-1. For example, one group immunized rhesus monkeys with a mixture of two allelic forms

of PfAMA-1 (designated AMA1-C1) or the component alleles and measured responses in vitro using GIA and ELISA (Kennedy et al. 2002; Malkin et al. 2005; Miura et al. 2007). The resulting antibodies were similarly effective regardless of whether immunization was with a single variant or AMA1-C1. Another group immunized mice and rabbits with two allelic variants of domain I and II of AMA-1 ectodomain from *P. falciparum* isolates. The anti-AMA-1 antibodies obtained with both proteins were active in an in vitro parasite growth invasion/inhibition assay, but to no greater extent than with either of the variants alone (Lalitha et al. 2008). Together these results have raised questions about the necessity of using multi-allele vaccines.

Here I use the rodent malaria *P. chabaudi* and two alleles of the blood-stage malaria vaccine candidate AMA-1 to investigate (i) whether immunization with a single or bi-allelic AMA-1 variant formulation afforded the host the greatest protection from morbidity and parasite infection, (ii) how these different vaccination regimes can alter clonal genotype frequencies in mixed infections, and (iii) whether more virulent genotypes are better at evading heterologous vaccine-induced protective responses.

4.3. Materials and methods

4.3.1. Parasites and hosts

Isolates containing *P. chabaudi adami* were originally collected from wild-caught thicket rats (*Thamnomys rutilans*) in the Congo (Brazaville). In this experiment we used *P. c. adami* genotype DS_{500} and DK_{122} originally genotyped from isolates 408XZ and 556KA respectively (Carter and Walliker 1976). The nucleotide sequence of *P. chabaudi* AMA-1 (PcAMA-1) genotype DS differs from genotype DK (556KA) by 79 amino acids (Marshall et al. 1989; Crewther et al. 1996). Hosts were inbred female C57BL/6J mice age 6-8 weeks (Harlan England) maintained as described previously (de Roode et al. 2004a). Studies by others (Crewther et al.

1996) and my own pilot studies showed that these genotypes differ in virulence during infection of C57BL/6 female mice, with genotype DS generating substantially more parasites and inducing greater weight and red blood cell loss relative to DK.

4.3.2. Immunizations and isotype ELISA

Here I used an immunization protocol adapted from Anders et al. 1998 (Anders et al. 1998). Prior to immunization, mice were randomized into four groups of eighteen (Table 4.1). Immunization was with the highly immunogenic ectodomain of the full AMA-1 protein termed AMA-1B. For mono-allelic immunizations (hereafter referred to as DS AMA-1 or DK AMA-1), groups of mice were injected intraperitoneally with 10 μg of the appropriate protein emulsified in 100 μl of the adjuvant Montanide ISA 720 (Seppic, France). For bi-allelic immunizations, mice were injected with a mixture of 5 μg of both DS and DK AMA-1, giving the same total dose of antigen as for the single antigen immunizations, again emulsified in ISA 720. Control mice were injected with 100 μl emulsion of PBS in ISA 720. Mice were given a single booster immunization with the same amount of antigen emulsified in Montanide ISA 720 4 weeks after the primary immunization.

	No. mice per immunization	Infecting clone	No. mice per parasite infection	No. deaths	No. euthanized
Sham-immun		DS	6	3	2
Sham-immun	18	DK	6		
Sham-immun		DS+DK	6		
DK AMA-1		DS	6		
DK AMA-1	18	DK	6		
DK AMA-1		DS+DK	6		
DS AMA-1		DS	6	1	
DS AMA-1	18	DK	6		
DSAMA-1		DS+DK	6	1	
Bi-allelic		DS	6	2	1
Bi-allelic	18	DK	6		
Bi-allelic		DS+DK	6		
Total			**72**	**7**	**3**

Table 4.1. Experimental design. Immunization was either with DK AMA-1, DS AMA-1, a formulation containing an equal mix of both forms of AMA-1 (bi-allelic), or immunization with adjuvant only ('sham-immune'). Groups of 18 mice were immunized with one of the four treatments before being separated into groups of 6. Infection was with parasites of genotype (clone) DK alone, genotype DS alone or a mixture of both. During the experiment

7 mice were found dead and 3 had to been euthanized due to severe morbidity. Euthanization was at predetermined levels of morbidity prescribed by animal care protocols.

To ensure that antigen immunization successfully generated antibody responses, and to determine whether there was any cross-reactivity between the antibodies generated to the different immunizing antigens, I first carried out a pilot experiment. A total of 11 mice were immunized with DS AMA-1, 11 with DK AMA-1, and 10 were sham-immunized. I estimated the quantity of IgG2b antigen-specific antibodies in all mice sera 11 days after the booster immunisation by ELISA using wells coated with DS AMA-1 or DK AMA-1. Thus the sera from 32 mice were tested in 64 wells. I used IgG2b as previous work in my laboratory showed that C57BL/6 produce this isotype in response to *P. chabaudi* infection (K. Grocock, A. Graham unpublished). Protection induced by immunisation with recombinant AMA-1 is isotype independent (Burns et al. 2004). Given the lack of cross-reactivity I observed in this pilot experiment (see Results below), in the main experiment, I measured IgG2b isotype antibodies to each antigen separately only from the sera of mice immunized with a mixture of DS and DK AMA-1 and in sham-immunized control mice.

In both the pilot and main experiments, serum fractions were separated by centrifugation from 20 µl of blood taken from a tail snip and were stored at -80^{o}C. High binding 96 well ELISA Maxisorb immunoplates (Nunc) were coated with either DS AMA-1 or DK AMA-1 at a concentration of 1 µg /ml in 0.06M carbonate buffer (0.04M $NaHCO_3$, 0.02M $NaCO_3$, pH 9.6) in a final volume of 50 ul per well. Plates were stored at 4^{o}C overnight to allow the antigen to bind. Non-specific binding was blocked by incubating wells with 5% BSA: carbonate buffer (200ul/well) for 2 hours at 37^{o}C. Wells were then washed three times in Tris buffered saline with 0.01% Tween 20 (TBST). We used end-point dilution methods to detect IgG2b titres: serum samples were detected in a serial dilution 1/100-1/204800 using TBST as a diluent, in a final volume of 50 µl per well and incubated for 2 hours at 37^{o}C. Wells were washed three times in TBST. HRP conjugated goat anti-mouse IgG2b detection antibody (Southern Biotech 1100-05) was diluted 1/4000 in TBST to a final volume of 50 µl per well. Plates were incubated for 1 hour at

37°C. Wells were washed three times in TBST followed by a final wash in distilled water. ABTS peroxide substrate (Insight Biotechnology) was added at 100 µl per well and allowed to develop at room temperature for 20 minutes. Optical density was read at 405 nm using a spectrophotometer. IgG2b isotype antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D.) was greater than the mean (plus 2 standard deviations) O.D values observed for naïve mouse sera assayed against both DS and DK AMA-1 at 1/100.

4.3.3. Parasite challenge and monitoring of within host dynamics

Two weeks after the boost immunisation, groups of immunized mice (18 per group) were further randomized into groups of six and challenged with 10^5 parasites of either genotype DS alone, genotype DK alone or a mixture of genotype DS and DK (Table 4.1). Thus, mice infected with both genotypes received twice as many parasites as those infected with one genotype. A two-fold difference in infective dose has negligible effects on the population dynamics of the parasite (Timms et al. 2001). During the course of infection, we measured body weights and took blood samples from the tail to (i) make Giemsa-stained blood smears, (ii) estimate red blood cell density by flow cytometry (Beckman Coulter), and (iii) for genotype-specific real-time quantitative PCR (qPCR) assays as described previously (Bell et al. 2006). For amplification of the DK genotype, we used primers previously designed to amplify AS/AJ genotypes as described elsewhere (Bell et al. 2006). DS genotype-specific primers were as follows: DS forward 5' GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA 3'; DS reverse 5' CAG GAG AAA TGT TTA CAT CTG CTT T 3'.

4.3.4. Trait definition and statistical analyses

Since *P. chabaudi* has a 24 hour replication cycle, the total number of parasites present in any period can be estimated by summing the daily parasite counts. Data were analysed using General Linear Models (GLMs) in MINITAB (Minitab, version

14). To meet normality and homogeneity of variance assumptions, data on antibodies, weight and red blood cell density were log transformed while all parasite densities and proportions were square root transformed. GLMs were used to test whether the magnitude of protection differed between the three antigen immunizations (DK AMA-1, DS AMA-1, or the bi-allelic form); that is whether there was a statistical interaction between infecting genotype and immunizing treatments. Maximal models (response variable = infecting genotype + immunizing treatment + infecting genotype x immunization treatment) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. For analyses of within host selection, we asked for mixed genotype infections, whether the frequency of genotype DS in the parasite population differed between the sham-immunized controls and the antigen immunizations.

4.4 Results

Table 4.1 gives details of the immunization treatments, infecting genotype and sample size of the experiment. Some mice died; these were included in the calculation of daily densities until death, and in the analyses of peak parasite densities since death always occurred as initial parasitaemias were declining.

4.4.1. Pre- challenge anti-AMA-1 IgG2b antibodies

Figure 4.1 illustrates the data from a pilot experiment where IgG2b antigen-specific antibodies were measured to each of the immunizing antigens and the cross-reactivity between them. All antigen immunization treatments generated antibody titres that were higher than those present in sham-immunized controls (sham-immunized versus antigen immunized: $F_{1,62}$=8.92, p=0.004). IgG2b antibodies were

specific for the antigen they had been exposed to during immunization (immunizing treatment x ELISA antigen: $F_{1,40=}9.99$, p=0.003). For example, anti-DS AMA-1 IgG2b antibody titres were higher when assayed against the homologous DS antigen than the heterologous DK antigen and vice versa. Thus, neither antigen elicited a stronger response overall.

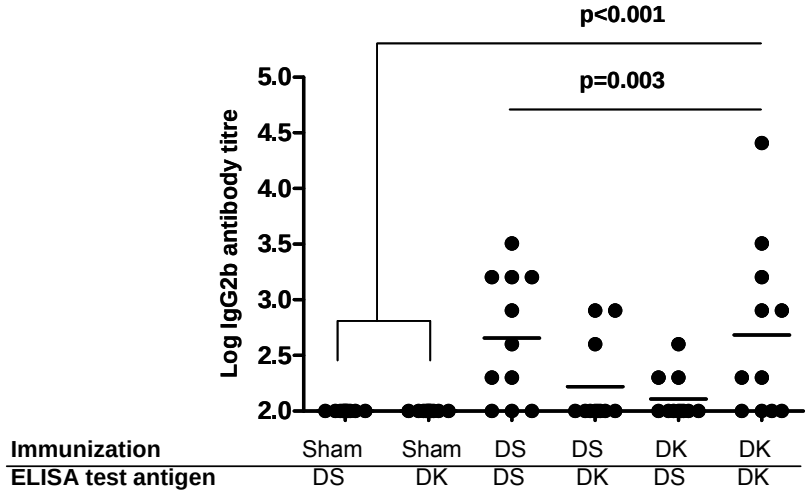

Figure 4.1. IGg2b antibody levels from the serum of mice in the pilot experiment. Mice were either sham-immunized or immunised with one of the two antigens (DS AMA-1, DK AMA-1). Each of the treatments used to immunize mice and the AMA-1 test antigen used to coat ELISA plates are shown on the x axis. Dots represent the antibody titre against a particular immunizing antigen. Horizontal lines indicate mean antibody levels. Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls ($p<0.001$) and, among the immunized mice, the levels induced between the antigen immunized groups differed (immunizing treatment x ELISA antigen: p=0.003) with higher titres against the homologous antigen. Neither of the immunising antigens induced higher titres ($p>0.05$).

Figure 4.2 illustrates the IgG2b antibody titres in mice from the main experiment three days prior to parasite infection. All antigen immunization treatments induced antibody titres that were higher than those present in sham-immunized mice (sham-

immunized v immunized: $F_{1,106}$=58.89, p<0.001). Among antigen-immunized groups, titres did not differ ($F_{2,69}$=2.56, p=0.085) In those mice which had been immunized with the bi-allelic form, antibodies were not more specifically recognising either component antigen ($F_{1,35}$=0.61, p=0.44). These data show that immunization successfully elicited antibody responses, and that, at least as measured by IgG2b titres, these responses were of equal magnitude in all immunized groups. Among antigen-immunized mice, antibody titres prior to challenge did not predict subsequent parasite intensities, weight loss or anaemia (all correlations, p >0.2).

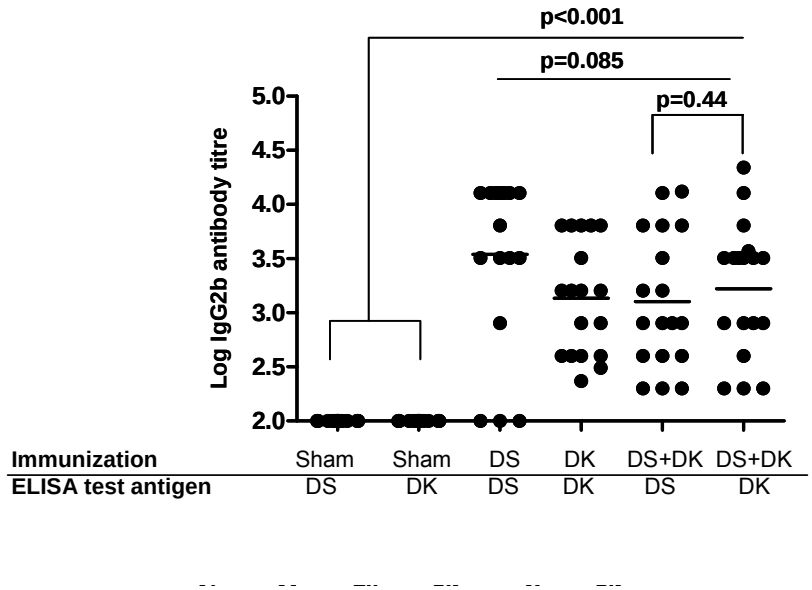

Figure 4.2. IgG2b antibody levels from the serum of mice in the main experiment. Mice were either sham-immunized, or immunized with one of the antigen immunization treatments (DS AMA-1, DK AMA-1 or the bi-allelic formulation). Each of the treatments used to immunize mice and the AMA-1 test antigen used to coat ELISA plates are shown on the x axis. Dots represent the antibody titre for individual mice against a particular immunizing antigen. Mice that were sham-immunized or immunized with the bi-allelic formulation were assayed for antibody responses against both DS and DK AMA-1 antigens.

Horizontal lines indicate mean antibody levels. Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls (p<0.001), and among the antigen immunized mice, antibody titres did not differ (p=0.085). The antibody titres in

animals immunized with both antigens were not dominated by responses to either one ($p=0.44$).

4.4.2. Bi-allelic immunization did not generate a greater anti-morbidity response than did mono-allelic immunization

Red blood cell density and weight kinetics following parasite challenge for each of the immunization treatments are illustrated in Fig. 4.3A-F, and the minimum red blood cell density and minimum weight reached are illustrated in Fig. 4.3G-H. In sham-immunized control mice, genotype DK was less virulent than genotype DS, induced less anaemia and less weight loss (Fig. 4.3A-H; anaemia: $F_{2,14}=6.29$, $p=0.011$; weight loss: $F_{2,14}=9.97$, $p=0.002$).

Immunization protected mice against anaemia induced by infection with any of the genotypes (Fig. 4.3A-C, G; sham-immunized v immunized: $F_{1,69}=16.94$, $p<0.001$). Bi-allelic immunization reduced anaemia no more than did immunization with either of the alleles alone (Fig. 4.3G; immunizing treatment x infecting genotype: $F_{4,44,}=0.71$, $p=0.59$). All pairwise immunization comparisons were non-significant ($p>0.5$ in all cases).

As infection with genotype DK did not induce any weight loss in sham-immunized controls (Fig. 4.3D) the protective effects of immunization were analyzed only for infections that contained genotype DS (Fig. 4.3E-F). We found that all immunizations protected mice against weight loss due to DS infections (Fig. 4.3E-F, H; sham-immunized v immunized: $F_{1,45}=11.13$, $p=0.002$). Similar to the anaemia data, we found that immunization with either the bi-allelic form or either of the alleles alone afforded similar levels of protection against weight loss (Fig. 4.3H; immunizing treatment x infecting genotype: $F_{2,29}=2.43$, $p=0.11$). All pairwise immunization comparisons were non-significant ($p>0.5$ in all cases).

Together, these results show that immunization with the bi-allelic vaccine does not afford the host greater protection from morbidity, as measured by anaemia and weight loss. Immunization with either of the variants alone provided protection which was as effective as that induced by the two variants together.

4.4.3. Bi-allelic immunization did not generate greater anti-parasite response than did mono-allelic immunization

Parasite dynamics under each of the treatments are illustrated in Fig. 4.4. Genotype DS achieved higher parasite density in sham-immunized control mice than did genotype DK (infecting genotype: $F_{1,10}$=7.03 =0.024).

All three immunizations reduced peak parasite densities relative to those which had received a sham inoculation (Fig. 4.4D, sham-immunized v immunized: $F_{1,69}$= 11.55, p=0.001). The extent of anti-parasite protection depended on the identity of the immunising antigen and the identity of the challenge genotype (Fig. 4.4D; immunizing treatment x infecting genotype: $F_{4,44}$=8.71, p <0.001). We found that protection was genotype-specific: immunization with DS AMA-1 antigen reduced DS parasite densities more than it reduced the densities of genotype DK, and vice versa (among single antigen immunized groups, immunizing treatment x infecting genotype: $F_{1,19}$=36.26, p<0.001).

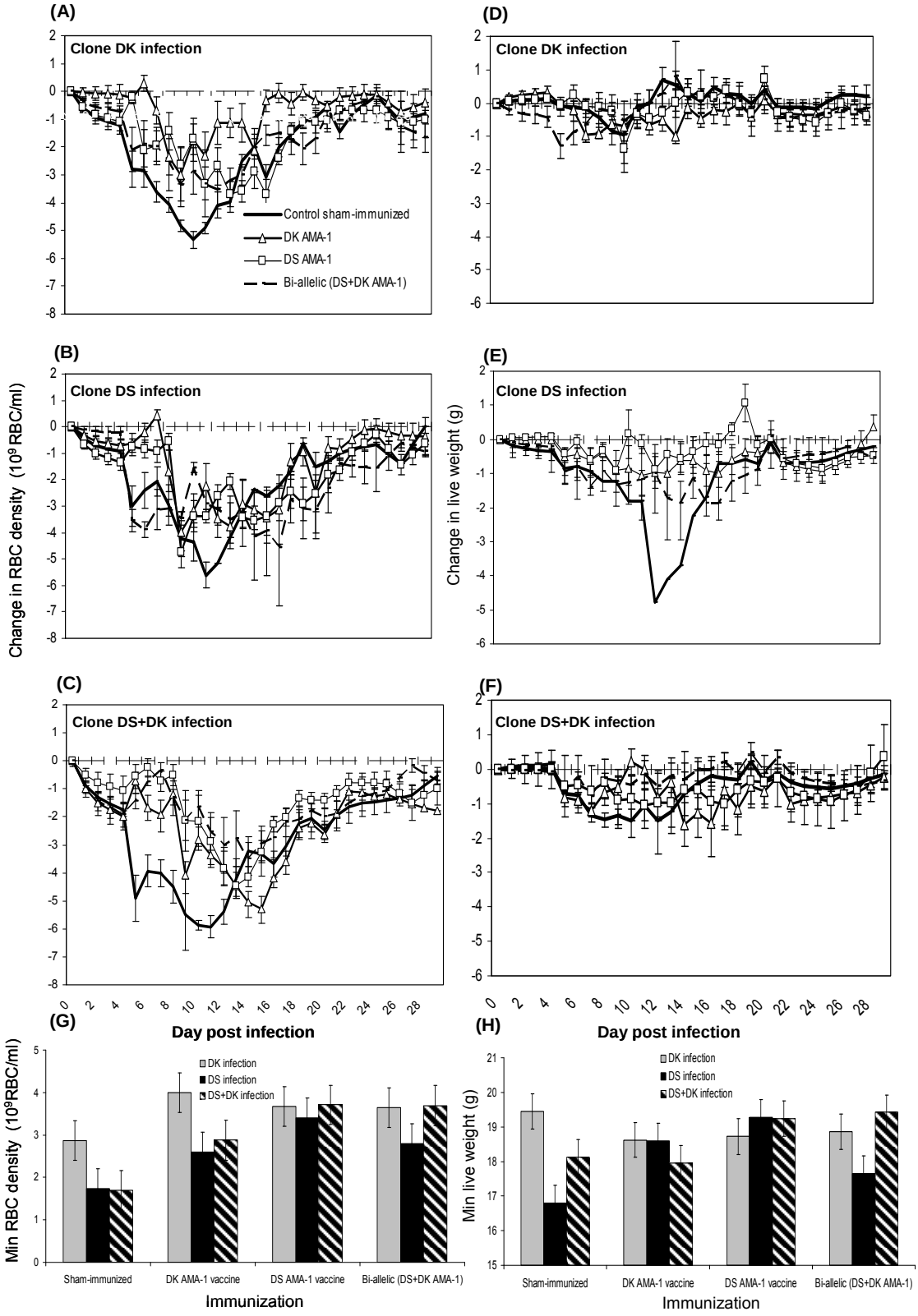

Figure 4.3. Legend on next page

Figure 4.3. Effect of *Plasmodium chabaudi* infection (genotype (clone) DK alone, DS alone or DS+DK) and immunization (sham-immunized control, DK AMA-1, DS AMA-1, or bi-allelic form) on the kinetics of minimum red blood cell density (left panels) and minimum weight (right panels). In A-F lines represent the change in RBC density (left panels) and weight (right panels) over time. Each line represents the mean of up to 6 mice (+/- 1 s.e.m) that were infected with DK alone (A and D), DS alone (B and E) or a mixed genotype (C and F) infection during immunization with either a sham-inoculation control (solid thick black line), DK AMA-1 (open triangle), DS AMA-1 (open squares), or the bi-allelic mixture (dotted black line). In G-H bars represent the minimum red blood cell density (left panel) and minimum weight (right panel) reached during infection with genotype DK alone (grey bars), DS alone (black bars) or a mixture of both genotypes (black and white bars) under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m).

When I compared the extent of anti-parasite protection between the immunized groups we found that under no circumstances did the bi-allelic immunization afford greater protection than did immunization with a single allele. For example, immunization with DS AMA-1 reduced the peak density of DK infections and infections with both genotypes together, but the bi-allelic immunization did not protect against DS alone (Fig. 4.4D; immunizing treatment x infecting genotype: $F_{2,30}$=9.84, p=0.001). Although the bi-allelic immunization reduced the densities of genotype DK, reduction was no greater than with a single DK AMA-1 immunization (Fig. 4.4D; immunizing treatment x infecting genotype $F_{2,29}$=4.09, p=0.027).

Together these results show that bi-allelic immunization did not afford the host greater anti-parasite protection than did mono-allelic immunization. Unlike morbidity, where protection was induced regardless of the antigen used in immunization, we found that immunization with a single allele achieved better protection against the homologous genotype, and bi-allelic immunisation never did as well. Indeed, I found just one of the variants (DS AMA-1) to be the most effective at reducing parasite densities.

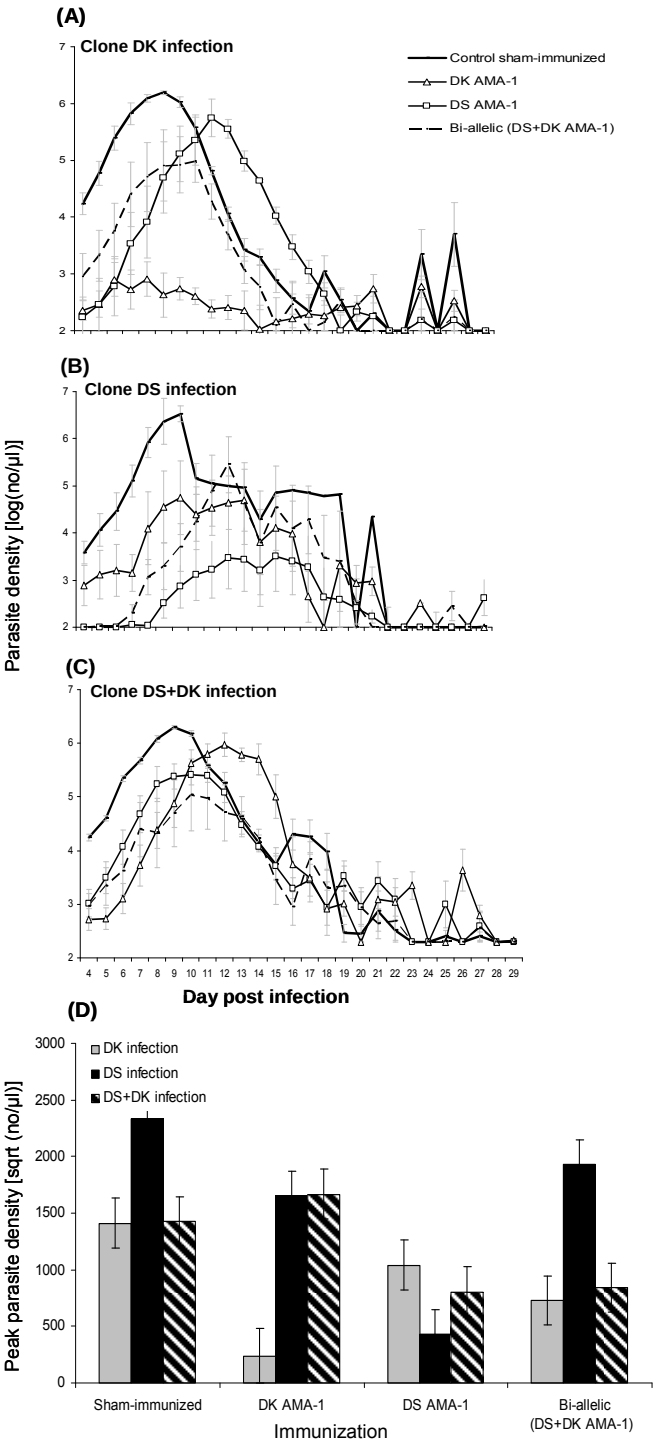

Figure 4.4. Legend on next page

Figure 4.4. Kinetics of *Plasmodium chabaudi* infections (genotype (clone) DK alone, DS alone or both together) following immunization (DK AMA-1, DS AMA-1, or bi-allelic formulation or sham-immunized control). In A-C, lines represent the change in parasite density over time. Each line represents the mean of up to 6 mice (+/- 1 s.e.m) that were infected with DK alone (A), DS alone (B) or a mixed genotype (C) infection during immunization with either a sham-inoculation control (solid thick black line), DK AMA-1 (open triangle), DS AMA-1 (open squares), or the bi-allelic mixture (dotted black line). In (D), bars represent peak parasite densities reached during infection with genotype DK alone (grey bars), DS alone (black bars) or a mixture of both genotypes (black and white diagonal) under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m).

4.4.4. Vaccine-induced anti-parasite protection was genotype specific in mixed infections and independent of genotype virulence

To examine how the antigenic composition of the immunising formulation affects within-host selection (relative frequency) in mixed genotype infections, and whether heterologous immunity less effectively controlled the virulent genotype, I compared the frequency of genotype DS in mixed infections (Fig. 4.5).

I found that antigen immunization altered genotype frequencies. In sham-immunized mice, and those immunized with the bi-allelic formulation, DS made up about 60% of all the parasites present in the infections. Thus, immunization with a mixture of DS and DK AMA-1 had negligible effect on genotype frequency and thus within host selection (Fig. 4.5A; sham-immunized versus bi-allelic immunization: $F_{1,10}$= 2.02, p=0.19). In contrast, immunization with a single antigen reduced parasites in a genotype-specific manner, facilitating the heterologous genotype (Fig. 4.5A,B; immunizing treatment $F_{1,10}$= 105.54, p<0.001). Immunization with DK AMA-1 increased the frequency of genotype DS, while DS AMA-1 immunization increased the frequency of DK. These effects were essentially symmetrical. Thus, there was

no evidence that the more virulent genotype, DS, was less affected by heterologous immunization than was the less virulent genotype DK.

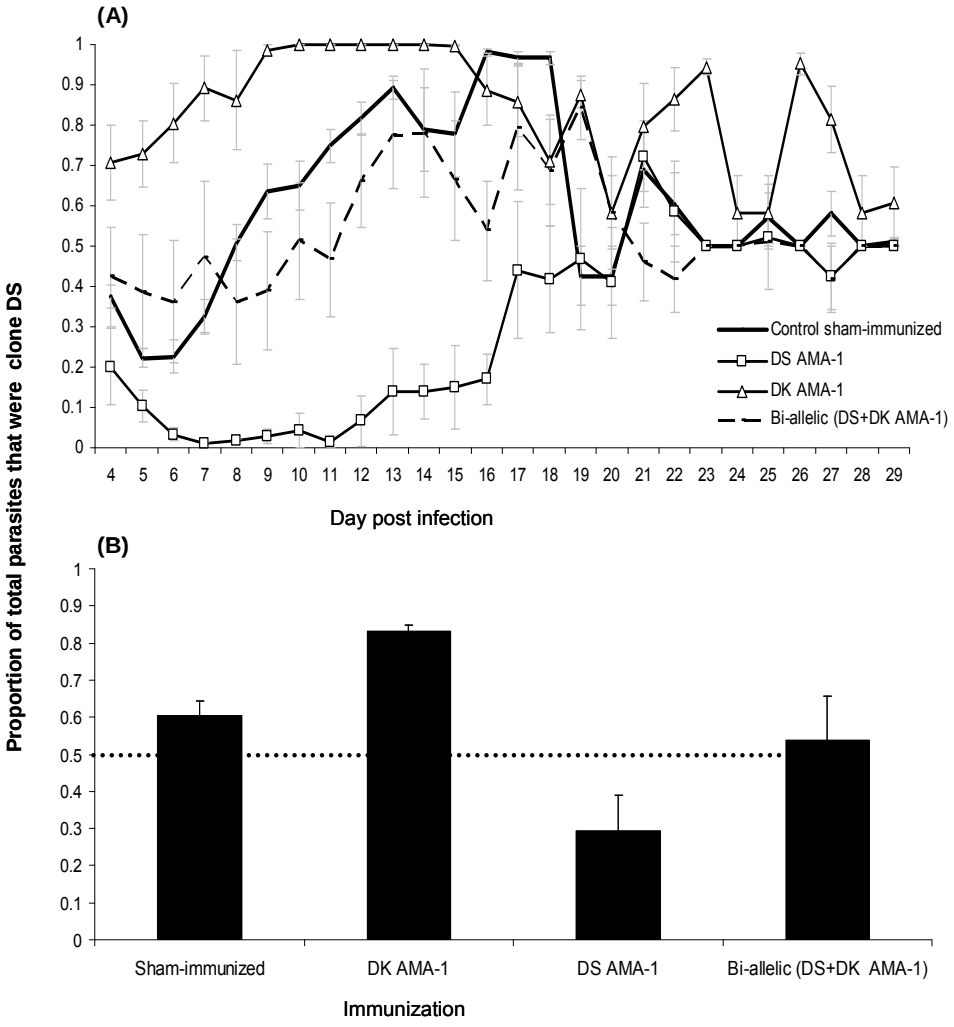

Figure 4.5. Proportion of genotype DS in mixed DS and DK infections following immunization with DK AMA-1, DS AMA-1, the bi-allelic formulation, or in sham-immunized controls. (A) Lines represents the proportion of genotype DS through time in control (solid black line), DK AMA-1 (open triangles), DS AMA-1 (open diamonds) or bi-allelic (dotted black line) immunized mice. Each line represents the mean of up to 6 mice (+/- 1 s.e.m). (B) Bar graphs represent the proportion of total parasites in a mixed infection that were DS under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice with 95% confidence intervals. The black horizontal dotted line represents the proportion of DS present in the inoculum.

4.5. Discussion

In this study, I investigated (i) whether immunization with a single or bi-allelic AMA-1 formulation afforded the host the greatest protection from morbidity and parasite infection, (ii) how these different vaccination regimes altered clonal compositions in mixed infections, and (iii) whether a more virulent genotype was less successfully controlled by vaccine-induced protective responses. Addressing each of these in turn, I found the following. (i) Bi-allelic immunization did not generate better anti-morbidity or anti-parasite protection than did single allele immunization. Rather, immunization with one of the two variants alone (DS) provided the best protection. (ii) Both single variant immunizations reduced the frequency of homologous genotypes in mixed infections; bi-allelic immunization had no impact on within-host selection. (iii) There was no evidence that the more virulent genotype (DS) was better at evading vaccine-induced immunity than was the less virulent genotype.

Rightly, protecting individual hosts from morbidity is one of the goals of malaria vaccines directed against the blood-stage of infection. If infection densities are positively correlated with host morbidity (virulence) (Mackinnon et al. 2008) multi-allele vaccines could potentially improve the health of the host by suppressing more of the parasite population and reducing strain-specific responses. Subject to the usual cautions about generalising from animal models (reviewed in this context by Råberg et al. 2006 and Wargo et al. 2007), the results presented here argue against that, and suggest that protective efficacy may not be increased by including alternative variants of AMA-1. My in vivo observations are consistent with previous results showing that immunization of rhesus monkeys with only one of two PfAMA-1 variants is sufficient to induce cross protective antibody responses as measured by GIA and ELISA assays in vitro (Miura et al. 2007). My results are also consistent with another study which demonstrated that mice and rabbits immunized with two allelic variants of domain I and II of the full length AMA-1 ectodomain from Indian *P. falciparum* isolates were able to inhibit in vitro parasite growth, but to no greater extent than with either of the allelic variants alone (Lalitha et al. 2008).

My results also demonstrate strain-specific anti-parasite responses (Fig. 4.4D) need not result in strain-specific protection against disease (Fig. 4.3G,H). The observation that there are two different types of anti-malarial responses – immunity against the parasite itself and immunity against disease – is poorly understood on a molecular basis although the distinction is widely appreciated (Schofield & Grau 2005). An explanation for the two different responses observed here could be that the specificity of the anti-AMA-1 antibody response lies with the generation of inhibitory antibodies which may target the hypervariable region located around a conserved hydrophobic pocket on domain I (Coley et al. 2007). The presence of such antibodies could determine the observed parasitaemias. For bi-allelic immunizations there may exist a dominant epitope in one allelic form of AMA-1. Thus, high titres of cross-reactive antibodies may be sufficient to lessen morbidity (hence the similar effects for mono-and-bi-allelic vaccination on morbidity) but the inhibitory antibodies are more effective at controlling parasite numbers by inhibiting invasion. In my pilot studies I did not observe a disproportionate IgG2b antibody response to one of the immunizing antigens (Fig. 4.1). However, since immunization with AMA-1 is likely to induce a repertoire of IgG isotypes (Burns et al. 2004; Eisenhut 2007; Gray et al. 2007; Osier et al. 2008) some of the other isotypes may be sufficiently cross-reactive. An implication of this may be that while strain-specific immunization may alter allele frequencies in parasite populations, this need not have clinical consequences in a vaccinated host. Changes in allele frequencies without public health consequences have been seen in some other diseases, such as pertussis (reviewed in Read and Mackinnon 2008).

The 'Combination B' malaria vaccine, one of the few to reach field trials, demonstrated strain specific anti-parasite effects despite being comprised of an allele of each of 3 asexual blood stage proteins, MSP-1, MSP-2 and RESA (ring-infected erythrocyte surface antigen) (Genton et al. 2002). Of particular interest was that vaccination increased the frequency of parasites with an MSP-2 genotype belonging to the FC27 allelic family. No representatives of this allelic family, which had been found previously to be associated with severe morbidity, were included in the vaccine (Engelbrecht et al. 1995; Genton et al. 2002; Genton et al. 2003). Selection

for the FC27 form of MSP-2 could have been because of strain-specific protection (Fluck et al. 2004; Fluck et al. 2007), or because the vaccine was less effective at protecting against more virulent strains (Mackinnon & Read 2004a; Mackinnon et al. 2008; Read & Mackinnon 2008). In the study I report here, I looked at the relative proportion of the more virulent genotype in a mixed infection under the different immunization compositions. In sham-immunized control mice and those which received the bi-allelic immunization, the more virulent genotype (DS) was proportionally the most dominant. Thus, bi-allelic immunization did not alter within host selection. On the other hand, immunization with a single AMA-1 variant did facilitate evasion of the heterologous genotype in mixed infections. In my experiments, this effect was symmetrical (Fig. 4.5), so that immunization with AMA-1 appears to induce protective responses that are strain-specific and evasion is independent of parasite virulence.

Nevertheless, selection for virulence could be an inadvertent consequence of including just one allele from a given locus in a vaccine, as apparently happened in the Combination B trial. As far as I am aware, there are no reports that variants of AMA-1 have different intrinsic virulence, so that the strain specific immunity against this locus I report here and that has been seen by others (Crewther et al. 1996; Healer et al. 2004), should not directly alter virulence. But caution is necessary for all antigens involved in processes like cell invasion which are associated with pathogenesis. Population-level association studies for disease severity should be performed for all antigens included in candidate vaccines. Should associations like that for MSP-2 be found (Engelbrecht et al. 1995), I suggest on the basis of my results that there would be a strong case for including all known variants at that locus in the vaccine. This would not confer short term clinical advantage, but it would be the safest way to avoid inadvertent selection for virulent variants, which would put unvaccinated people at greater risk.

More generally, though, we still have some way to go to understand the potential for vaccine-driven virulence evolution, even in the *P. chabaudi* model. One experimental study demonstrated that parasites from a single *P. chabaudi* genotype

serially passaged through whole-parasite immunized mice evolved to be more virulent than parasite lines that those evolved in naïve hosts (Mackinnon & Read 2004a). That study was the first to show under controlled conditions that immunization can favour the evolution of more virulent parasites. The implication was that more virulent variants had a selective advantage in immunized hosts. In the study I report here, which did not involve serial passage, I saw no signs of such an advantage. DS, the more virulent genotype, dominated in mice immunized with the bi-allelic form, but to the same extent as in non-immunized mice. In single antigen immunized mice, strain-specific immunity dominated with symmetrical effects for both genotypes. Competition experiments with other *P. chabaudi* genotypes also failed to find an increased advantage to virulence in immunized hosts (Grech et al. 2008). It may be that the accelerated evolution of virulence seen during serial passage in immunized hosts (Mackinnon & Read 2004a; Mackinnon and Read 2004b) is a feature of selection of virulence variants on an antigenically identical background. In future experiments, I will serially passage single *P. chabaudi* genotypes through AMA-1 immunized and naïve mice to determine whether vaccination can drive the evolution of virulence to be greater when measured in naïve hosts.

My experiments concerned antigenic polymorphism at a single target antigen. Considerably more work has focused on vaccines combining single variants from multiple antigenic loci (Richie & Saul 2002; Heppner et al. 2005; Garcia et al. 2006). For example, animal and human phase I trails have shown safety, tolerability and immunogenicity of formulations containing AMA-1 and MSP-1 (2002; Burns et al. 2003; Pan et al. 2004; Faber et al. 2007; Hu et al. 2008). Moreover, such 'multivalent' vaccines have been shown to reduce parasitaemias in mice of distinct MHC haplotypes (Doolan et al. 1996) and against infections with different parasite strains as well as subspecies of different virulence (Scorza et al. 2008). Thus, multi-valency may be required to induce antibody responses against a repertoire of polymorphic parasite antigens (Bouharoun-Tayoun et al. 1995; Bull & Marsh 2002; Wipasa et al. 2002a; Wipasa et al. 2002b; Doolan et al. 2003; Moorthy et al. 2004; Eisenhut 2007; Osier et al. 2008) in the human outbred population exposed to

multiple parasite genotypes (Doolan & Hoffman 2001; Genton et al. 2002; Plebanski et al. 2002; Cortes et al. 2003; Doolan et al. 2003). I suspect that multi-valent vaccines will prove to be a more efficient means of generating protection against the widest range of parasite genotypes. Certainly, I found no evidence that the anti-morbidity and anti-parasitic potency of a malaria vaccine would be enhanced by increasing the number of variants of a particular antigen.

5. Serial passage of *Plasmodium chabaudi* promotes virulence and parasite evasion of vaccine–induced immunity

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5.1. Abstract

Theory and some data suggest that more virulent malaria parasites may be less readily controlled by immunization, raising the possibility that a malaria vaccine could select for more virulent parasites. In this study, we tested whether the protective effects of vaccination with the malaria vaccine candidate apical membrane antigen (AMA-1) depended on the virulence of the challenge parasites. A single *Plasmodium chabaudi* genotype was serially passaged through immunologically naïve mice which generated parasites that caused more anaemia and weight loss, and which were able to achieve higher densities and persist for longer before immune clearance. These more virulent parasites were less readily controlled by immunity induced by immunization with homologous recombinant AMA-1 than were the less virulent parasite from which they were derived. Thus, vaccination has the potential to selectively favor more virulent parasites. These results may go some way to explaining why parasites serially passaged through whole-parasite immunized mice become virulent more rapidly than those evolved through naïve mice, and an observed example of selection imposed by malaria vaccines during a field trial. The

data argue for further consideration of the evolutionary consequences of pathogen virulence on vaccination.

5.2. Introduction

Any form of medically therapeutic intervention has the potential to impose selection on pathogen populations. The fact that malaria parasites developed drug resistance within 5-30 years of first using a drug is indicative of their potential to rapidly evolve (Peters 1987; Hyde 2005). Currently, there is no vaccine against malaria, and therefore we do not know formally how vaccines may impose selection on *Plasmodium* populations.

The trial of the malaria vaccine known as 'Combination B', provided the first field evidence that a vaccine against this disease could impose selection (Genton et al. 2002; Genton et al. 2003). One component of the vaccine was the Merozoite Surface Protein 2 (MSP-2). MSP-2 is known to exist as two allelic families, the 3D7 type and the FC27 type. In follow up surveillance studies, the prevalence of parasites with the 3D7 type, included in the vaccine, was reduced in vaccinees compared to those which had received a placebo (Genton et al. 2003). The prevalence of parasites which carried the FC27 type was unaffected. Interestingly, the FC27 type had previously been associated with more virulent infections (Engelbrecht et al. 1995). This trial raised the question as to whether vaccination had imposed selection that was strain (genotype)-specific (Fluck et al. 2004; Fluck et al. 2007) or whether the vaccine was just less effective at controlling more virulent variants.

Immunization has been shown to impose selection on malaria parasites in a strain (genotype)-specific manner (Crewther et al. 1996; Fluck et al. 2004; Darko et al. 2005; Martinelli et al. 2005; Cheesman et al. 2006). However, evidence to support the possibility that vaccination may select for more virulent genotypes has also started to appear. Evolutionary theory suggests there are two possible advantages to virulence. First, higher virulence is associated with a greater total transmission potential across the course of infection. This could be achieved by more transmissible forms per day of infection, a longer transmission period due to reduced

immune clearance, or a combination of both processes (Anderson & May 1982; Mackinnon & Read 2004a; Mackinnon & Read 2004b; Frank & Schmid-Hempel 2008; Schmid-Hempel 2008). Second, competition experiments between genotypes which differ in virulence have shown a transmission advantage to virulence (de Roode et al. 2005; Bell et al. 2006). It is possible that vaccination could alter one or more of the parasite benefits of virulence and thus enhance selection for more virulent genotypes.

In line with predictions, when single *P. chabaudi* genotypes are serially passaged (by syringe transfer of parasites in blood from one host to another) through whole-parasite immunized or naïve mice, genotypes evolved through immunized animals have higher virulence (here defined as harm to host following infection) when measured on naïve mice, even after mosquito transmission (Mackinnon & Read 2004b). That study was the first to show under controlled conditions that immunization can favour the evolution of more virulent parasites. In another study, which did not involve serial passage, selection for virulence was measured by comparing vaccine efficacy of AMA-1 derived from *P. chabaudi* genotype DK against two antigenically distinct parasites which differed in virulence but were identical at the *ama-1* sequence (Grech et al. 2008 *in prep*). The more virulent genotype was found to be less well controlled. Similarly, in the same experiment when DK AMA-1 vaccine efficacy was tested against a virulent lineage and the ancestral genotype from which it was derived by serial passage through immunologically naïve mice, the virulent lineage was less well controlled by heterologous immunization. Those studies have suggested that there is a within-host advantage to virulence in immunized hosts.

However, other studies have presented conflicting data. AMA-1 derived from *P. chabaudi* genotype DS tested against the same *P. chabaudi* parasites used by Grech et al (2008) found that the more virulent genotype was not harder to control (Chapter 3). Furthermore, reciprocal DK AMA-1/DS AMA-1 immunization and parasite challenge trails appeared to induce protective responses that were genotype-specific, independent of parasite virulence [Chapter 4, Appendix 1 (Barclay et al. 2008a)].

Competition experiments have also failed to find an increased advantage to virulence in immunized hosts (Grech et al. 2008). Additionally, $CD4^+$ T cells were shown not to enhance selection for virulence during competitive interactions [Chapter 6, Appendix 2 (Barclay et al. 2008b)]. Those results suggested that the $CD4^+$-dependent immune response, and mechanisms that act to enhance it such as vaccination, may not have the undesirable affect of strengthening selection for virulence.

One of the explanations for the contrasting results above has been that the selection imposed by vaccination may depend on the fine specificity of the protective response induced by alternative vaccine antigens (Chapter 3). Another explanation has been that accelerated evolution of virulence seen during serial passage (Mackinnon & Read 2004a; Mackinnon & Read 2004b) may be a feature of selection of virulence variants on antigenically identical backgrounds [Chapter 4, Appendix 1 (Barclay et al. 2008b)]. The aim of this study was to directly test whether there was a within-host advantage to parasite virulence between two parasites with antigenically identical backgrounds. A virulent lineage was derived by serial passage of a single *P. chabaudi* genotype through immunologically naïve mice. The more virulent lineage and the less virulent genotype from which the line was derived were then used to infect AMA-1 immunized mice. Morbidity measurements as well as the parasitological status of the host were used to determine whether there was within-host advantage to virulence in immunized hosts.

Since there are currently at least six different vaccines based on the AMA-1 allele from the *P. falciparum* 3D7 strain currently in efficacy trials in humans (Polhemus et al. 2007; Maher 2008; Thera et al. 2008), deciphering whether AMA-1 has the potential to select for more virulent parasites is of critical importance.

5.3. Material and Methods

5.3.1. Parasites, hosts and serial passage

Isolates containing *Plasmodium chabaudi adami* were originally collected from thicket rats (*Thamnomys rutilans*) in the Congo (Brazzaville) (Cater and Walliker 1976). *P. c. adami* were cloned, genotyped and are stored in liquid nitrogen with subscript codes used to identify their position in clonal history. The protocol for serial passage followed that of Mackinnon and Read (2004a). In this study I used DK_{122} as the ancestral genotype. Ancestral parasites were repeatedly passaged in mice (female C57BL/6) that were naïve to malaria infection for 30 mouse generations to derive a parasite line known as DK_{294}. Herein, the ancestral DK genotype will be referred to as $DK_{ancestral}$ and the line derived by serial passage as $DK_{derived.}$ Passages involved the syringe transfer to a fresh mouse of 0.1 ml of diluted blood containing $5x10^5$ parasites from a donor mouse that had been infected 7 days previously. Day 7 post infection is during the period of rapid population growth, and is about 2 days prior to peak parasitaemia, after which population size rapidly declines.

5.3.2. Immunization and experimental set-up

Immunization protocols followed those of (Anders et al. 1998) and (Crewther et al. 1996). Rabbits were immunized with the highly immunogenic ectodomain of the full AMA-1 protein derived from parasite DK emulsified in Montanide ISA 720. For primary immunizations, rabbits received a total of 100-200 μg of emulsified antigen between two intramuscular sites. Rabbits were boosted at 4-8 week intervals with the same amount of antigen injected intramuscularly and subcutaneously. Immunoglobulin G (IgG) was isolated from the sera of rabbits by affinity chromatography on protein A-Sepharose columns (Pharmacia/Amrad). Prior to immunization experimental mice were randomized into four groups (Table 5.1). For

the transfer of antibodies to mice, three milligrams of purified anti-DK AMA-1 IgG (hereafter referred to as DK AMA-1) was administered intraperitoneally one day before parasite infection and again 3 hours before parasite infection. Control mice were injected with a sham inoculation of rabbit serum of the same isotype (IgG Sigma I5006).

	Immunization	
Parasite	Control	DK AMA-1
DK ancestral	4	4
DK derived	4[1]	4[1]

Table 5.1 Experimental design. Groups of mice (4 per group) either sham-immunized (control) or immunized with recombinant DK AMA-1. Infection was with $DK_{ancestral}$ or $DK_{derived}$. The number of mice found dead during the experiment are indicated by the number in brackets.

Groups of treated mice (control sham-immunized or DK AMA-1) were infected either with $DK_{ancestral}$ or $DK_{derived}$ 3 hours after the boost immunization. Mice were 6-8 week old female C57BL/6J and were injected intraperitoneally with 10^5 parasites as described elsewhere (Mackinnon & Read 1999).

I followed the infection dynamics for 29 days. From the first day of parasite infection (day 0), I daily measured body weights and took blood samples from the tail of experimental mice to estimate RBC density (by flow cytometry; Beckman Coulter). From day 4 post parasite infection onwards, I daily took blood samples from the tail to make Giemsa-stained blood smears for estimating daily parasite densities. I chose day 4 as this is around the first day that parasites can be detected in the blood using microscopy. Since *P. chabaudi* has a 24 hour replication cycle, the total number of parasites in any period can be estimated by summing the daily parasite counts.

5.3.3. Sequencing of the AMA-1 alleles of P. chabaudi

Primers were designed to amplify the ama-1 gene as two fragments. Primer sequences were: Forward 5' GGG TCC AAG ATA TTG TAG 3' and reverse 5' TGG TGT TTG TGT GTG ATG C 3'. Primer sequences for fragment 2 were: Forward 5' CTT GGG TAA TTG TTC CGA 3' and reverse 5' GGT TTC CCA ATC TTC ACG 3'. PCR was performed using Plantinum® Tag DNA polymerase (Invitrogen), with the thermocycle profile; 95^{o}C for 7 mins, 50 ^{o}C for 1 min, 72 ^{o}C for 2 min, followed by 95 ^{o}C for 3 mins, 51 ^{o}C for 1 min, 72 ^{o}C for 2 min, followed by (93 ^{o}C for 1 min, 52 ^{o}C for 1 min, 72 ^{o}C for 1 min) x 28, ending at 72 ^{o}C for 10 mins. DNA was amplified using Immolase^{TM} DNA polymerase (Bioline). Sequencing reactions were performed using a Prism BigDye Terminator Cycle Sequence Kit Version 3.1 (ABI) and the University of Edinburgh SBS Sequencing service ran the products of the reactions in-house on an ABI 3730 sequencer. Sequences were analyzed using Lasergene 7 (DNAstar, Inc).

5.3.4. Trait definition and statistical analyses

Here I define virulence as the maximum change in red blood cells (anaemia) and weight, as well as the total parasite density reached across the course of an infection. Thus, an increase in virulence is defined as more severe anaemia, weight loss, and a greater number of parasites during infection of naïve mice with a derived line compared to an ancestral genotype.

I used General Liner Models (GLM) in MINITAB (release 14, MINITAB Inc.) to test whether serial passage had derived a line with increased virulence and whether those traits also accrued under AMA-1 immunization; that is whether there was a statistical interaction between immunization and infecting parasite. The response variables included minimum red blood cell density, minimum weight and total parasite density. Explanatory variables included infecting parasite ($DK_{ancestral}$ or $DK_{derived}$) and immunization (control sham-immunized or DK AMA-1). Initial red cell density and weights were used as a co-variate. Maximal models (response variable =

immunization + infecting parasite + all higher order interactions) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with the highest order interactions, to obtain the significant minimal model. All data were log transformed to meet normality assumptions of the model.

During the experiment two mice died; these were included in the calculation of daily densities until death, and in the analyses of peak parasite densities since death always occurred as initial parasitaemias were declining.

5.4. Results

The treatment groups including number of mice per group and number of deaths during the experiment are shown in Table 5.1.

In sham-immunised mice, infections with $DK_{derived}$ induced higher parasite densities (Fig 1; $F_{1,6}$=19.74, p=0.004), caused more anaemia (Fig. 5.2, 3; $F_{1,5}$ =8.31, p=0.034) and weight loss (Fig. 5.2,3; $F_{1,5}$ =14.50, p=0.013) than did infections with $DK_{ancestral}$. Thus, serial passage resulted in more virulent parasites which were less easily controlled by immunologically naive mice. The AMA-1 nucleotide sequence of the $DK_{ancestral}$ and the $DK_{derived}$ genotype were unaffected by serial passage, with identity at all nucleotides (Fig.5.4)

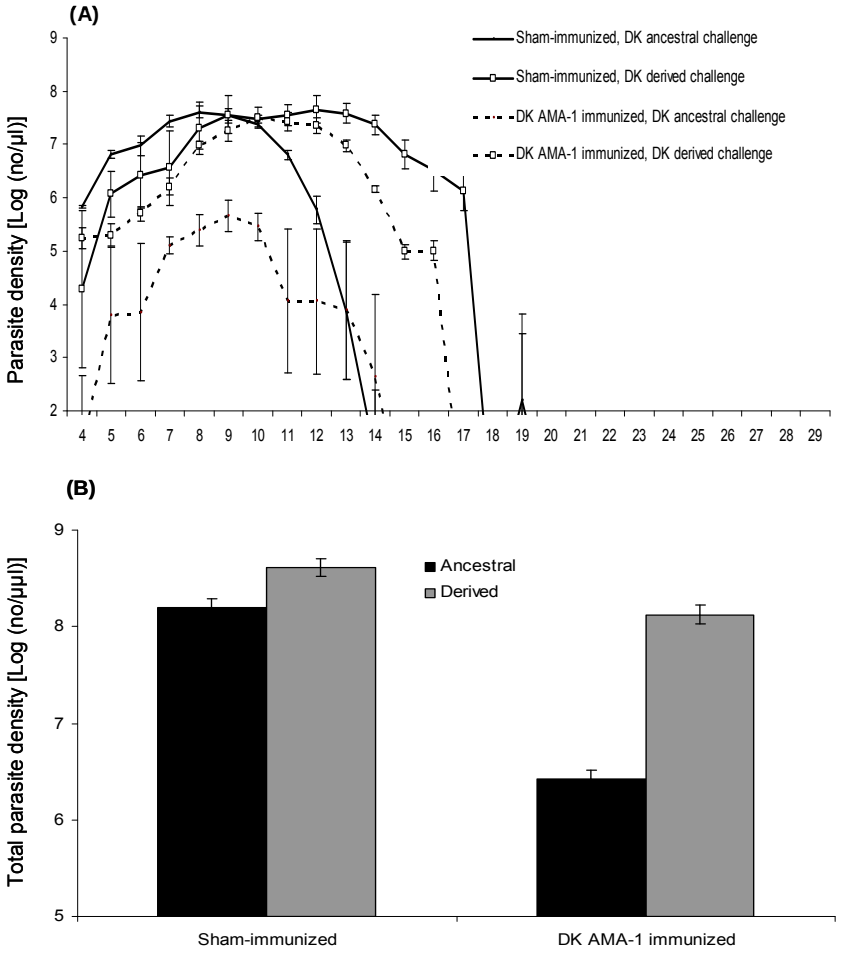

Figure 5.1. Effect of *Plasmodium chabaudi* infection ($DK_{ancestral}$ or $DK_{derived}$) and immunization (sham control or recombinant DK AMA-1) on the kinetics of total asexual parasite density. Lines (A) represent the change in parasite density over time. Each line represents the mean of 4 mice (+/- 1 s.e.m) that were sham-immunized and infected with $DK_{ancestral}$ (solid black line), or $DK_{derived}$ (solid black line open square), or immunized with recombinant DK AMA-1 and infected with $DK_{ancestral}$ (dotted black line), or $DK_{derived}$ (dotted black line open square). The bar graph (B) represents the total parasite density reached during infection with genotype $DK_{ancestral}$ (black bars) or $DK_{derived}$ (grey bars). Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m).

Parasite density kinetics under AMA-1 immunization and in sham-immunized control mice are illustrated in Fig. 5.1. Immunization reduced parasite densities for the ancestral and derived genotypes ($F_{1,6}$ = 190.60, p =<0.001 and $F_{1,6}$=20.24,

p=0.004 respectively), but the extent of the reduction differed between them (immunization x infecting parasite interaction: $F_{1,12}$ = 53.61, p =<0.001), with the protection less effective against $DK_{derived}$ (Fig. 5.1). Thus, serial passage generated parasites which were less well controlled by immunization with recombinant homologous AMA-1 than were the parasites from which they were derived.

The kinetics of red blood cell density and weight loss of the ancestral and derived genotype under AMA-1 immunization and in sham-immunized mice are illustrated in Figures 5.2 and 5.3. Immunization protected against anaemia (Fig. 5.2A,3A; $F_{1,12}$ = 8.65, p=0.012), and the magnitude of protection was similar against $DK_{ancestral}$ and $DK_{derived}$ (immunization x infecting parasite interaction $F_{1,11}$ = 0.37, p= 0.55). Immunization also protected against weight loss (Fig. 5.2B,3B; $F_{1,12}$ =7.61, p=0.017), almost more effectively against the ancestral genotype than against the more virulent derived line (immunization x infecting parasite interaction: $F_{1,11}$ = 4.71, p=0.053). Thus, in contrast to the anti-parasite protection, AMA-1 immunization was equally protective against anaemia induced by the two lines. Protection against weight loss, on the other hand was more ambiguous, being almost significantly more effective against the ancestral genotype.

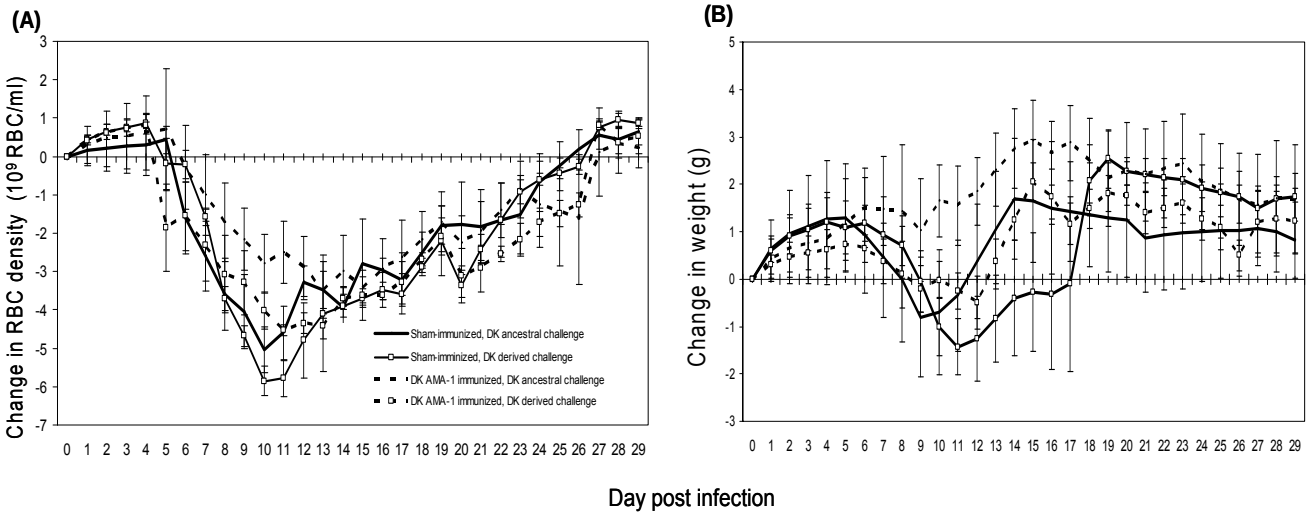

Figure 5.2. Legend on next page

Figure 5.2. Effect of *Plasmodium chabaudi* genotype infection ($DK_{ancestral}$ or $DK_{derived}$) and immunization (sham or with recombinant DK AMA-1) on red blood cell density and weight kinetics. Lines represent the change in RBC density (A) and weight (B) over time. Each line represents the mean of 4 mice (+/- 1 s.e.m) that were treated with a control sham-immunization and infected with $DK_{ancestral}$ (solid black line) or $DK_{derived}$ (solid black line open square), or immunized with DK AMA-1 and infected with $DK_{ancestral}$ (dotted black line), or $DK_{derived}$ (dotted black line open square).

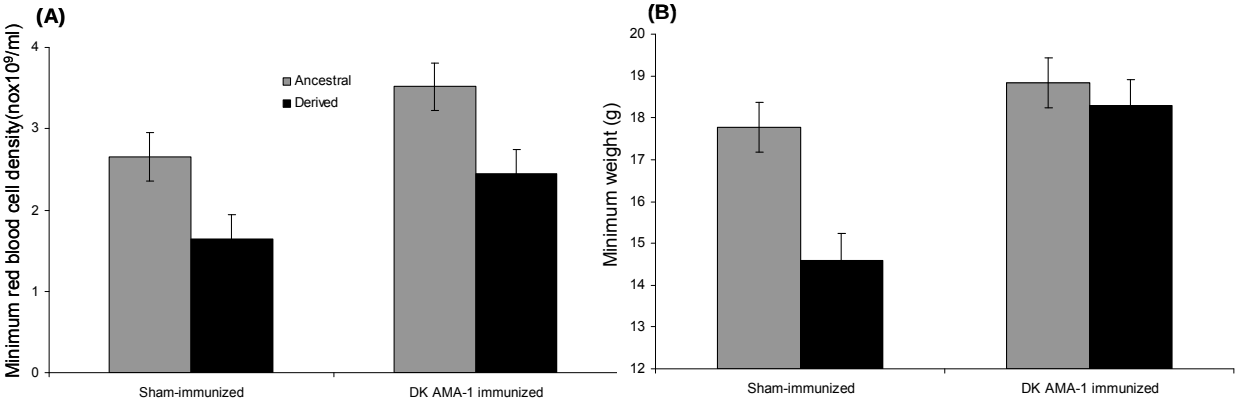

Figure 5.3. Minimum red blood cell density, and minimum weight reached during infection with $DK_{ancestral}$ or $DK_{derived}$ in sham-immunized or DK AMA-1 immunized mice. Each bar represents the minimum red blood cell density (A) or minimum weight (B) reached during infection with $DK_{ancestral}$ (black bars) or $DK_{derived}$ (grey bars). Each bar represents the least squares mean of up to 6 mice (+/- 1 s.e.m).

```
DKancestral
DYRIPSGKCPVMGKGITIQNSKVSFLTRVATGNQKVREGGLAFPQTDVNISPITIDNLKL 60
DKderived
DYRIPSGKCPVMGKGITIQNSKVSFLTRVATGNQKVREGGLAFPQTDVNISPITIDNLKL 60

************************************************************

DKancestral
MYKDHKEILALNDMSLCAKHASFYVPGTNVNTAYRHPAVYDKSNKTCYILYVAAQENMGP 120
DKderived
MYKDHKEILALNDMSLCAKHASFYVPGTNVNTAYRHPAVYDKSNKTCYILYVAAQENMGP 120

************************************************************

DKancestral
RYCSNEEDNENQPFCFTPEKKDEYKNLSYLTKNLREDWETSCPNKSIQNAKFGVWVDGYC 180
DKderived
RYCSNEEDNENQPFCFTPEKKDEYKNLSYLTKNLREDWETSCPNKSIQNAKFGVWVDGYC 180

************************************************************

DKancestral
SEYQKKEVHDNKTLLECNQIVFNESASDQPKQYEKHLEDTAKIRRGIVDRNGKLIGEALL 240
DKderived
SEYQKKEVHDNKTLLECNQIVFNESASDQPKQYEKHLEDTAKIRRGIVDRNGKLIGEALL 240

************************************************************

DKancestral
PIGSYRADQVKSKGKGYNWANYDKKTKKCYIFNKKPTCLINDKDFVATTALSSLEEGPQE 300
DKderived
PIGSYRADQVKSKGKGYNWANYDKKTKKCYIFNKKPTCLINDKDFVATTALSSLEEGPQE 300

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DKancestral
SFPCDIYKKKIAEEIKVMNVNRNNNGNDTIKFPRIFISDDKESLNCPCEPTQLTQSTCKF 360
DKdeived
SFPCDIYKKKIAEEIKVMNVNRNNNGNDTIKFPRIFISDDKESLNCPCEPTQLTQSTCKF 360

************************************************************

DKancestral      FVCNCVEKRQFISENNEVEIKDEFKSEYESPINQ 394
DKderived         FVCNCVEKRQFISENNEVEIKDEFKSEYESPINQ 394
                  *********************************
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Figure 5.4. Protein alignment of AMA-1 fragment from $DK_{ancestral}$ and $DK_{derived}$ and. The stars indicate amino acid identity between the two.

5.5. Discussion

In this study, as in many others (Ebert 1998; Mackinnon & Read 2004a; Mackinnon & Read 2004b), serial passage produced parasites that in naïve mice were able to induce more anaemia and weight loss, and achieve higher parasite densities than were the ancestral parasites. The derived lineage was less effectively controlled by immunity induced by AMA-1 vaccination. This decreased sensitivity to vaccination was not associated with the presence at detectable levels of mutations in AMA-1. Thus determinants at sites other than the target of vaccination must affect vaccine efficacy. Whether these are virulence determinants remains to be formally determined. Nonetheless, the data do show that selection imposed by immunity

induced by recombinant antigen immunisation has the potential to increase the frequency of virulent parasites.

In the trial of the Combination B malaria vaccine it was unclear whether parasites which were carrying the more virulent MSP FC27 allele were unaffected by vaccination because of strain-specificity or whether the vaccine was just less effective at controlling those more virulent parasites. This study demonstrates that the latter is possible: immunization may result in a within-host advantage to virulence. My results support the implication from serial passage experiments through whole-parasite immunized hosts (Mackinnon & Read 2004b) and suggest the same selection for virulence may occur with immunity generated by candidate malaria vaccines.

There are two possible within host advantages to virulence in an immunized host: enhanced competitive interactions in mixed infections and/or increased transmission potential. In mixed *P. chabaudi* infections, competitive suppression of a less virulent genotype by a more virulent genotype has now explicitly been shown to be a positive selector for virulence (de Roode et al. 2005; Bell et al. 2006). In one study, competitive interactions were shown to be weakly immune-mediated, raising concerns that vaccination may enhance competitive interactions and thus the force of this selection for virulence (Råberg et al. 2006). However, another study convincingly demonstrated competitive interactions not to be immune-mediated [Chapter 6, Appendix 2 (Barclay et al. 2008b)]. Moreover, direct immunization with AMA-1 found no evidence that competitive suppression was enhanced by vaccination (Grech et al. 2008). These results have suggested that the selection imposed by vaccination may not select for virulence by enhancing competitive interactions. On the other hand higher parasite virulence may result in an increased transmission potential (Frank 1996; Mackinnon et al. 2008). This could be achieved either by increasing the number of transmissible unit forms per day, by reducing immune clearance resulting in a more durable infection, or a combination of both mechanisms (Anderson & May 1982; Mackinnon & Read 2004a; Mackinnon & Read 2004b; Frank & Schmid-Hempel 2008; Schmid-Hempel 2008). In this study I

observed that the derived lineage reached higher parasite densities and persisted for longer compared to ancestral genotype. An explanation for this could be that that the advantage to virulence in an immunized host was avoidance of immune clearance.

However, the result that a more virulent strain may have a selective advantage in immunized hosts may only go some way to explaining the selection observed with the Combination B vaccine trial. For instance, immunization of mice with an AMA-1 antigen derived from a more virulent *P. chabaudi* parasite observed anti-parasitic heterologous protection, independent of virulence (Chapter 3) Furthermore, reciprocal DK AMA-1/DS AMA-1 immunization and parasite challenge trails appeared to induce protective responses that were strain (genotype)-specific [Chapter 2; Chapter 4, Appendix 1 (Barclay et al. 2008b)]. Thus it is possible that both strain-specificity and virulence differences have the potential to mediate vaccine-induced selection and this may depend on a number of factors including the fine specificity of the immune response induced by vaccination, the antigenic identity of the infecting parasites and the nature of the virulence difference.

In this study I saw no difference in AMA-1 sequences between the derived lineage and the ancestral genotype. Thus, serial passage through naïve mice did not select for variants at the AMA-1 locus (Fig. 5.4). Clearly, the derived lineage caused more aggressive infections (Fig. 5.2, 5.3) and was less well controlled under AMA-1 vaccination (Fig. 5.1). Whether these phenotypic changes are heritably stable could be determined by passage through mosquitoes and/or by genomic comparison of the ancestral genotype and the derived lineage. There is evidence for parasite heritable differences associated with virulence. For example, molecular studies in *P. falciparum* have identified a sequence type called 'sy2' associated with rosetting, dominantly expressed in parasite isolates from children with severe malaria (Rowe et al. 1995; Chen et al. 1998; Bull et al. 2005). Parasites sequestering in the placenta and giving rise to pregnancy-associated malaria share a common phenotype (adherence of infected red blood cells to chondroitin sulphate A via PfEMP-1) and some candidate *var* genes responsible for this phenotype have been identified (Salanti et al. 2004; Francis et al. 2007). In the case of the Combination B malaria

vaccine the MSP2 FC27 allele, the type not included in the vaccine formulation, had itself been previously associated with more morbid infections (Engelbrecht et al. 1995; Genton et al. 2002). Population-level association studies for disease severity will be of importance for monitoring selection imposed in future vaccine field trials.

Of notable interest, I found that AMA-1 immunization was stronger against the ancestral genotype densities but equally protective against anaemia. The notion that there are two different types of anti-malarial immunity -immunity against the parasite itself and immunity against disease- is poorly understood on a molecular basis although the distinction is widely appreciated (Schofield & Grau 2005). Mathematical models of epidemiological data suggest that immunity to clinical disease develops faster (and thus earlier in life) than does anti-parasitic immunity. I view the fact that the two types of immunity may develop on different time scales to be of importance on two fronts. First, both the parasitological status of the host as well as measurements of disease should be monitored during vaccine efficacy trials as the outcome of a trail could be interpreted differently depending on the measurement used. Second, mathematical models of virulence evolution caution against malaria vaccines which reduce symptomatic disease while still maintaining parasite transmission (Mackinnon & Read 1999; Gandon et al. 2001; Gandon & Day 2003; Gandon & Day 2007). Such vaccines are expected to select for more virulent strains as the cost of virulence (host death) is removed by vaccination. Those evolved strains are expected to cause more severe morbidity if transmitted to an unvaccinated host. Since the work presented here and that by others (Mackinnon & Read 2004b; Grech et al. 2008 *in prep*) suggest that more virulent parasites may have a selective advantage in immunized hosts, one should be aware that the selection imposed by vaccination on individuals may have important evolutionary consequences for future host population disease morbidity. Evolved virulence in response to vaccination has been described for two poultry diseases: Marek's disease and Infectious Bursal Disease (van den Berg et al. 2000; Witter 2001; Davidson & Nair 2004; Rautenschlein & Haase 2005; Nouen et al. 2006). In both these diseases failed vaccines were undermined by strains that were more virulent.

In these experiments I looked at whether heterogeneity induced by immunization affected selection for virulence. Of course, heterogeneity exists on a number of levels which may influence how any within-host and between-host selection for virulence plays out (Garamszegi 2006; Williams & Day 2008). For example, host susceptibility can depend on a range of factors including age, gender, nutritional status and concomitant infection with other pathogens (Williams & Day 2008). Thus, considering all aspects of host-pathogen interactions will be of importance in predicting pathogen evolution.

6. $CD4^+$ T cells do not mediate within-host competition between genetically diverse malaria parasites

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6.1. Abstract

Ecological interactions between microparasite populations in the same host are an important source of selection on pathogen traits such as virulence and drug resistance. In the rodent malaria *Plasmodium chabaudi* in laboratory mice, parasites which are more virulent can competitively suppress less virulent parasites in mixed infections. There is evidence that some of this suppression is due to immune-mediated apparent competition, an immune response elicited by one parasite population suppress the population density of another. This raises the question whether enhanced immunity following vaccination would intensify competitive interactions, thus strengthening selection for virulence in *Plasmodium* populations. Using the *P. chabaudi* model, I studied mixed infections of virulent and avirulent genotypes in mice depleted of CD4+ T cells. Enhanced efficacy of CD4+ T-cell dependent responses is the aim of several candidate malaria vaccines. I hypothesized that if immune-mediated interactions were involved in competition, removal of CD4+ T cells would alleviate competitive suppression of the avirulent genotype. Instead, I found no alleviation of competition in the acute phase, and significant enhancement of competitive suppression after parasite densities had peaked. Thus the host immune response may actually be alleviating other forms of competition, such as that over red blood cells. My results suggest that the CD4+-dependent immune

response, and mechanisms which act to enhance it such as vaccination, may not have the undesirable affect of exacerbating within-host competition and hence the strength of this source of selection for virulence.

6.2. Introduction

Parasitic infections are often genetically diverse, with hosts concurrently infected by more than one genotype. Crowding, where pathogen populations within a host are suppressed by the presence of competitor strains, can affect the health and infectiousness of individual hosts, as well as the evolution of medically relevant traits such as virulence and drug resistance (Read & Taylor 2001) . For example, selection for increased virulence is expected when a slower growing parasite is out-competed by a faster growing, more virulent parasite (Bremermann & Pickering 1983; van Baalan & Sabelis 1995; Frank 1996; Gandon et al. 2001; Alder & Losada 2002). Similarly, the relative fitness of drug resistant strains, and hence their rate of spread in a population, can be substantially enhanced when co-infecting drug-sensitive competitors are removed by chemotherapy (Bremermann & Pickering 1983; van Baalan & Sabelis 1995; Frank 1996; Hastings 1997 ; Mackinnon & Hastings 1998; Hastings & D'Alessandro 2000; Gandon et al. 2001; Alder & Losada 2002; Hastings 2003; Mackinnon 2005a; Hastings 2006). Analogous evolutionary processes can affect the rate of evolution of epitope variants in response to strain (genotype)-specific vaccination (Lipsitch & Samore 2002; Read & Mackinnon 2008).

Infections with the human malaria parasite *Plasmodium falciparum* frequently consist of more than one genotype (Walliker et al. 1976; Anderson et al. 2000; Awadalla et al. 2001; Jafari et al. 2004), and a variety of epidemiological evidence is consistent with crowding (Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006). In the rodent malaria model *Plasmodium chabaudi* in laboratory mice there is a strong relationship between parasite virulence and crowding such that more virulent genotypes have a competitive advantage (de Roode et al. 2003; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006).

A number of biological mechanisms may underlie competition between genotypes within hosts (Read & Taylor 2001). One of these is immune-mediated apparent competition (Holt 1977), where increasing densities of one pathogen population elicits a host response which suppresses the population of another. T-cell dependent immune-mediated competition has been demonstrated in *P. chabaudi* (Råberg et al. 2006): in nude mice, which cannot produce mature T cells, competition was less severe than in nude mice reconstituted with T cells. Because many malaria vaccines currently under trial are aimed at inducing T cell-dependent responses, that experiment raised the question of whether vaccination might exacerbate in-host competition and thus affect pathogen evolution, for instance by strengthening selection for competitive ability and hence virulence.

The effects of immunity on in-host competition are unlikely to be simple. The immune response to *Plasmodium* infection has both pathogen genotype transcending (non-specific) and genotype specific components. Protection is generally thought to become more specific during later stages of infection (Jarra & Brown 1989; Buckling & Read 2001; Mackinnon & Read 2003; Stevenson & Riley 2004; Martinelli et al. 2005; Cheesman et al. 2006). Thus, in contrast to non-specific immunity, which could generate immune-mediated apparent competition, specific immunity could in principle act to alleviate competition (Råberg et al. 2006). Here I extend the study by Råberg et al. (2006) by focusing on a specific subset of T-cells, in order to further investigate the importance of immunity in determining competitive outcomes within hosts.

T cells can be divided into two major categories, $CD4^+$ and $CD8^+$ cells. It is well established from both experimental animal models and field studies in humans that $CD4^+$ T cells play a pivotal role in the development of blood stage immunity to *Plasmodium* infection (Good & Doolan 1999; Pombo et al. 2002). They are initially required to produce cytokines which amplify the phagocytic and parasitocidal response of the innate immune response and later on to dampen this response to limit immunopathology. As the response becomes more adaptive they are required to help

B cells produce antibodies which are essential for parasite clearance (Urban et al. 2005; Stephens & Langhorne 2006).

Since $CD4^+$ T cells have been described as having such a crucial role in natural immunity to the blood stage of infection, and vaccine programmes strive to mimic and enhance this response (e.g. Stephens and Langhorne 2006), I have begun to investigate the specific role of these cells during competition in mixed infections of *P. chabaudi.* Specifically I looked at the acute phase of infection, where any interactions between the parasite and the host immune response can strongly influence host health (Urban et al. 2005). I chose two parasite genotypes which had been shown previously to differ in competitive ability and compared the extent of competition in immunocompetent and $CD4^+$ T cell depleted mice. I hypothesised two possible scenarios: (i) if T-cell dependent immunity induces a non-specific response, then a numerically subdominant genotype would experience a stronger immune response in a mixed infection than when on its own. Thus, competition should be eased in $CD4^+$ T cell depleted mice; (ii) if the immune response is largely genotype-specific and primarily elicited against the numerically dominant genotype, then $CD4^+$ T cell depletion may exacerbate other forms of competition, such as competition for limited resources such as red blood cells.

6.3. Material and methods

6.3.1 Parasites and hosts

Isolates containing *Plasmodium* were originally collected from wild caught thicket rats (*Thamnomys rutilans*) from different geographical areas of the African continent (Carter and Walliker 1976; Beale et al. 1978). *P. chabaudi chabaudi* were isolated from the blood of rats from the Central African Republic, while *P. c. adami* were isolated from the blood of rats from the Congo (Brazzaville). Laboratory rodents were infected with sub-inoculations of blood and the parasites were genotyped. Genotyped genotypes were stored as frozen stabilites in liquid nitrogen with

subscript codes used to identify their position in clonal history (Beale et al. 1978; Mackinnon & Read 1999b).

In this experiment I used *P. c. chabaudi* genotype AS_{12062} and *P. c. adami* genotype $DK_{108.}$ Herein these genotypes will be referred to as AS and DK. These genotypes were chosen based on their relative virulence and non-lethality. Pilot studies showed that genotype DK achieved higher parasite densities when genotype AS was absent than when AS was present. In contrast, genotype AS was not competitively suppressed by DK. Hosts were inbred female C57BL/6J mice age 6-8 weeks (Harlan England) maintained as described previously (de Roode et al. 2004b).

6.3.2. Depletion of $CD4^+$ T lymphocytes in vivo

A rat monoclonal antibody, GK1.5, was used to deplete $CD4^+$ T cells. A non-depleting rat monoclonal antibody of the same isotype (IgG 14131, Sigma) was used as a control. Experimental mice were injected intra-peritoneally with 500 μg of the appropriate purified antibody in phosphate-buffered saline (PBS) 5 days before parasite challenge, and then by 250 μg 4 days and 1 day before parasite challenge and weekly after challenge.

A fluorescent activated cell sorter (FACS) was used to confirm $CD4^+$ T depletion. 20 μl of blood was taken from a tail snip 1 day prior to parasite challenge and once a week throughout the experiment. Single cell suspensions were made by removing red blood cells using Lympholyte according to the manufactures instructions (Cedarlane, Canada). Approximately 1 x 10^6 cells were then transferred to a round bottomed plate and re-suspended in FACS buffer (PBS with 2% FCS with 0.05% sodium azide) before incubation for 20 minutes at 4^oC with Allophycocyanin (APC) labeled anti-$CD4^+$ antibody (Pharmingen). Cells were washed 3 times in FACS buffer. Samples were collected on a FACSCalibur, and 10,000 live events were collected for the majority of samples. FlowJo (TreeStar, Ca) was used to analyse the data.

6.3.3. Experimental setup and sampling

Groups of five mice were treated as follows: (i) control antibodies and challenged with 10^6 AS parasites; (ii) control antibodies and challenged with 10^6 DK parasites; (iii) control antibodies and challenged with 10^6 AS and 10^6 DK; (iv) anti-CD4 antibodies and challenged with 10^6AS; (v) anti-CD4 antibodies and challenged with 10^6 DK, (vi) anti-CD4 antibodies and challenged with 10^6 AS and 10^6 DK.

Parasites were delivered by intra-peritoneal injection. I used the same dose of each genotype in single and mixed infections (rather than the same total dose in single and mixed infections) because the aim of the study was to compare the performance of a genotype when it is on its own with its performance when it is in a mixed infection. A two-fold difference in infective dose has negligible effects on the population dynamics of the parasite (Timms et al. 2001). In addition I included 2 extra control groups, each of two mice, which were not challenged with malaria, one group treated with anti-CD4 antibodies and one group with control antibodies. These mice were used to check that $CD4^+$ T cell depletion was continuous throughout the experiment as the number of peripheral T cells are lower than normal during the acute stage of disease (Hviid et al. 1997).

During the course of infection I measured body weights and took blood samples from the tail to make Giemsa-stained blood smears and to estimate RBC density (by flow cytometry; Beckman Coulter) and for genotype-specific real-time quantitative PCR (qPCR) assays.

One mouse died during the experiment ($CD4^+$ T cell depleted, mixed infection) and was included in the analyses only where possible. For unknown reasons, two infections (both non-depleted, a DK-only and an AS-only) achieved a peak parasite density two orders of magnitude lower than all others, and these were excluded from all analyses.

6.3.4. Quantitative PCR

Samples were taken in the morning as this is the stage when most parasites are in the ring or early trophozoite stage in the peripheral blood (de Roode et al. 2004). From each mouse, 5 µl of tail blood was taken and added to 100µL of citrate saline on ice. Samples were subsequently pelleted by centrifugation and the citrate saline was removed. Blood was stored at -80^{o}C until required. DNA extraction was performed using the BloodPrepkit (Applied Biosystems) on the ABI prism 6100 Nucleic Acid prep-station according to manufacturer's instructions. DNA was eluted in a total volume of 200 µL and stored at -80^{o}C until quantification. Genotype-specific qPCR was performed as described previously (Bell et al. 2006) with the addition of the DK specific reverse primer: 5' AGG CAT GTT TTG CAC ACA ATG A 3'.

6.3.5. Trait definition and statistical analyses

I define competitive suppression to be a reduction of parasite numbers when another genotype is present, which I tested for by comparing the performance of a genotype in single and mixed infections. Performance was measured as the clonal density summed over a defined time period. *P. chabaudi* has a 24 hour replication cycle, so the total number of parasites present in any period can be estimated by summing the daily parasite counts. Thus, to test whether competitive suppression was CD4+ T-cell mediated, I asked, for each genotype, whether the magnitude of any competitive suppression differed between intact control and CD4+ T cell-depleted hosts; that is, whether there was a statistical interaction between immune treatment (intact control vs CD4+ T cell-depleted hosts) and infection type (single vs mixed).

The effects of competition and CD4+ depletion on the performance of individual genotype and red blood cell density were first examined by using general linear models (GLM) in the statistical package MINITAB (release 14, MINITAB Inc.). For GLM analysis, response variables included mean total parasite density and mean RBC density, with initial RBC as a covariate. Explanatory variables for GLM

included CD4+ depletion (depleted or intact control) and competition (genotype alone or in mixed infection). Maximal models (response variable = CD4+ depletion + competition + all higher order interactions) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. Second, we used repeated measures analyses, which take into account the importance of day post-infection. These analyses were performed as described by Råberg et al. (2006) using the statistical package SAS 9.1 (SAS Institute 1999). Briefly, the analyses were performed with PROC MIXED, using the REPEATED statement (subject=mouse), the Satterthwaite approximation of the denominator degrees of freedom, and the autoregressive covariance structure AR(1). Within each treatment group, the peak day varied ± 2 days, presumably as a result of slight differences in inoculation dose. To control for this variation, I centered the peak day at the median peak day within each treatment group. All density data was transformed using [log10 (density + 10)].

6.4. Results

Mice treated with the anti-CD4+ T cell antibody were successfully depleted of CD4+ T cells, both prior to parasite challenge and during the whole course of the experiment (Fig. 6.1). CD4+ T cell depletion resulted in more parasites of both genotypes (Fig.6.2 A,B; Table 1).

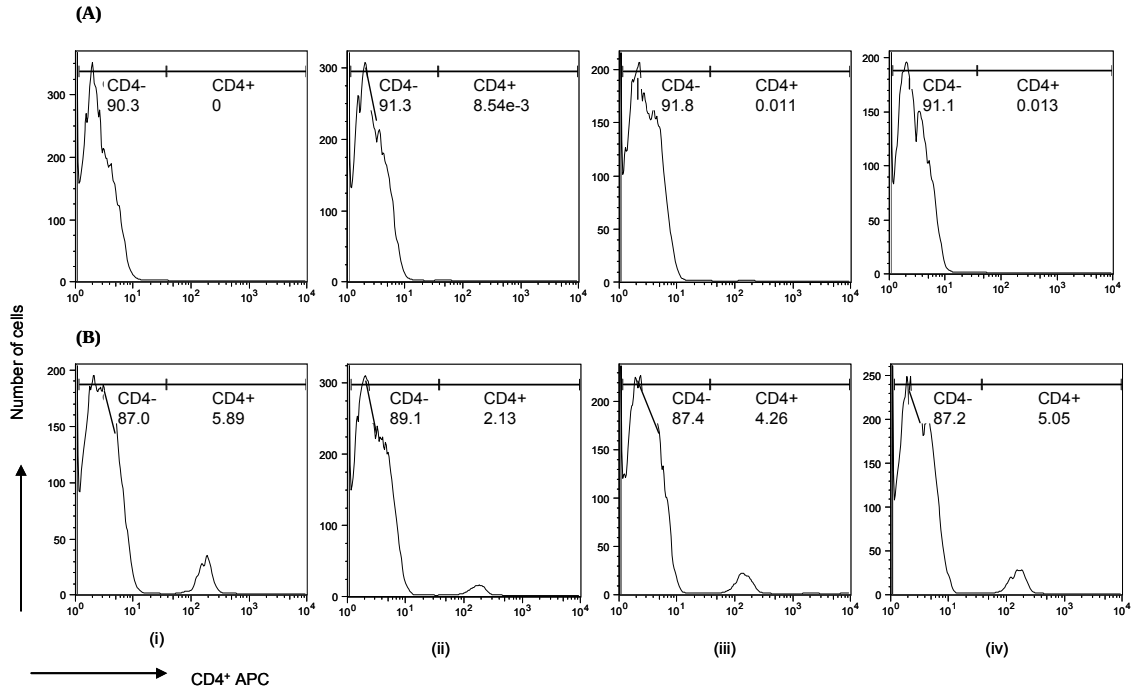

Figure 6.1. FACS plots of number of cells analyzed and percentage of $CD4^+$ T cells in (A) $CD4^+$ T cell depleted and (B) intact control mice. Percentage of $CD4^+$ T cells was analyzed: (i) one day before parasite challenge and; (ii-iv) once a week throughout the experiment. Each graph is a representative of one mouse from either the $CD4^+$ T cell depleted or immuno-competent control group.

6.4.1. Genotype DK

As found with other pairs of genotypes (de Roode et al. 2003; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006), I found here that the relatively avirulent genotype was competitively suppressed by the more virulent genotype, with DK achieving lower parasite densities when AS was present than when it was absent (Fig. 6.2A; Table 6.1). However, the extent of competitive suppression of genotype DK was similar regardless of $CD4^+$ T cell depletion (Table 6.1; depletion x competition interaction n.s.). Thus, there was no evidence that the competitive

suppression of the total number of DK parasites present in an infection was mediated by $CD4^+$ T cell dependent immunity.

However, repeated-measures analysis of the period where $CD4^+$ T cell depletion affected parasite densities (day 6 onwards) showed a weak but significant three-way depletion x competition x day interaction (Table 6.2). To investigate this further, and following Råberg et al (2006), I divided the data into three parts: days 6-8; days 9-11; and days 12-14, and repeated the analyses with each of these (Fig. 6.2A,C-E; Table 6.3). During each of these time periods there were significant depletion x competition or depletion x competition x day interactions. Inspection of Fig. 2A and C shows that the three-way interaction in the first period is a very weak effect from which it is difficult to conclude much, given the rapid alterations in infection kinetics during that period caused by depletion. In the other two periods, there are significant competition x depletion interactions (Fig. 6.2D,D; Table 6.3), with more severe competitive suppression in $CD4^+$ T cell depleted mice than in control mice. Thus, there was no evidence that competitive suppression is $CD4^+$ T cell-mediated: once the initial wave of parasitaemia began to subside, competitive suppression was exacerbated rather than alleviated in $CD4^+$ depleted mice.

6.4.2. Genotype AS

There was no evidence of competitive suppression of AS by DK, irrespective of immune treatment (Fig. 6.2B; Table 6.1). Repeated-measures analysis from day 6 onwards, when $CD4^+$ depletion had an effect, revealed no evidence of interactions between depletion and competition (Table 6.2). However for comparison with the analysis of genotype DK, I repeated the same analyses for AS on days 6-8, 9-11 and 12-14 (Table 6.4). In none of these time periods was there any evidence of competitive suppression (in all cases, competition main effect, and depletion x competition, $p>0.15$).

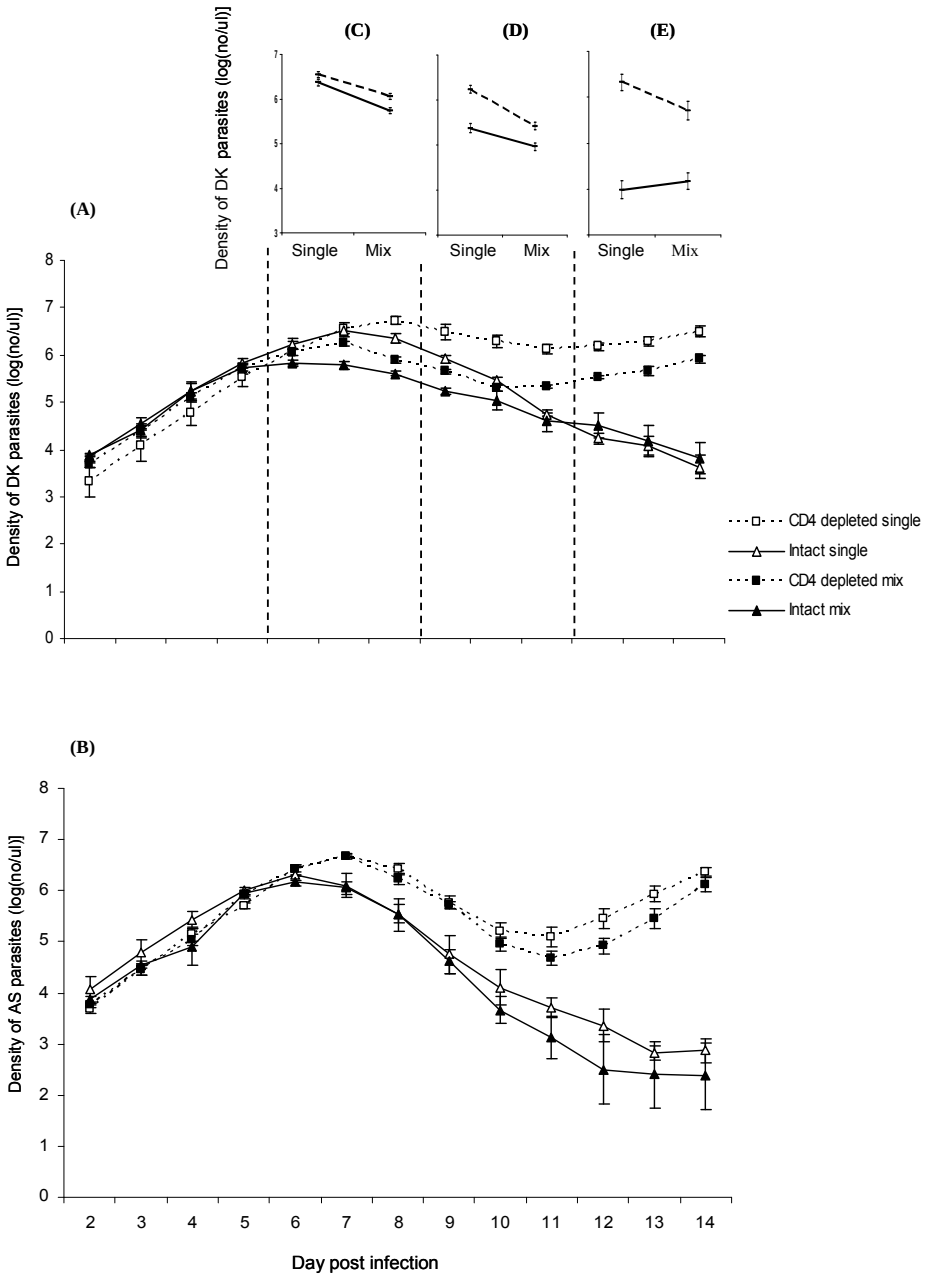

Figure 6.2. Parasite densities through time of (A) genotype DK and (B) genotype AS, and (C-E) average densities of DK during the periods denoted by the vertical dotted lines. Asexual density from qPCR of $CD4^+$ T cell depleted mice and intact control (normal) mice with single infections in $CD4^+$ T cell depleted mice and intact control (normal) mice with mixed infections. Mean densities (± 1 S.E.M) were calculated from all mice which were alive on the respective day of sampling (up to five). The interaction plots (C-E) show total numbers of DK parasites when the competitor genotype AS is absent (single infection) or present (mixed infection) in CD4+ depleted mice (dotted lines) and normal animals (solid

lines). Competition x depletion interactions: (c) $F_{1,16} = 1$, P=0.33, (d) $F_{1,17} = 5.11$, P=0.0372, (e) $F_{1,14} = 4.57$, P=0.0499.

	DK days 2-14			AS days 2-14		
Effect	df	F	P	df	F	P
$CD4^+$ depletion	1,15	82.7	<0.001	1, 15	93.36	<0.000
Competition	1,15	84.2	<0.001	1, 15	2.05	0.17
$CD4^+$ depletion x competition	1,15	0.6	0.74	1, 15	0.94	0.94

Table 6.1. GLM analyses of the effects of $CD4^+$ T cell depletion, competition (presence/absence of co-infecting clonal genotype) and their interaction on the total number of parasites across the course of infection for parasite genotype DK and AS.

	DK days 6-14			AS days 6-14		
Effect	df	F	P	df	F	P
$CD4^+$ depletion	1, 16	74.52	<.0001	1, 17	51.58	<.0001
Competition	1, 16	17.92	0.0006	1, 17	1.86	0.19
Day	8, 101	30.80	<.0001	8, 108	40.85	<.0001
$CD4^+$ depletion x competition	1, 16	2.65	0.12	1, 17	0.02	0.88
$CD4^+$ depletion x day	8, 101	22.35	<.0001	8, 108	15.67	<.0001
Competition x day	8, 101	3.67	0.0009	8, 108	0.91	0.51
$CD4^+$ depletion x competition x day	8, 101	2.10	0.042	8, 108	0.47	0.87

Table 6.2. Repeated measure analyses of the effects of $CD4^+$ depletion, competition (presence/absence of coinfecting clonal genotype) and day post-infection on the daily densities of the two parasite genotypes DK and AS for days 6-14 post-infection.

Clone DK		Days 6-8			Days 9-11			Days 12-14	
Effect	df	F	P	df	F	P	df	F	P
$CD4^+$ depletion	1, 16	12.61	0.0026	1, 17	54.79	<.0001	1, 14	105.95	<.0001
Competition	1, 16	62.31	<0.001	1, 17	47.53	<.0001	1, 14	1.33	0.26
Day	2, 29	15.32	<0.001	2, 30	39.12	<.0001	2, 28	2.22	0.12
$CD4^+$ depletion x competition	1, 16	1.00	0.33	1, 17	5.11	0.037	1, 14	4.57	0.049
$CD4^+$ depletion x day	2, 29	2.88	0.071	2, 30	11.87	0.0002	2, 28	23.78	<.0001
Competition x day	2, 29	12.34	0.0001	2, 30	2.32	0.11	2,28	0.64	0.53
$CD4^+$ depletion x competition x day	2, 29	3.78	0.034	2, 30	2.30	0.11	2,28	0.17	0.84

Table 6.3. Repeated measure analyses of the effects of $CD4^+$ depletion, competition (presence/absence of coinfecting clonal genotype) and day post-infection on the daily parasite density of genotype DK for days 6-8, 9-11 and 12-14 post-infection.

Clone AS		Days 6-8			Days 9-11			Days 12-14	
Effect	df	F	P	df	F	P	df	F	P
$CD4^+$ depletion	1, 14	17.58	0.0008	1, 15	31.83	<0.001	1, 14	58.71	<0.001
Competition	1, 14	0.3	0.58	1, 15	2.08	0.16	1, 14	1.60	0.22
Day	2, 28	39.82	<0.001	2, 29	89.06	<0.001	27	6.52	0.0049
$CD4^+$ depletion x competition	1, 14	0.01	0.91	1, 15	0.11	0.74	1, 14	0.03	0.86
$CD4^+$ depletion x day	2, 28	12.17	0.0001	2, 29	3.03	0.06	27	15.34	<0.001
Competition x day	2, 28	0.22	0.80	2, 29	3.13	0.058	27	0.76	0.47
$CD4^+$ depletion x competition x day	2, 28	1.20	0.31	2, 29	0.08	0.92	27	0.70	0.50

Table 6.4. Repeated measure analyses of the effects of $CD4^+$ depletion, competition (presence/absence of coinfecting clonal genotype) and day post-infection on the daily parasite density of genotype AS for days 6-8, 9-11 and 12-14 post-infection.

6.4.3. Red blood cells

Red blood cell density over time for the different treatment groups are shown in fig 3. Uninfected red blood cells form an important resource for malaria parasites. To assess whether the potential for competition over this resource differed between $CD4^+$ depleted and intact control mice I compared the red blood cell densities in mice with mixed infections. Repeated measures analysis of days 6-14 revealed that CD4+ depleted mice had significantly lower red blood cell densities during this time period (Fig. 3C: $F_{1,34.7}$=4.35, p=0.045). There was also a significant depletion x day interaction ($F_{8,94.1}$=2.58, p=0.014). Separate analyses of days 6-8, 9-11, and 12-14 showed that the difference in RBC density was most pronounced during days 9-14 (Table 6.5).

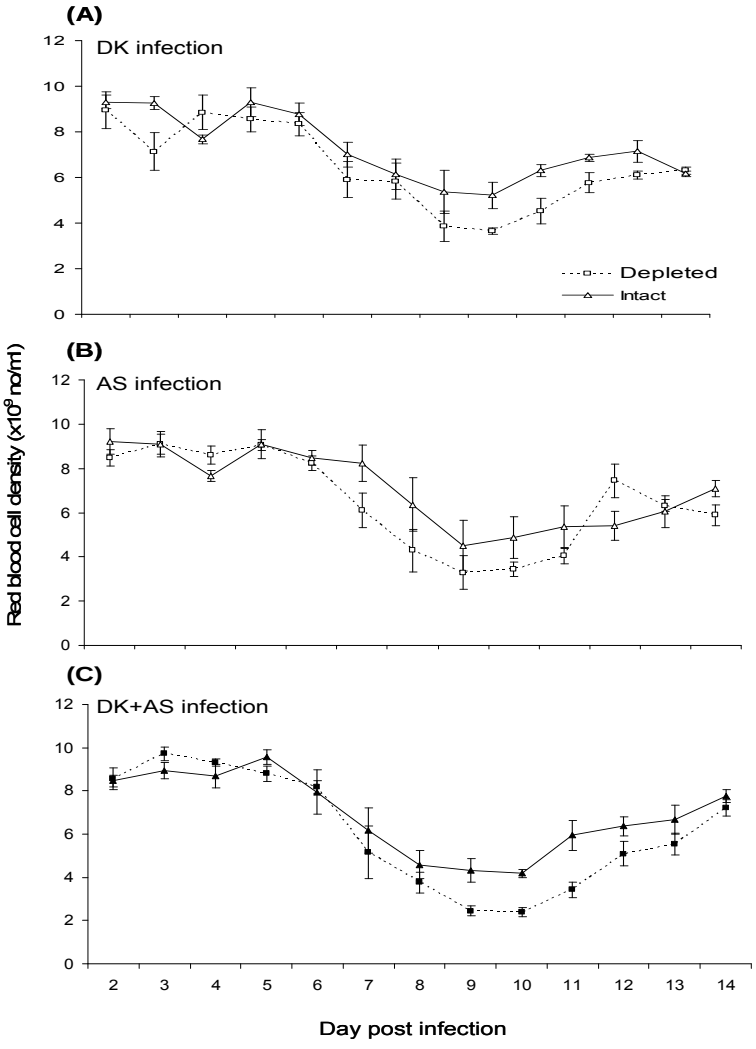

Figure 6.3. Legend on next page

Figure 6.3. Mean red blood cell densities (± 1 S.E.M) over time. (A) $CD4^+$ T cell depleted and intact mice infected with DK. (B) Depleted and intact mice infected with AS. (C) Depleted and intact mice infected with DK and AS.

		Days 6-8			Days 9-11			Days 12-14	
Effect	df	F	P	df	F	P	df	F	P
$CD4^+$ depletion	1, 21	1.77	0.14	1, 23	10.75	0.0033	1, 19	4.57	0.045
day	2, 36	42.18	<0.001	2,37	11.98	<.0001	2,38	0.33	0.72
$CD4^+$ depletion x day	2, 26	3.18	0.05	2, 37	0.65	0.52	2, 28	0.08	0.92

Table 6.5. Repeated measure analyses of the effects of $CD4^+$ depletion and day post-infection on the mean red blood cell density in mixed infections during days 6-8, 9-11 and 12-14.

6.5. Discussion

I found no evidence that $CD4^+$ T cells enhanced competition during mixed genotype infections with *P. chabaudi* (Fig. 6.2A; Table 6.1). Specifically, during the peak stages of acute infection (days 6-8) suppression was independent of $CD4^+$ T cells (Fig. 6.2C; Table 6.3). After the peak of infection (day 9+), $CD4^+$ T cells acted to alleviate competition, such that upon their removal competitive suppression was enhanced (Fig. 2D, E; Table 6.3). In addition, the presence of $CD4^+$ T cells did not cause suppression of the dominant genotype (Fig. 6.2B; Table 6.4).

The immune response to *Plasmodium* infection has both pathogen genotype transcending (non-specific) and genotype specific components, with protection becoming more specific during later stages of infection (see Introduction). Here I found that after the peak of acute infection (day 9 onwards), there was no competitive suppression of DK parasites in intact control mice, whereas in $CD4^+$ T cell depleted mice there was still evidence of competition (Fig. 6.2D,E; Table 6.3). Both genotypes did better in depleted mice, probably because of an impaired early antibody production through lack of T cell help and possibly the reduced recruitment and activation of macrophages for the uptake of infected cells. Thus, in normal hosts, a largely genotype-specific adaptive immune response towards a numerically dominant genotype may act to alleviate competition by regulating clonal genotype populations and limiting other forms of competition, for example competition for red blood cells.

During the peak of infection (days 6-8), competition was $CD4^+$ T cell-independent so that the extent of competitive suppression of genotype DK was similar in intact control and $CD4^+$ T cell depleted mice (Fig. 6.2A,C; Table 6.3). A number of biological mechanisms could be the proximate cause of competitive suppression during the peak of infection. First, there may be direct interference between two infecting strains. This has not yet been demonstrated in any parasites, but pathogenic bacteria can produce allelopathic substances that actively suppress competitors (Riley & Gordon 1999) and competing viruses can produce interference molecules (Hart & Cloyd 1990). Second, competition may be influenced by non-specific components of the innate immune response ($CD4^+$ T cell-independent). Third, there may be competition for resources as genotypes infecting mice simultaneously must divide the available red blood cells and other resources such as blood glucose between them (Hellriegel 1992; Hetzel & Anderson 1996; de Roode et al. 2005b; Gurarie et al. 2006). $CD4^+$ T cell depleted mice were more anaemic than control mice (Fig. 6.3; Table 6.5), so that if red cells are limiting, there is more potential for competition for that resource in depleted mice. Mathematical models have suggested that during mixed-infection the proximate cause of competitive advantage may be attributable to an earlier and wider red blood cell preference of dominant genotypes

(Hellriegel 1992; Gravenor et al. 1995; McKenzie & Bossert 1997; Jakeman et al. 1999; Mason & McKenzie 1999; McQueen et al. 2004; Antia et al. 2007). Because these predictions are based on data from rodent malaria, they could be tested directly by transferring red blood cells of different ages into a single mouse and determine their loss following infection, or indirectly by measuring competition in untreated mice, and mice treated with erythropoietin (Suzuki et al. 2006).

My conclusion that competition is not $CD4^+$ T cell mediated apparently contradicts the recent finding of T-cell mediated apparent competition (Råberg et al. (2006). In that study, the authors looked at mixed infections with *P. chabaudi* in nude mice (which lack the ability to produce mature T cells) and compared the extent of competition with that in nude mice re-constituted with T cells. There was still pronounced competition in all animals, but there was some alleviation of competitive suppression in nude mice towards the end of the acute phase of infection, when the initial wave of parasitaemia was waning. This period corresponds roughly to days 9-14 in fig 2. A number of experimental differences could explain the contrasting results of Råberg et al. (2006) and the present study. First, different mouse strains were used in the two studies and host genotype has previously been shown to quantitatively affect the outcome of competition (de Roode et al. 2004b). Second, different pairs of genotypes were used, and *P. chabaudi* genotypes can induce different levels of strain-specific immunity (Cheesman et al. 2006). Third, there was a difference in the method used to modulate T cell-dependent immunity. Nude mice lack the ability to produce any mature T cells, including both $CD4^+$ and $CD8^+$ T cells. The role of $CD8^+$ T cells during malaria infection in mice is still unclear (Lamb et al. 2006), but it could be that they are involved in the relatively small component of competition which was shown to be immune-mediated competition in reconstituted nude mice (Råberg et al. 2006). In addition, the repertoire of serum antibodies (including both natural antibodies and antigen elicited antibodies) in the $CD4^+$ T cell depleted mice will be different from that in nude mice. Nude mice grow up producing only T-cell independent antibodies, while in the $CD4^+$ T cell depleted mice there will be both T-cell independent and persisting T-cell dependent antibodies

(produced by existing plasma cells in the bone marrow) and these may cross react with the parasite.

Taken together, the present study and that of Råberg et al. (2006) show that the effect of T-cell dependent immunity on competition is relatively weak, and may be either positive or negative depending on specific details of host and parasite. Rather than further dissection of any immune mechanism mediating competition, one could use this malaria model system to look at the strength of competition in hosts immunized by a variety of different candidate vaccines towards the blood stage of infection. Meanwhile, the result I report here suggest that vaccines which enhance $CD4^+$-dependent immunity will not increase the selection in favour of virulence arising from in-host competition.

7. Vaccine-induced strain specific immunity does not enhance competitive interactions between genetically diverse malaria parasites

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7.1. Abstract

Given that a large majority of infections with human malaria in the field constitute more than one genotype, it is likely that competitive interactions, where more virulent genotypes competitively suppress less virulent genotypes, are a driving force for virulence in natural *Plasmodium* populations. Medical intervention strategies such as drugs and vaccination are likely to alter parasite interactions and thus the force of this selection for virulence. The malaria vaccine candidate Apical Membrane Antigen 1 (AMA-1) has a number of polymorphisms, so that immunization induces protective responses that are strain-specific. A proposed solution to overcome strain-specificity has been to design vaccines which contain more than one allelic form of AMA-1 (multi-allelic vaccines). AMA-1 vaccination could affect the rate of virulence evolution if strain-specificity enhances the competitive suppression of a less virulent genotype, or the competitive release of a more virulent genotype. In this study, I investigated how vaccine-induced strain-specific immunity altered within host competition between genetically diverse *P.*

chabaudi infections. I also tested whether a bi-allelic AMA-1 vaccine may alter such interactions. I found no evidence that any type of immunization altered within-host competitive interactions. The results from this study further suggest that the competitive benefits associated with virulence should not result in a selective advantage in an immunized host.

7.2. Introduction

Within-host competition among different parasite genotypes is predicted to affect the epidemiology and evolution of many important parasite traits such as virulence, drug resistance and vaccine escape (Read & Taylor 2001). For instance, competitive interactions within hosts alter the costs and benefits of drug resistance and hence the spread of resistance in a population. In the absence of drug treatment, small costs of resistance can be magnified if they result in competitive suppression; in the presence of drug treatment, survival benefits of resistance can be magnified when sensitive competitors are removed. (Hastings 1997 ; Hastings & D'Alessandro 2000; Hastings 2003; Mackinnon 2005a; Hastings 2006; Wargo et al. 2007)). Competitive interactions also affect the evolution of virulence (Bremermann & Pickering 1983; van Baalan & Sabelis 1995; Frank 1996; Gandon et al. 2001; Alder & Losada 2002). This is especially so if more virulent strains have higher competitive ability. Vaccination programmes have the potential to alter the strength of this force of selection for virulence, potentially weakening or strengthening it. If competition is immune-mediated, vaccination could make competition more intense. Alternatively, by suppressing parasite densities, immunization could weaken resource-based competition. These forces would have alternate effects on virulence evolution (Read & Mackinnon 2008). In contrast, if vaccination has a strain (genotype)-specific component, it could facilitate the performance of heterologous genotypes (Lipsitch & Samore 2002), which need have little to do with virulence. Here I experimentally test which of these occurs following AMA-1 immunization.

In nature, human infections with malaria are often genetically diverse (Babiker et al. 1991; Conway et al. 1991; Arnot 1998; Babiker et al. 1999; Smith et al. 1999a;

Tanner et al. 1999; Jafari et al. 2004). Mixed infections can arise due to the bite of a single mosquito inoculating more than one genotype or via successive bites from multiple mosquitoes infected with different genotypes. A variety of non-experimental observations imply that competition occurs in human malaria (Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith et al. 1999b; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006). In the rodent malaria model *Plasmodium chabaudi* virulence is strongly correlated with competitiveness (Taylor et al. 1997; de Roode et al. 2003; de Roode et al. 2004a; de Roode et al. 2004b; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006; Råberg et al. 2006). In this system, avirulent genotypes tend to suffer more than virulent genotypes in mixed infections; their replicative and transmission stage densities are severely suppressed when co-infecting strains are present.

The impact of vaccination on within-host competition and hence virulence evolution could be better predicted if we knew the factors mediating competition. In theory, competitive interactions between different genotypes could be mediated by a number of different biological processes, including the host immune response (Holt 1977; Read & Taylor 2001). To date, one experimental study has indicated that competition could be immune-mediated (Råberg et al. 2006). In those experiments competitive suppression of an avirulent genotype was significantly less in nude mice, which cannot produce mature T cells, than in control mice. That study raised the question of whether current vaccination programmes, which are aimed at inducing T cell-dependent responses, might exacerbate this source of selection for virulence. Consistent with this, serial passage (i.e. the syringe transfer of malaria infected blood from one host to another) of *P. chabaudi* through whole-parasite immunized hosts increased virulence more rapidly than serial passage through immunologically naïve mice (Mackinnon & Read 2004a). Furthermore, more virulent *P. chabaudi* have been shown to be less well controlled by the malaria vaccine candidate Apical Membrane Antigen 1 (AMA-1) (Grech et al. 2008 *in prep*). An implication of those studies was that immunization may enhance the selective advantage of high virulence.

However, there are also two studies showing that competition may not be immune-mediated. First, competitive interactions were not alleviated during experiments which depleted mice of $CD4^+$ T cells, a subset of the immune response identified as playing a pivotal role in protection against malaria [Chapter 6, Appendix 2 (Barclay et al. 2008a)]. In that study competitive suppression of the avirulent genotype was exacerbated in the absence of CD4 T cells, suggesting CD4 T cells may be alleviating other forms of competition, such as that for red blood cells. That study highlighted that CD4+-dependent immune responses, and mechanisms that act to enhance it such as vaccination, may not have the undesirable affect of exacerbating within-host competition. Second, a direct immunization study also failed to find to any evidence that vaccination might exacerbate competitive interactions (Grech et al. 2008). In that study immunization of mice was either with the malaria vaccine candidate AMA-1 or with live parasite. Neither type of immunization altered the strength of selection within mixed infections in favour of virulence. In this study I have extended on those previous experiments, where the combination of AMA-1 antigens and parasites genotypes did not involve SSI, to test whether strain-specific immunity induced by immunization with AMA-1 is more likely to facilitate the release of a more virulent genotype in a mixed *P. chabaudi* infection than a less virulent genotype.

AMA-1 is a well-characterized and functionally important merozoite protein and is currently considered a major candidate for a malaria vaccine [reviewed by Remarque et al. (2008a)]. Immunoepidemiological studies have shown that AMA-1 is highly immunogenic in natural infections (Thomas et al. 1994; Rodrigues et al. 2005; Wickramarachchi et al. 2006). Immunization with AMA-1 confers protection against parasite challenge in a number of animal models, probably by inducing antibodies which inhibit invasion (Deans et al. 1988; Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998; Stowers et al. 2002; Healer et al. 2004). However, a number of studies have demonstrated that protection elicited by this vaccine is strain-specific (Crewther et al. 1996; Anders et al. 1998; Hodder et al. 2001; Polley et al. 2003; Cortes et al. 2005; Polhemus et al. 2007). In theory, vaccine-induced strain-

specific immunity could enhance competitive interactions by facilitating the competitive release of more virulent genotypes in a mixed infection.

One approach to minimizing vaccine-induced strain-specificity has been to design vaccines which combine more than one allele of an antigen (Hoffmann & Miller 1996; Bolad & Berzins 2000; Richie & Saul 2002; Polley et al. 2007). A growing body of evidence suggest that multi-allele vaccines do not afford the host more protection from morbidity than do single antigen vaccines (Kennedy et al. 2002; Malkin et al. 2005; Miura et al. 2007) but that multi-allelic vaccines should not enhance the strength of selection for more virulent genotypes [Chapter 4, Appendix 1 (Barclay et al. 2008b)]. What remains to be determined is whether multi-allelic vaccines will alter competitive interactions between diverse genotypes in mixed infections, which we test in this study.

Thus, the aim of this study was to test whether strain-specific immunity, induced by AMA-1 vaccination, exacerbated the competitive suppression of a less virulent genotype, and/or the competitive release of a more virulent genotype in a mixed *P. chabaudi* infection. I also investigated how a bi-allelic AMA-1 vaccine may alter competitive interactions. This paper reports further analyses of the experiment described by Barclay et al. (2008) (Chapter 4, Appendix 1), which were designed to test the within host effects of mono and bi-allelic AMA-1 on host protection and parasite selection. That previously published analysis showed that the immunization protocols generated SSI, but did not test for the effects of this on competition[1].

7.3. Material and methods

[1] This chapter is split off from Chapter 4 both for clarity and because that chapter and this address different issues, and are likely to be for different audiences. Because this chapter is written as a stand alone paper for publication, some repetition of methods is necessary.

7.3.1. Parasites and hosts

P. chabaudi adami (*P.c.a.*) were originally isolated from wild-caught thicket rats (*Thamnomys rutilans*) in the Congo (Brazzaville) (Carter & Walliker 1976). Isolates were genotyped and stored as frozen stabilites in liquid nitrogen with subscript codes used to identify their position in clonal history. In this experiment we used *P.c.a.* genotypes DS_{500} and DK_{122}. Studies by others (Crewther et al. 1996) and my own pilot studies showed that these genotypes differ in virulence during infection of C57BL/6 female mice, with genotype DS generating substantially more parasites and inducing greater weight and red blood cell loss relative to DK [see Chapters 3 and 4]. Hosts were inbred female C57BL/6 mice age 6-8 weeks (Harlan England) maintained as described previously (de Roode et al. 2004a).

7.3.2. Immunizations and isotype ELISA

Here I used an immunization protocol adapted from Anders et al. 1998. Prior to immunization, mice were randomized into four groups of eighteen (Table 4.1). Immunization was with the highly immunogenic ectodomain of the full AMA-1 protein termed AMA-1B. For mono-allelic immunizations (hereafter referred to as DS AMA-1 or DK AMA-1), groups of mice were injected intraperitoneally with 10µg of the appropriate protein emulsified in 100 µl of the adjuvant Montanide ISA 720 (Seppic, France). For bi-allelic immunizations, mice were injected with a mixture of 5µg of both DS and DK AMA-1, giving the same total dose of antigen as for the single antigen immunizations, again emulsified in Montanide ISA 720. Control mice were injected with 100µl emulsion of PBS in Montanide ISA 720. Mice were given a single booster immunization with the same amount of antigen emulsified in Montanide ISA 720 4 weeks after the primary immunization.

	No. mice per immunization	Infecting clone	No. mice per parasite infection	No. deaths	No. euthanized
Sham-immun		DS	6	3	2
Sham-immun	18	DK	6		
Sham-immun		DS+DK	6		
DK AMA-1		DS	6[2]		
DK AMA-1	18	DK	6[2]		
DK AMA-1		DS+DK	6		
DS AMA-1		DS	6	1	
DS AMA-1	18	DK	6[1]		
DSAMA-1		DS+DK	6	1	
Bi-allelic		DS	6	2	1
Bi-allelic	18	DK	6		
Bi-allelic		DS+DK	6		
Total			**72**	7	3

Table 7.1. Experimental design. Immunization was either with DK AMA-1, DS AMA-1, a formulation containing an equal mix of both forms of AMA-1 (bi-allelic), or immunization with adjuvant only ('sham-immunization'). Groups of 18 mice were immunized with one of the four treatments before being separated into groups of 6. Infection was with parasites of genotype (clone) DK alone, genotype DS alone or a mixture of both. During the experiment 7 mice were found dead and 3 had to been euthanized due to severe morbidity. Euthanization was at predetermined levels of morbidity prescribed by animal care protocols. The numbers in brackets highlight those mice that had to be excluded during repeated measures analyses due to unknown reasons.

To ensure that antigen immunization successfully generated antibody responses, and to determine whether there was any cross-reactivity between the antibodies generated to the different immunizing antigens, I first carried out a pilot experiment. A total of 11 mice were immunized with DS AMA-1, 11 with DK AMA-1, and 10 were sham-immunized. We estimated the quantity of IgG2b antigen-specific antibodies in all mice sera 11 days after the booster immunization by ELISA using wells coated with DS AMA-1 or DK AMA-1. Thus the sera from 32 mice were tested in 64 wells. We used IgG2b as previous work in my laboratory showed that C57BL/6 produce this isotype in response to *P. chabaudi* infection (K. Grocock, A. Graham unpublished). Protection induced by immunization with recombinant AMA-1 is isotype independent (Burns et al. 2004). Given the lack of cross-reactivity we observed in this pilot experiment (see Results below), in the main experiment, we measured IgG2b isotype antibodies to each antigen separately only from the sera of mice

immunized with a mixture of DS and DK AMA-1 and in sham-immunized control mice.

In both the pilot and main experiments, sera fractions were separated by centrifugation from 20 µl of blood taken from a tail snip and were stored at -80^{o}C. High binding 96 well ELISA Maxisorb immunoplates (Nunc) were coated with either DS AMA-1 or DK AMA-1 at a concentration of 1 µg /ml in 0.06M carbonate buffer (0.04M $NaHCO_3$, 0.02M $NaCO_3$, pH 9.6) in a final volume of 50ul per well. Plates were stored at 4^{o}C overnight to allow the antigen to bind. Non-specific binding was blocked by incubating wells with 5% BSA: carbonate buffer (200ul/well) for 2 hours at 37^{o}C. Wells were then washed three times in Tris buffered saline with 0.01% Tween 20 (TBST). We used end-point dilution methods to detect IgG2b titres: serum samples were detected in a serial dilution 1/100-1/204800 using TBST as a diluent, in a final volume of 50 µl per well and incubated for 2 hours at 37^{o}C. Wells were washed three times in TBST. HRP conjugated goat anti-mouse IgG2b detection antibody (Southern Biotech 1100-05) was diluted 1/4000 in TBST to a final volume of 50 µl per well. Plates were incubated for 1 hour at 37^{o}C. Wells were washed three times in TBST followed by a final wash in distilled water. ABTS peroxide substrate (Insight Biotechnology) was added at 100 µl per well and allowed to develop at room temperature for 20 minutes. Optical density was read at 405nm using a spectrophotometer. IgG2b isotype antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D.) was greater than the mean (plus 2 standard deviations) O.D values observed for naïve mouse sera assayed against both DS and DK AMA-1 at 1/100.

7.3.3. Parasite challenge and monitoring of within host dynamics

Two weeks after the boost immunization, groups of immunized mice (18 per group) were further randomized into groups of six and challenged with 10^5 parasites of either genotype DS alone, genotype DK alone or a mixture of genotype DS and DK (Table 7.1). I used the same total dose of each genotype in single and mixed infections (rather than the same total dose in single and mixed infections) because the

aim of the study was to compare the performance of a genotype when on its own, with its performance when in a mixed infection. A twofold difference in infective dose has negligible effects on the population dynamics of the parasites (Timms et al. 2001).

I recorded the infection dynamics between days 6 and 14. During that time we daily measured body weights and took blood samples from the tail to estimate red blood cell density (by flow cytometry; Beckman Coulter) and for genotype-specific real-time quantitative PCR (qPCR) assays (Bell et al. 2006). The genotype specific primers for genotype DS and DK are described elsewhere [Chapter 4, Appendix 1 (Barclay et al. 2008b). *P. chabaudi* has a 24 hour replication cycle therefore the total number of parasites can be estimated daily.

7.3.4. Trait definition and statistical analyses

I define competitive suppression to be a reduction of parasite numbers when another genotype is present, and competitive release is improved genotype performance after the removal of a competitor. Evidence for both suppression and release are tested for by comparing the performance of a genotype in single and mixed infections. Thus, for each genotype, we asked whether there was a statistical interaction between immunizing treatment (sham-immunized control versus DK AMA-1, DS AMA-1 or DS+DK AMA-1) and infection type (single versus mixed).

The effects of competition and immunizing treatment on the performance of individual genotype and red blood cell density were first examined by using general linear models (GLM) in the statistical package MINITAB (release 14, MINITAB Inc.). For GLM analysis, response variables included mean total parasite density and mean RBC density, with initial RBC as a covariate. Explanatory variables for GLM included immunizing treatment (sham-immunized control, versus DK AMA-1, DS AMA-1 or DS+DK AMA-1) and competition (genotype alone or in mixed infection). Maximal models (response variable= immunizing treatment + competition + all higher order interactions) were tested in the first instance, and minimal models were

obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. Second, we used repeated measures analyses, which take into account the importance of day post-infection. These analyses were performed as described by Råberg et al. (2006) using the statistical package SAS 9.1 (SAS Institute 1999). All density data was transformed using [log10 (density + 10)] to meet the assumption of normality and homogeneity of variance. Consistent with my previous studies, [Chapter 6, Appendix 1 (Barclay et al. 2008a)] analyses focused on the acute phase of infection (days 4-14) as it is during the phase that competitive effects are most pronounced (de Roode et al. 2005b; Bell et al. 2006).

7.4. Results

The experimental set-up, including immunizing treatments, infecting genotypes and the number of mice that died during the experiment or had to be euthanized due to severe morbidity are described in Table 7.1. Since death always occurred as initial parasitaemias were declining these were included in the calculation of daily densities. Four out of the five control-sham immunized mice infected with genotype DS died by day 10, thus from day 10 onwards this group was removed from the analyses. Because of missing values due to failed PCR, 5 mice and these had to be excluded from the repeated measures analyses. All antigen immunizations reduced parasite densities compared to control infections (DS; control sham-immunized versus antigen immunized: $F_{1,44}$=8.84, p=0.005; DK; control sham-immunized versus antigen immunized: $F_{1,43}$=11.53, p=0.001). All antigen immunized mice raised antibodies that were higher than sham-immunized controls (Fig. 7.1A,B)

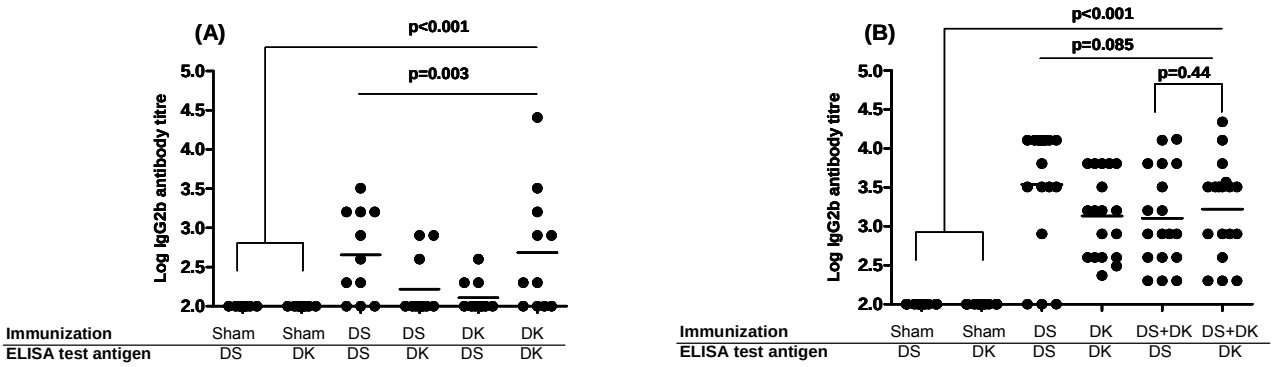

Figure 7.1. IGg2b antibody levels from the serum of mice in the pilot experiment (A) and the main experiment (B). Mice were either sham-immunized or immunised with one of the two antigens (DS AMA-1, DK AMA-1). Each of the treatments used to immunize mice and the AMA-1 test antigen used to coat ELISA plates are shown on the x axis. Dots represent the antibody titre against a particular immunizing antigen. Horizontal lines indicate mean antibody levels. (A) Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls ($p<0.001$) and, among the immunized mice, the levels induced between the antigen immunized groups differed (immunizing treatment x ELISA antigen: $p=0.003$) with higher titres against the homologous antigen. Neither of the immunizing antigens induced higher titres ($p>0.05$). (B) Mice that were sham-immunized or immunized with the bi-allelic formulation were assayed for antibody responses against both DS and DK AMA-1 antigens. Horizontal lines indicate mean antibody levels. Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls ($p<0.001$), and among the antigen immunized mice, antibody titres did not differ ($p=0.085$). The antibody titres in animals immunized with both antigens were not dominated by responses to either one ($p=0.44$).

7.4.1. Genotype DK

As found with other pairs of genotypes (de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006; Grech et al. 2008), here I found in control sham-immunized

mice that the less virulent genotype DK was competitively suppressed, achieving half parasite densities when DS was present than when it was absent (Fig.7.2; Table 7.2). Antigen immunization suppressed parasite replication in both single and mixed infections, but there was no evidence that the magnitude of competitive suppression was affected by immunization (interaction n.s.; Table 7.2)**.**

Repeated-measures analysis revealed that effects of immunization on competition varied by day (significant immunizing x competition x day interaction; Table 7.3). Visual inspection of Figs 7.2A-D shows that this is due to possible competitive release of genotype DK during DS AMA-1 immunization during early infection (Fig. 7.2C)

The repeated measures analyses also revealed some evidence of immune-mediated competition during DK AMA-1 immunization (Table 7.4; immunization x competition interaction, but not for DS AMA-1 or DS+DK AMA-1 immunizations). Visual inspection of Fig. 7.2B indicates that competitive suppression was slightly more intense in the presence of DK AMA-1 earlier in infection compared to control sham-immunized infection, where competitive suppression was most intense immediately after peak parasite density (around day 10).

	DK days 4-14			**DS days 4-14**		
Effect	d.f.	F	p	d.f.	F	p
immunizing treatment	3,37	38.7	<0.001	3,38	20.2	<0.001
competition	1,37	6.67	0.014	1,38	1.28	0.26
immunizing treatment x competition	3,37	0.91	0.44	3,38	0.35	0.78

Table 7.2. GLM analyses of the effects of immunizing treatment (control sham-immunized, DS AMA-1, DK AMA-1 or DS+DK AMA-1) competition (presence/absence of co-infecting clonal genotype) and their interaction on the total number of parasites for days 4-14 post-infection for parasite genotype DK and DS.

	DK days 4-14			DS days 4-14		
Effect	d.f.	F	p	d.f.	F	p
Immunizing treatment	3,51	43.3	<0.001	3,52	17.9	<0.001
Competition	1,51	7.72	0.008	1,52	1.56	0.22
Day	8,249	6.45	<0.001	8,256	8.15	<0.001
Immunizing x competition	3,51	2.17	0.1	3,52	0.87	0.46
Immunizing x day	24,249	2.98	<0.001	24,257	4.15	<0.001
Competition x day	8,249	1.38	0.2	8,256	0.2	0.99
Immunizing x competition x day	24,249	1.61	0.039	24,257	3.05	<0.001

Table 7.3. Repeated-measures analyses of the effects of immunizing treatment (control sham-immunized, DS AMA-1, DK AMA-1 or DS+DK AMA-1) competition (presence/absence of co-infecting clonal genotype) and day on the total number of parasites for days 4-14 post-infection for parasite genotype DK and DS.

Clone DK	DK AMA-1 immunization			DS AMA-1 immunization			DS+DK AMA-1 immunization		
Effect	d.f.	F	p	d.f.	F	p	d.f.	F	p
Immunizing treatment	1,34	287	<0.001	1,23	4.54	0.044	1,238	10.3	0
Competition	1,34	18.1	0.002	1,23	0.36	0.556	1,238	2.39	0.13
Day	8,107	5.96	<0.001	8,140	30.5	<0.001	8,139	9.59	<0.001
Immunizing x competition	1,34	6.79	0.014	1,23	2.05	0.166	1,23.8	0.5	0.49
Immunizing x day	8,107	3.34	0.002	8,140	21	<0.001	8,139	1.69	0.11
Competition x day	8,107	2.06	0.046	8,140	1.78	0.086	8,139	0.47	0.87
Immunizing x competition x day	8,107	1.95	0.06	8,140	1.68	0.108	8,139	0.46	0.89

Table 7.4. Repeated-measures analyses of the effects of each antigen immunizing treatment (DS AMA-1, DK AMA-1 or DS+DK AMA-1) compared to control sham-immunized, competition (presence/absence of co-infecting clonal genotype) and day, on the daily parasite density of genotype DK for days 4-14 post-infection.

Clone DS	DS AMA-1 immunization			DK AMA-1 immunization			DS+DK AMA-1 immunization		
Effect	d.f.	F	p	d.f.	F	p	d.f.	F	p
Immunizing treatment	1,29	37	<0.001	1,26.2	0.3	0.59	1,29.8	5.74	0.0228
Competition	1,29	1.59	0.22	1,26.2	3.14	0.088	1,29.8	3.86	0.06
Day	8,134	3.78	0.005	8,120	7.7	<0.001	1,133	5.29	<0.001
Immunizing x competition	1,29	2.2	0.15	1,26.2	1.86	0.184	1,29.8	1.36	0.25
Immunizing x day	8,134	6.32	<0.001	8,120	5.95	<0.001	8,133	8.21	<0.001
Competition x day	8,134	0.89	0.53	8,120	2.49	0.015	8,133	1.78	0.08
Immunizing x competition x day	8,134	4.46	<0.001	8,120	1.29	0.28	8,133	3.43	0.0013

Table 7.5. Repeated-measures analyses of the effects of each antigen immunizing treatment (DS AMA-1, DK AMA-1 or DS+DK AMA-1) compared to control sham-immunized, competition (presence/absence of co-infecting clonal genotype) and day, on the daily parasite density of genotype DS for days 4-14 post-infection.

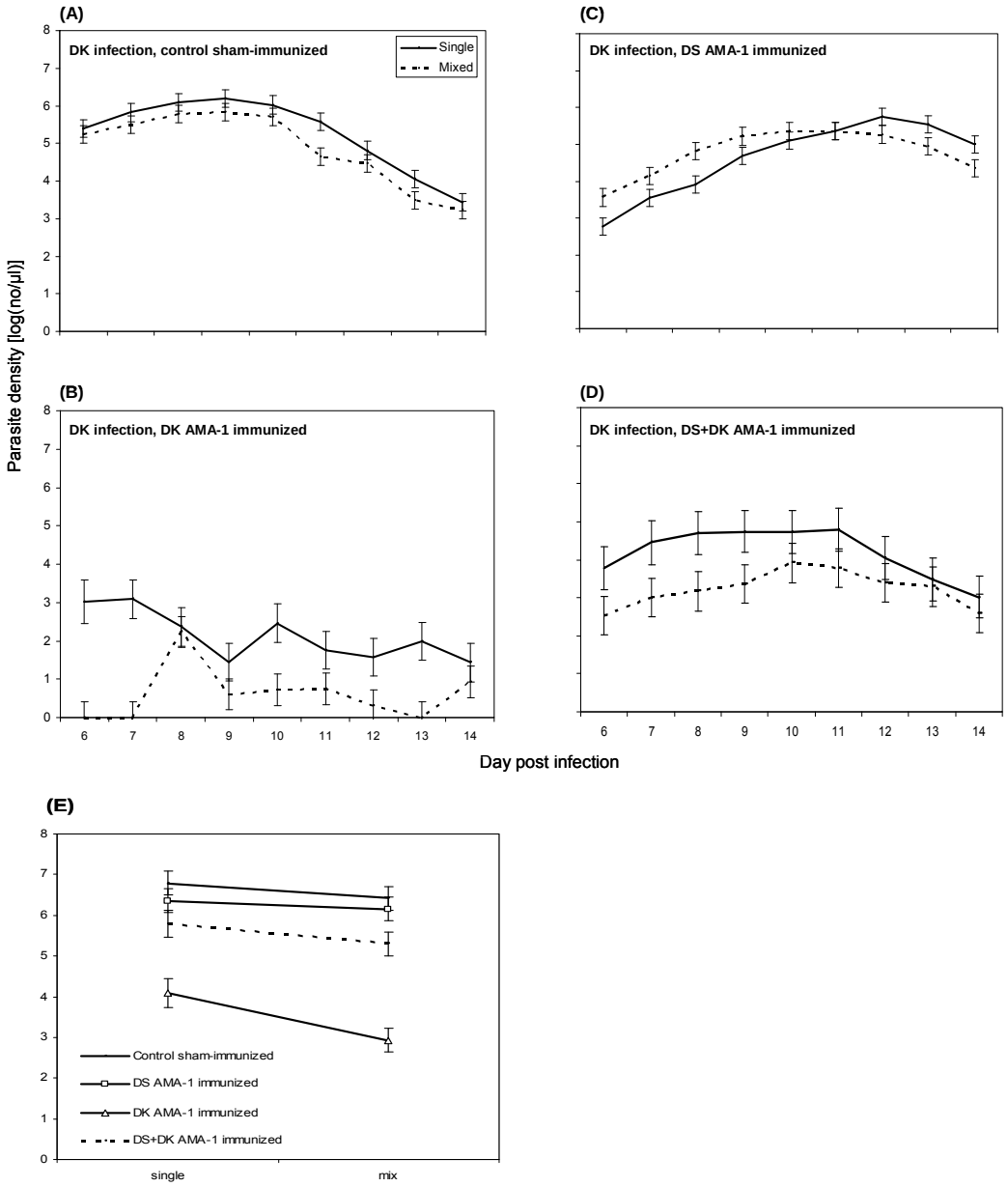

Figure 7.2. Parasite densities through time of genotype DK under the different immunization treatments (A-D) and the interaction of total densities across the course of infection (E). A-D represent the asexual parasite density from qPCR in sham-immunized controls (A), DK AMA-1 immunized (B), DS AMA-1 immunized (C), and DS+DK AMA-1 immunized (D), in single (solid black line) or mixed (dotted black line) infections. Mean densities (± 1 s.e.m.) were calculated from all mice that were alive on the perspective day of sampling (up to six). The interaction plot (E) shows the total numbers of DK parasites when the competitor genotype DS is absent (single infection) or present (mixed infection) in sham-immunized controls (solid black line), DS AMA-1 immunized (black line open boxes), DK AMA-1 immunized (black line open triangles) or DS+DK AMA-1 immunized (dotted black line).

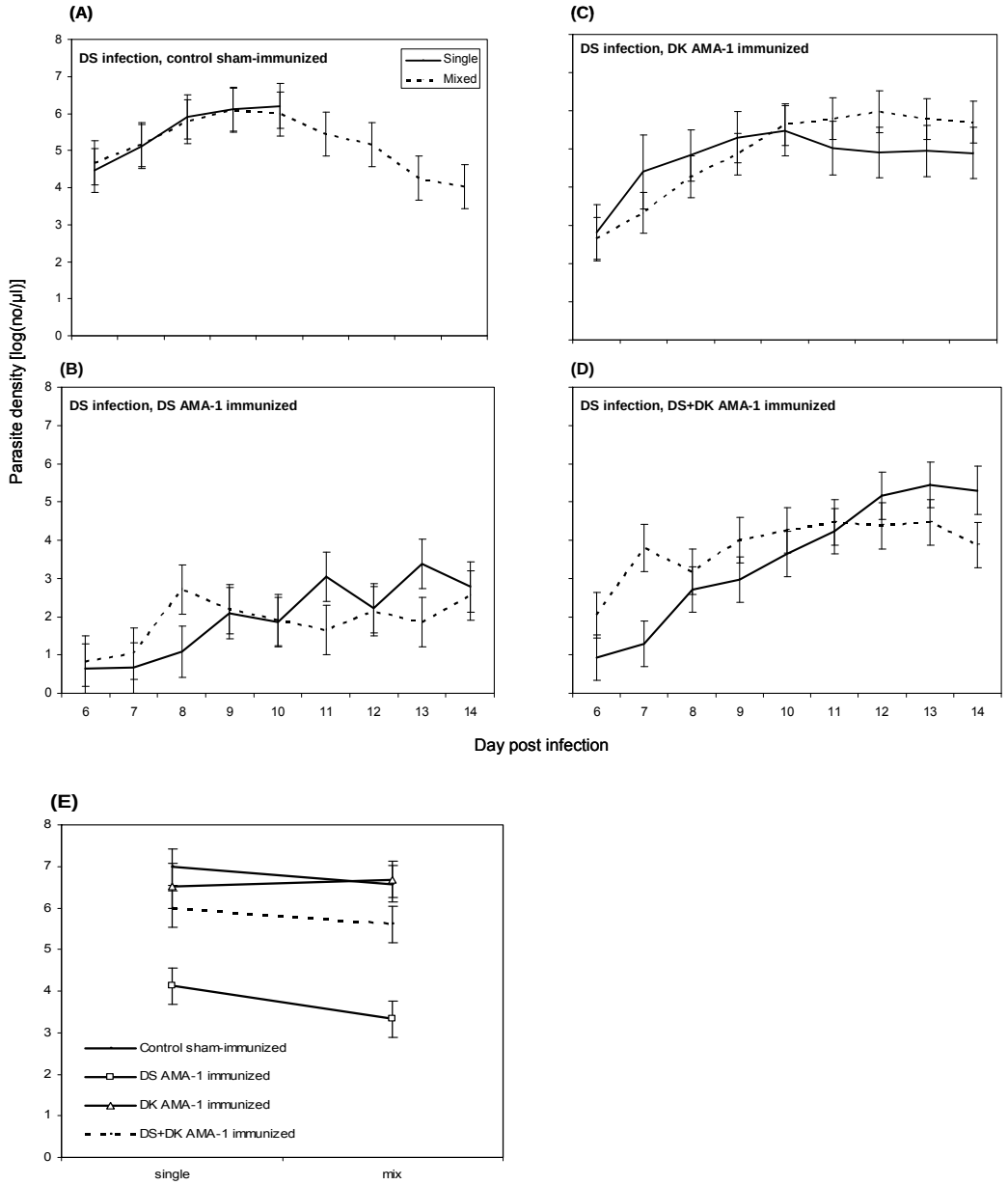

Figure 7.3. Parasite densities through time of genotype DS under the different immunization treatments (A-D) and the interaction of total densities across the course of infection (E). A-D represent the asexual parasite density from qPCR in sham-immunized controls (A), DS AMA-1 immunized (B), DK AMA-1 immunized (C), and DS+DK AMA-1 immunized (D), in single (solid black line) or mixed (dotted black line) infections. Mean densities (± 1 s.e.m.) were calculated from all mice that were alive on the perspective day of sampling (up to six). The interaction plot (E) shows the total numbers of DD parasites when the competitor genotype DK is absent (single infection) or present (mixed infection) in sham-immunized controls (solid black line), DS AMA-1 immunized (black line open boxes), DK AMA-1 immunized (black line open triangles) or DS+DK AMA-1 immunized (dotted black line).

7.4.2. Genotype DS

In control sham-immunized infection there was no evidence of competitive suppression of genotype DS by DK (Fig. 7.3A,E; Table 7.2). As with genotype DK, antigen immunization suppressed parasite replication/densities in both single and mixed infections, but there was no evidence immunization facilitated the competitive release of genotype DS (interaction n.s.; Table 7.2).

Repeated-measures analysis revealed that the effects of immunization on competition varied by day (significant immunizing x competition x day interaction; Table 7.3). Visual inspection of Figs 7.2A-D shows that this is due to possible competitive release of genotype DS during DS+DK AMA-1 immunization during early infection (Fig. 7.2D).

The repeated measures analyses also revealed some evidence of immune-mediated competition during DS AMA-1 and DS+DK AMA-1 immunization (Table 7.5; immunization x competition x day interaction, but not for DK AMA-1 immunization). Visual inspection of Fig. 7.3A-D show competitive release of genotype DS early in infection (Fig. 7.3B,D) compared to control sham-immunized where infection dynamics were similar during single and mixed infection.

Overall, these results demonstrate that neither type of immunization enhanced the competitive suppression of the less virulent genotype (DK), or facilitated the competitive release of the more virulent genotype (DS) in a mixed infection. If anything, a single heterologous DS AMA-1 immunization may facilitate competitive release of the less virulent genotype by removing more of the virulent genotype in mixed infection (Fig. 7.2C). There was very weak contradictory evidence of immune-mediated competition (Fig. 7.2B, 3B). However, since these effects were day-specific I conclude that strain-specific immunity does not markedly alter competitive interactions.

7.5 Discussion

In this study the less virulent *P. chabaudi* genotype (DK) was suppressed by the presence of a co-infecting more virulent genotype (DS). Immunization with the two variant rodent analogues of the malaria vaccine candidate AMA-1 reduced parasites in a strain-specific manner. However, I found no evidence that immunization enhanced competitive suppression of the less virulent genotype or facilitated the competitive release of the more virulent genotype. Thus, there was no evidence that strain-specific immunization exacerbated or alleviated competition and thus the force of this component of selection on virulence. Furthermore, I found no evidence that a bi-allelic AMA-1 vaccine should enhance or alleviate competitive interactions.

The experiments presented in this study contribute towards a growing body of evidence which suggest that the competitive benefits associated with virulence need not result in a selective advantage in an immunized host [Chapter 6, Appendix 2 (Barclay et al. (2008a)] (de Roode et al. 2005b; Bell et al. 2006; Grech et al. 2008). The finding that strain-specific immunization does not exacerbate within host competition is consistent with two experimental studies which suggest that vaccination should have negligible impact on this source of selection on virulence [Chapter 6, Appendix 2 (Barclay et al. 2008a)] (Grech et al. 2008)]. The first study came from my own work, where I indirectly tested whether CD4-T cell dependent immunity, or mechanisms which act to enhance it such as vaccination, might exacerbate within host competition [Chapter 6, Appendix 2 (Barclay et al. 2008a)]. In that study, I found no evidence to suggest that vaccination should exacerbate this source of selection for virulence. If anything, the data from that study suggested that, after the peak of infection, CD4-dependent immunity may act to alleviate other forms of competition, such as resource-based competition (e.g. for red blood cells) [Appendix 3 (Mideo et al. 2008b) (Taylor et al. 1997; Antia et al. 2007)]. The second study (Grech et al. 2008), used a more direct approach to test whether vaccination could exacerbate competitive interactions. In experiments similar to those presented here, mice were immunized with either AMA-1 or with live parasites. Unlike in this study, neither of the genotypes used to infect the AMA-1

immunized mice were homologous at the target antigen, therefore the strain-specific affect of vaccination was not tested. The live parasite immunizations involved a strain specific component, as one of the genotypes in the infection was always identical to the immunizing genotype but because of the experimental design strain-specific immunity could not be tested for. That study also found no evidence that either type of immunization alleviated or exacerbated competitive suppression.

My previous studies [Chapter 6, Appendix 2 (Barclay et al. 2008a)] and the data presented here show no consistency with one study which showed weak immune-mediated competitive interactions (Råberg et al. 2006)]. In that study, competitive suppression of a less virulent *P. chabaudi* genotype was alleviated during infection (post peak days 10-12) of 'nude' mice, which have no mature T cells. The authors implied that T-cell dependent immunity, and mechanisms which act to enhance it such as vaccination, may mediate competition and thus selection for virulence. However, given the relatively small immune-mediated component, and the accumulating evidence against this as a mediator of competition, I am inclined to think the results from the Råberg et al. 2006 study, were a possibly unique attribute of using nude mice.

Previous studies involving serial passage found that genotypes passaged through whole-parasite immunized mice evolved virulence faster than those passaged through naïve mice, even after mosquito transmission (Mackinnon & Read 2004a). That study implied that there was a selective advantage to virulence; immunity selected for those genotypes which proliferated ahead of the immune response and were thus most likely to win the race into the syringe for passage. If this is not a result of a competitive advantage, what other mechanisms could explain the selection for more virulent genotypes? In *P. chabaudi* there are a number of other advantages associated with virulence. For instance, more virulent genotypes are also found to be less rapidly cleared by the host (Mackinnon & Read 1999; de Roode et al. 2003; Ferguson et al. 2003; de Roode et al. 2004a; de Roode et al. 2004b; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006). Thus, in the case of serial passage experiments, immunity may select for those genotypes which are able to evade

protective responses, for instance, by undergoing antigenic variation, secreting immunomodulatory products or by accessing immunologically privileged sites (Rowe et al. 1995; Chen et al. 1998; Salanti et al. 2004; Bull et al. 2005; Francis et al. 2007). Immunization may have enhanced natural-immunity, or imposed some unique form of selection, which caused those parasite passaged through whole immunized mice to evolve faster. Thus, in light of the results from my experiments, selection imposed by immunity observed during serial passage experiments was likely independent of competition (Mackinnon & Read 2004a).

Enhanced immune evasion may go some way to explaining the within host advantage to virulence observed during other immunization experiments with AMA-1 which did not involve serial passage (Chapter 5; Grech et al. 2008 *in prep).* In those experiments, *P. chabaudi* genotypes which differed in virulence but were identical at the *ama-1* sequence to each other and to the vaccine antigen were used to infect AMA-1 immunized mice. The more virulent genotypes were less well controlled by vaccine-induced immunity. Those studies suggested that virulence determinants, different from the vaccine target, were likely to be involved in selection. Some of these virulence determinants may be involved in immune evasion. Full genomic analysis of *P. chabaudi* genotypes may identify such virulence genetic determinants.

Immunity against malaria parasites has strain-transcending and strain-specific components (Martinelli et al. 2005). It is generally assumed that during the acute phase of infection in naïve animals the response is largely a non-specific cellular immune response (Taylor-Robinson 1995; Li et al. 2001). During the chronic phase, the response becomes far more specific as antibody-mediated immunity is deployed (Jarra & Brown 1985; Phillips et al. 1997; Mota et al. 1998; Buckling & Read 2001). In this study, the presence of a heterologous AMA-1 vaccine appeared to slow parasite growth compared to a sham-immunized infection, in what could be described as a strain-transcending response (Fig. 7.2A,C, 7.3A,C). The growth dynamics of the virulent genotype were similar regardless of whether the less virulent genotype was present or not (Fig. 7.3C). However, despite no statistical

significance, it appeared as though the less virulent genotype gained some advantage during heterologous AMA-1 immunization when the more virulent genotype was present (Fig. 7.2C). Thus, the data I present here indicate that analogous processes to those observed with anti-malarial drug use are not likely to occur during vaccination; strain-specific removal of less virulent genotypes by vaccination should not result in competitive release of more virulent genotypes.

The fact I found strain-specific immunity not to alter within host competition should not preclude vaccination as a source of selection for virulence. The use of any form of therapy that selectively removes some parasite strains but not others should be implemented with caution. For instance, the trail of the Combination B malaria vaccine exerted selective pressure by reducing those parasite strains which carried vaccine alleles, but with no affect on parasites which carried vaccine allelic variants (Genton et al. 2002; Genton et al. 2003). Those variants which remained in the population were also associated with more severe disease (Engelbrecht et al. 1995). One solution to reduce vaccine-induced selection has been to design vaccines which contain more than one allelic variant (Bolad & Berzins 2000; Richie & Saul 2002; Polley et al. 2007). Studies so far have reported that these 'multi-allelic' vaccines may not enhance protection from disease (Miura et al. 2007; Lalitha et al. 2008), but should not result in the strain-specific selection induced by mono-allelic vaccines [Chapter 4, Appendix 1 (Barclay et al. 2008b)] (Miura et al. 2007; Lalitha et al. 2008). In the study I report here, bi-allelic vaccination did not alter competitive interaction and should thus not enhance this source of selection for virulence.

The mechanisms that mediate competition are likely to continue drawing interest from evolutionary biologists, who have so far identified resource-based competition and direct interference, in addition to the immune response, as potential mediators of competition. The current approach is dissecting each mediator appears most appropriate. For instance, the modelling of the data presented in this study has identified a possible resource-based mechanism in the strain-specific pattern of pathogenesis observed [Appendix 3 (Mideo et al. 2008b)]. Such studies merit the

continued collaboration between empiricists and mathematical modellers in studies of infectious disease.

In conclusion, the data I present here appear to contribute towards a growing body of evidence that vaccination is not likely to alter within host competition between genetically diverse malaria parasites, and thus the force of this selection for virulence. Rather than further dissection of competitive interactions with alternative vaccines, the most appropriate way forward will be to design experiments to test whether any of the other parasite benefits associated with virulence e.g. immune evasion, may enhance selection of these strains in an immunized host. One could envisage serial passage experiments through hosts vaccinated with a recombinant vaccine antigen such as AMA-1, paralleled by comparative molecular analysis studies of those genotypes to identify any adaptation in virulence markers.

8. General discussion

The aim of this thesis was to investigate whether parasite virulence could result in a within host selective advantage in an immunized host and hence whether vaccination could promote the evolution of virulence. I based my research on malaria, one of the world's most devastating diseases, to which there is currently no effective vaccine. The hope is that we can study the evolutionary consequences of vaccination before any wide-spread implementation. I used the rodent malaria *P. chabaudi* as an in vivo model system and the *P. chabaudi* analogue candidate malaria vaccine AMA-1 to test a number of my hypotheses. As each chapter has been written as a stand-alone paper, each has a discussion section with the specific findings. Here I summarise the main findings, the practical implications, and also the relevance of animal models in these studies. I also argue that collaborations between experimentalists and theoreticians should lead to advancements in the control of infectious diseases. I conclude by describing the future experiments which will no doubt complement my findings.

8.1. Findings

In Chapter 2, I started by exploring whether a more virulent *P. chabaudi* genotype was less well controlled by heterologous AMA-1 immunization than a less virulent genotype. I found no evidence of a selective advantage of virulence. Vaccination reduced parasites in strain-specific manner. The affect was asymmetrical in that the more virulent genotype was better controlled by vaccination. Similarly, in Chapter 3 I also found no selective advantage to virulence when the efficacy of AMA-1 was tested against a range of *P. chabaudi* genotypes which differed in *ama-1* sequence and/or in virulence. Vaccination reduced those parasites that were more similar to the vaccine target than those that were different. Together, the results from Chapters 2 and 3 suggested that vaccination with AMA-1 induces protective responses that are strain (genotype)-specific. Strain-specificity could lead to epitope evolution as the removal of one genotype creates a niche for another genotype to fill. One approach

to minimizing strain-specificity has been to design vaccines which contain more than one AMA-1 allelic form (multi-allelic vaccines). However, prior to my thesis there was little evidence that multi-allelic vaccines better protect hosts against parasites and/or disease, or how such vaccines may alter parasite within-host selection. In Chapter 4, I demonstrated in vivo that immunization with a bi-allelic AMA-1 did not afford the host greater protection from parasite infection or morbidity than did immunization with either of mono-allelic forms (Barclay et al. 2008b). I also found no evidence that any vaccination regime favored virulence. During the discussion of Chapter 4, I specifically highlight that selection for virulence could be in inadvertent consequence of including just one allele from a given locus in a vaccine, a possible explanation for the selection seen during the trial of the Combination B malaria vaccine. Since AMA-1 itself is not a known virulence factor, the strain-specific immunity induced by mono-allelic vaccines should not directly alter virulence. However, I indicate the importance of population-level disease association studies for all antigens included in vaccines in order to prevent inadvertent selection.

Up until this point the results from all my experiments indicated that there was no within-host advantage to virulence in an AMA-1 immunized host and that vaccine-efficacy more likely depended on *ama-1* sequence differences. Those data apparently contradicted a previous serial passage experiment, which demonstrated that parasites passed through whole-parasite immunized mice evolved virulence more rapidly than those passed through naïve mice (Mackinnon & Read 2004a). I thus hypothesized that the selective advantage to virulence may only be observed when parasites are from an antigenically identical background. To test this, in Chapter 5, a single *P. chabaudi* genotype was serially passaged through immunologically naïve mice to derive a line which contained parasites that caused more anaemia and weight loss, and which were able to achieve higher densities and persist for longer before immune clearance. These more virulent parasites were less readily controlled by immunity induced by immunization with homologous recombinant AMA-1 than were the less virulent parasite from which they were derived. One of the benefits associated with parasite virulence could be reduced immune clearance/immune evasion (Anderson & May 1982; Mackinnon & Read

2004a; Mackinnon & Read 2004b; Frank & Schmid-Hempel 2008; Schmid-Hempel 2008). An implication of the results from Chapter 5 was that those parasites had a selective advantage in an immunized host because they were less readily cleared by the immune response.

In Chapter 6, I studied whether vaccination has the potential to exacerbate the within-host competitive benefits associated with parasite virulence observed during mixed *P. chabaudi* genotype infections (de Roode et al. 2005; Bell et al. 2006). I found that when mice were depleted of $CD4^+$ T cells, the competitive suppression of a less virulent genotype by a more virulent genotype was not alleviated. Thus CD4-dependent immune responses, and mechanisms which act to enhance it such as vaccination, may not have the undesirable affect of exacerbating within-host competition. Interestingly, I found after the peak of parasite infection that there was a significant enhancement of competitive suppression in depleted animals, presumably because CD4-dependent responses alleviate other forms of competition, such as that of red blood cells.

In Chapter 7, I directly tested whether immunization with any of the mono-allelic AMA-1 vaccines used throughout this thesis could alter the competitive interactions between malaria parasites and thus the force of this selection on virulence. I also used this experiment to explore how a bi-allelic AMA-1 vaccine may alter competitive interactions. I found no evidence that any vaccine regime enhanced the competitive interactions compared to control infections.

8.2. Implications

8.2.1. Vaccine alleles and their association with virulence

The main question of this thesis was whether more virulent *P. chabaudi* genotypes had a selective advantage in an immunized host. The motivation for this work was

based on the Combination B malaria vaccine, where only one allelic type of MSP-2 (from the 3D7 family) was included in the three component vaccine. The vaccine induced a shift in the population towards parasites expressing MSP-2 proteins from the alternative allelic family FC27 (Genton et al. 2002). The FC27-type had been reported to be associated with symptomatic (more morbid) infections (Egelbrecht et al. 1995; Ofosu-Okyere et al. 2001).

However, a number of other studies have raised concerns to the validity of those associations between the FC27-type allele and more morbid infections. For example, the combination of two studies conducted in Senegal found a lower prevalence of parasites containing FC27-type alleles among clinical malaria infections than among asymptomatic infections, but the symptomatic and asymptomatic groups were not matched and sample size was small (Ntoumi et al. 1995; Robert et al. 1996). Furthermore, a recent study in Tanzania found that the presence of 3D7-type was predictive of clinical symptoms, but again the sample size was very small (Magesa et al. 2002). More recently, one study compared the allelic frequency, which is not affected by the multiplicity of infection, of the 3D7 and FC27 allelic families in the Wosera region of Papua New Guinea, the same study area used by Egelbrecht et al (1995). The allelic frequency of the 3D7-type and the FC27-type alleles was very similar between asymptomatic and symptomatic infections, with the FC27-type alleles being slightly more common in the asymptomatic group than the symptomatic group (Cortes et al. 2004). Based on those results the authors suggest that selection for parasites expressing FC27-type MSP-2 might not necessarily be associated with increased morbidity, but that it will be important to understand the possible effects on morbidity that interventions might have on the natural balance between allelic types. Even if allelic-types of the MSP-2 allele are not associated with virulence, as mentioned in the conclusion of Chapter 4 [Appendix 1 (Barclay et al. 2008a)], caution is necessary for all antigens involved in processes like cell invasion which are associated with pathogenesis, and one of the safest ways to avoid inadvertent selection would be to include all known variants of that locus in the vaccine.

8.2.2. The potential efficacy of multi-allelic vaccines

In Chapter 4 [Appendix 1 (Barclay et al. 2008a)], I found that the vaccination with a bi-allelic AMA-1 formulation did not afford the host greater protection from parasite infection than did mono-allelic AMA-1 immunization. However, these results by no means preclude the possibility that a multi-allelic AMA-1 vaccine may reduce vaccine-induced strain-specificity. For example, in one study the analysis 355 PfAMA-1 sequences found that around 10% of the 622 amino acid residues can vary between alleles and that linkages between polymorphic residues occur (Remarque et al. 2008b). Using this analysis, the authors designed three diversity-covering (DiCo) PfAMA-1 sequences that take account these linkages, and taken together incorporate 97% of the amino acid variability observed. Rabbits were then either immunized with FVO strain PfAMA-1 or with the DiCo proteins either individually or as a mixture. Animals immunized with the DiCo mix raised antibody titres and inhibited parasite growth similarly to animals immunized with strain FVO AMA-1 when measured against FCR3 strain parasites. However, the DiCo mix outperformed animals immunized with FVO AMA-1 when assessed against other strains. Those results suggest that including a number of polymorphic residues may increase the potency of an AMA-1 vaccine. Similarly, another study found enhanced protection from in vitro parasite invasion of red blood cells when a vaccine contained PfAMA-1 sequences representing a mixture of major haplotypes from around the world that could be grouped into six populations (Duan et al. 2008).

In comparison, MSP-2 is an example of a highly polymorphic merozoite antigen. Despite the Combination B vaccine containing the MSP-2 3D7-type, some 3D7-type infections still established themselves in immunized children (Genton et al. 2002). One study sequenced MSP-2 alleles detected in study participants after vaccination to identify breakthrough genotypes, and thus try and identify the regions of the protein under selection (Fluck et al. 2007). The analysis found that highly polymorphic non-repetitive blocks were not subject to selection in vaccinated individuals, and therefore assumed that responses against these regions were not protective. Instead, a 3D7 family-specific domain containing 50 residues of invariant

sequence was postulated as a likely candidate to account for selection observed on the level of the allelic family. Thus, unlike with AMA-1, if protective responses are directed against conserved regions, there may be no merit in including a number of 3D7-type variants in a MSP-2 containing vaccine.

Together these studies highlight that the efficacy of multi-allelic vaccines will not only depend on the degree of polymorphisms exhibited by a particular protein, but whether these are under selection and are thus important for generating protective immunity.

8.2.3. Malaria vaccine development

As mentioned at the beginning of my thesis, vaccines are a medical triumph and should be recognized as such. The aim of this thesis was by no means to belittle the success, or potential, of vaccines in controlling infectious disease. However, in view of my experiments I believe that future vaccines should not only depend on short-term individual responses, but should take into account and anticipate the population dynamical and evolutionary consequences of vaccination as well.

In Chapter 4 (Barclay et al. 2008b), I showed that enhanced protection from parasites or disease during vaccination with AMA-1 is not likely to be enhanced by including more than one allelic variant. This may just have been a result of the combination of AMA-1 alleles used in that experiment, and as mentioned above, does not preclude the potential increased efficacy of multi-allelic vaccines. However, considerably more data from other researchers have indicated that the potency of a malaria vaccine may be increased by combining single variants from multiple antigenic loci (Hoffmann & Miller 1996; Holder 1999; Richie & Saul 2002; Heppner et al. 2005; Garcia et al. 2006). For example, animal and human phase I trials have shown safety, tolerability and immunogenicity of formulations containing AMA-1 and MSP-1 (2002; Burns et al. 2003; Pan et al. 2004; Faber et al. 2007; Hu et al. 2008), and passive immunization studies of formulations containing AMA-1 support the concept of developing ‘multivalent’ blood-stage vaccines (Narum et al. 2006).

Moreover, such multivalent vaccines have been shown to reduce parasitaemias in mice of distinct MHC haplotypes (Doolan et al. 1996) and against infections with different parasite strains as well as subspecies of different virulence (Scorza et al. 2008). Thus, multi-valency may be required to induce antibody responses against a repertoire of polymorphic parasite antigens (Bouharoun-Tayoun et al. 1995; Bull & Marsh 2002; Wipasa et al. 2002a; Wipasa et al. 2002b; Doolan et al. 2003; Moorthy et al. 2004; Eisenhut 2007; Osier et al. 2008) in the human outbred population exposed to multiple parasite genotypes (Doolan & Hoffman 2001; Genton et al. 2002; Plebanski et al. 2002; Cortes et al. 2003; Doolan et al. 2003).

Of course, the erythrocytic stage of the life cycle is not the only stage that is targeted by vaccination. Several candidate malaria vaccines to the pre-erythrocytic and transmission stages are also progressing through clinical trails (Ballou et al. 2004; Moorthy et al. 2004). The most advanced malaria vaccine candidate is RTS,S, a pre-erythrocytic vaccine, for which pivotal phase III trial design and site preparation is underway (Stoute et al. 1997; Stoute et al. 1998; Bojang et al. 2001; Kester et al. 2001; Alonso et al. 2004; Alonso et al. 2005). However, it is still not obvious what level of efficacy needs to be achieved for a malaria vaccine to be worthwhile since even vaccines that only partially protect might offer substantial health benefits, given the enormous burden of morbidity and mortality in endemic areas (Greenwood et al. 2005). Even RTS, S induces only partial protection against infection and disease in clinical trials (Bojang et al. 2001; Alonso et al. 2004).

In Chapters 2-5 of my thesis I consistently observed a lack of correlation between protection from parasites and protection from disease (morbidity). The reasons for this are poorly understood at a molecular level but are likely to be due to the parasite invasion inhibitory antibodies depending on polymorphic epitopes, whereas protection against disease e.g. anaemia, may be more correlated with how fast the host can replace red blood cells during recovery. In view of my results, malaria vaccine trials should continue to monitor both the parasitological status of the host as well as measurements of disease which will hopefully result in the most accurate reflection of vaccine efficacy.

Mathematical models of virulence evolution caution against malaria vaccines which reduce symptomatic disease while still maintaining parasite transmission (Gandon et al. 2001; Gandon & Day 2003; Gandon & Day 2007). Such vaccines are expected to select for more virulent strains as the cost of virulence (host death) is removed by vaccination. Those evolved strains are expected to cause more severe morbidity if transmitted to an unvaccinated host. The results presented in Chapter 5 and that by others (Mackinnon and Read 2004a; Grech et al. 2008 *in prep*) suggest that more virulent parasites may have a selective advantage in immunized hosts. Thus, the selection imposed by vaccination on individuals may have important evolutionary consequences for future host population disease morbidity.

8.2.4. Evolution in biomedicine

This thesis started by describing how evolutionary biology has had little impact in biomedicine, and how an increased understanding of evolutionary processes may help prevent disasters such as the wide spread evolution of drug resistance. Thus, it appears appropriate that I should discuss how the work presented in this thesis may contribute towards the movement of evolutionary thinking into biomedicine.

The quest of my thesis was to investigate whether the parasite benefits associated with virulence in *P. chabaudi* could result in a selective advantage in an immunized host. Although my thesis was based on malaria, the questions that I asked are applicable to a number of other diseases (Day et al. 2008). I hope the results from my thesis prompt more evolutionary thinking in those working on malaria vaccines, but also during the design and implementation of vaccines against other diseases. In particular I have chosen to highlight Human Papillomavirus (HPV) as this vaccine has recently been approved for wide-spread use in young American females (FDA 2006). Monitoring the evolutionary consequences of HPV in the population will require collaborations between health worker, experimental biologists and bio-mathematicians. If these collaborations occur, there is great potential for HPV to be

one of the first diseases where the evolutionary consequences of vaccination can be determined.

Increased evolutionary thinking should prompt those working on vaccines against a number of diseases to ask the following questions:

- How should one balance the breadth against the depth of a vaccine i.e. should one include many polymorphic antigens or only a few monomorphic ones?
- Which vaccines, vaccination schemes and coverages minimize the probability of appearance of escape mutants?
- How does the evolutionary pressure on the parasite population depend on the relationship between natural and vaccine-induced immunity?
- Should the aim of vaccination be to reduce symptomatic disease while maintaining circulation, or should it be to reduce transmission?
- Will vaccination select for aggressive strains that efficiently exploit the host, or does it select for stealthy avirulent strains that are less visible to the immune system?

In view of my experiments I believe that the design of vaccines should not only be focused on short-term individual responses, but that it should take into account and anticipate the population dynamical and evolutionary consequences of vaccination as well.

8.2.5. Theorists and experimentalists

Mathematical models provide an invaluable tool for predicting and understanding the epidemiology of infectious disease. One of the limitations of modelling is that predictions and hypotheses usually well exceed the amount of experimental data available to which these models can test. Even when data are available there are often caveats, for instance, in sample sizes and data collection methods. Thus, strong collaborations between experimentalists and theorists are necessary if we are to

maximise the potential of these two disciplines for infectious disease studies. During my PhD I was lucky enough to work in an inter-disciplinary research group that I was exposed to the merits of working with theorists. In particular, I worked closely with Nicole Mideo, a graduate student of Troy Day from Queens University, Kingston, Canada. Nicole used the data I collected during Chapter 6 [Appendix 2 (Barclay et al. 2008a)] and was able to examine the role of resource alone (red blood cells) on the dynamics of two strains in the absence of the immune system. The model provided insights into the strain-specific pattern of pathogenesis, indicating that differences in virulence between strains could be explained by differences in red-blood-cell-age-specific invasion rates and burst sizes, being lower for the less virulent strain [Appendix 3 (Mideo et al. 2008b)]. Unfortunately, studies like this are very rare despite the great potential of this type of approach. An important goal of building mathematical models of malaria pathogenesis is to use them to evaluate interventions (Mideo et al. 2008a). For instance, models have demonstrated the potential for vaccination to select for increased virulence (Gandon et al. 2001; Ganusov & Antia 2006). There is still great potential for models to lead to better predictions about the effects of interventions. For example, models may help explain why passaging malaria parasites through immunized mice results in selection for more virulent parasites (Mackinnon & Read 2004a). In particular, what is the mechanism of this increased virulence, i.e. on what trait is selection acting? With a good model of mouse malaria (one that has been derived from, calibrated and validated with data), this experiment could be replicated *in silico* with the aim of predicting what kinds of malaria parasites (e.g. those that undergo rapid antigen switching, replicated at higher rates or infect RBCs at faster rates) have an advantage in immunized hosts (Mideo et al. 2008b). Achieving this, in studies of malaria and beyond, requires careful interactions between experimental biologists and biomathematicians.

8.3. Relevance of mouse models

In today's post-genomic era with *Plasmodium*, *Anopheles* and human genomes at hand, optimism abounds that developments in new drug therapies and vaccines will emerge (Carlton et al. 2002; Florens et al. 2002; Gardner et al. 2002; Holt et al. 2002; Maher 2002; Chauhan & Bhardwaj 2003) This has meant that researchers have become even more dependent upon models for screening molecules involved in protection. Animal models play a crucial position and thus an evaluation of their merits and limitations is warranted.

Merits of the rodent model systems are that they share many features with human malaria. For instance, the basic biology of rodent and human malaria is similar. In particular, *P. chabaudi* and *P. falciparum* both undergo synchronous schizogony, capillary sequestration and have preference for mature red blood cells (Mons & Sinden 1990). Genome size, the number of chromosomes and the G+C content are similar between the rodent and human parasites (Carlton et al. 2002; Gardner et al. 2002; Hall et al. 2005). Surface proteins are conserved between rodent and human parasites, with some with the exceptions such as MSP-2 (Marshall et al. 1989; Waters et al. 1990; Thompson et al. 2001). The molecular basis of drug sensitivity and resistance are similar between rodent and human parasites (Carlton et al. 1998; Carlton et al. 2001; Walliker et al. 2005). Another merit of the rodent malaria system is that it enables in vivo investigations of parasite-host interactions in well characterized host genetic backgrounds with a variety of parasite types (Grech et al. 2006). In particular, the *P. chabaudi* system is the only malaria model with a well characterized range of genetically distinct parasite clones and distinct virulence phenotypes (Mackinnon & Read 1999b; Ferguson & Read 2002). This well defined system allows the generation of results quickly under controlled laboratory settings.

Parallelisms between mice and humans would at a first glance provide support to the relevance of the rodent model. However, there are a number of limitations which cannot be ignored. For instance, unlike human malaria, rodent infections are usually short lived, reach high parasite densities and often result in host death (Stevenson et

al. 1982; Collins & Jeffery 1999; Mackinnon & Read 1999a). Furthermore, the rodent host and parasite are not a natural combination. The adaptation of a parasite to an artificial host actually translates in immunological terms into defense mechanism that in most circumstances are more effective than those seen in the natural host. Consequently vaccine candidates that show promise in a given model might fail in humans or worse, experimental results may lead to the rejection of vaccine candidates that would be effective in humans. For instance, the most successful *Plasmodium* vaccine used to date, RTS/S, relies on an adjuvant inducing optimal cytotoxic T lymphocyte (CTL) activity in models. In humans it induces no CTL activity, very high antibody levels, yet generates a degree of protection. (Alloueche et al. 2003). Thus, each transfer between species-from rodents to non-human primates to humans- cannot not be assumed to act uniformly (Chatterjee et al. 2006).

In malaria vaccine development a dilemma now exists: either we rely on direct testing on non-human primates or even humans and accept that progress will be slow, though results should be more relevant; or we continue to employ easier models (including rodents and in vitro culture), generating data which cannot necessarily be extrapolated fully to the human situation. However, modern genomics may provide a valuable link between models and human malaria. For instance, availability of the full genomes of most rodent plasmodia may lead to the selection of a preferred rodent *Plasmodium* species based on knowledge of molecular homologies with the corresponding human *Plasmodium* gene. The same can apply to the selection of the best fit between host molecules (Chatterjee et al. 2006). Another approach promoted by some groups is gene replacement to substitute *P. falciparum* genes for rodent *Plasmodium* genes and similarly, to substitute human genes for mouse ones (O'Donnell et al. 2000; McIntosh et al. 2007). But always there will be a real challenge measuring protection against disease.

Overall, rodent malaria systems have allowed huge advancements in drug and vaccine development as well as providing models for the study of virulence evolution. In vivo manipulations, such as those preformed in this thesis, provide

insights into potential vaccine protection efficacy in humans as well as the evolutionary consequences as predicted by mathematical models. Thus, I view rodent malaria as a continued necessity in the research of this disease, but extrapolations must of course be made cautiously.

8.4. Future directions

8.4.1. Virulence genes

In general the biomedical field is sometimes dominated by people looking for epitope evolution with little consideration for virulence. During this thesis I have tried to make it clear that these are not discrete non-overlapping hypotheses (virulence evolution could occur due to epitope changes). However, the virulence hypothesis is tested by determining whether vaccine-adapted strains cause more disease in unvaccinated individuals. In malaria, the mechanisms underlying virulence are probably complex, involving a large number of genes associated with parasite phenotypes such as cell invasion, resetting, cytoadherence and replication. In Chapter 5 I demonstrated that serial passage of *P. chabaudi* through naïve mice derived a parasite line which was better able to evade AMA-1 vaccine-induced immunity. One of the caveats of that study was that there was no transmission through mosquitoes, where recombination of the sexual stage of the parasite life cycle occurs. However, many laboratory studies in malaria have shown that high or low virulence phenotypes accrued through serial passage can be maintained upon transmission through mosquitoes (James et al. 1936; Coatney et al. 1961; Alger et al. 1971; Walliker et al. 1976; Barnwell et al. 1983), although occasional major losses (or gains) of virulence do occur (Alger et al. 1971; Walliker et al. 1976; Knowles & Walliker 1980).

When a single *P. chabaudi* genotype was serially passaged through whole-parasite immunized or immunologically naïve mice, there was a general reduction in

virulence across all lines following mosquito transmission (Mackinnon and Read 2004a). The virulence reduction observed in that study might be due to the deterministic forces of selection against virulent variants that have lost or reduced the ability to transmit through mosquitoes (Ebert et al. 1998). Another possibility is that virulence reductions observed following mosquito transmission are due to the systematic resetting during meiosis of the expression of genes that have been switched on or up-regulated during asexual serial passage (Mackinnon and Read 2004a). In order to replicate how virulence genes may be selected or maintained in real world sexual parasites, the most appropriate experimental design would include transmission through mosquitoes of selected lines between every passage.

Furthermore, selection for virulence may be an over adaptation to a single host genotype and therefore less adapted to outbred diverse population of host. Thus, it would be of particular interest to observe whether lines selected through one host genotype are also capable of inducing virulence in a different host genotype.

To confirm whether vaccination is likely to select for increased virulence, future experiments will serially passage *P. chabaudi* through AMA-1 immunized mice and test the virulence hypothesis in unvaccinated mice. That experiment will take all of the above into consideration to ensure the most accurate reflection of a real world scenario is obtained. Finally, the discovery of virulence genetic markers would allow one to track any adaptation of these genes in response to vaccination. The tools for doing this are being rapidly developed.

8.4.2 Vaccines

An obvious limitation of this thesis is that it is based on a limited number of parasite clones and only one candidate vaccine antigen. In Chapter 4 [Appendix 1 (Barclay et al. 2008b)], I show that the potency of a malaria vaccine may not be increased by including more than one antigen allele. However, combining different antigens directed against the same or different stages of the *Plasmodium* life cycle might increases vaccine efficacy (Richie & Saul 2002; Heppner et al. 2005; Garcia et al.

2006). It was also be interesting to investigate how these vaccines may alter the within host parasite dynamics.

8.4.3. Transmission

Parasite fitness is measured by parasite transmission. In *P. chabaudi*, asexual parasite density is generally found to be positively correlated with transmission success (Mackinnon & Read 1999b; de Roode et al. 2005) although there is one possible exception (Gadsby et al. 2008). One of the caveats of this thesis was that I did not measure gametocytes or transmission to mosquitoes. In future experiments it will be necessary to investigate not only how vaccination alters the selection of asexual parasites, but how vaccination impacts on transmission. This is possible due to advanced molecular techniques which can design gametocyte genotype-specific assays using real-time PCR (Wargo et al. 2006; Reece et al. 2008).

8.5. Concluding remarks

In conclusion, this thesis has contributed to the scientific community on how a malaria vaccine may impose selection on *Plasmodium* populations. As with all scientific research, my thesis has raised more questions. I hope that my work will be fruitful for those continuing after me, and that I can take forward the invaluable knowledge and techniques.

9. References

Alder, F. R. & Losada, J. M. 2002 Super-and co-infection: filling the range. In: Dieckmann,U Metz JAJ, Sabelis MW, Sigmund K, editors. *Adaptive Dynamics of Infectious Diseases. In Pursuit of Virulence Management*. Cambridge, Cambridge University Press.

Alger, N. E., Branton, M., Harvant, J. & Silverman, P. H. 1971 *Plasmodium berghei* NK65 in the inbred A/J mouse: variation in virulence in P. berghei demes. *J Protozoo* **18**, 598-601.

Alles, H. K., Mendis, K. N. & Carter, R. 1998 Malaria mortality rates in South Asia and in Africa: Implications for malaria control. *Parasitol Today* **14**, 369-375.

Anders, R. F. & Smythe, J. A. 1989 Polymorphic antigens in *Plasmodium falciparum*. *Blood* **74**, 1865-1875.

Anders, R. F., Crewther, P. E., Edwards, S., Margetts, M., Matthew, M. L. S. M., Pollock, B. & Pye, D. 1998 Immunisation with recombinant AMA-1 protects mice against infection with *Plasmodium chabaudi*. *Vaccine* **16**, 240-247.

Anderson, R. M. & May, R. M. 1982 Coevolution of hosts and parasites. *Parasitology* **85**, 411-426.

Anderson, R. M. & May, R. M. 1991 The ecology and genetics of host-parasite associations. *Infectious Diseases of Humans. Dynamics and Control.* Oxford University Press, Oxford.

Anderson, T. J. C., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I. D., Brockman, A. H., Nosten, F., Ferreira, M. U. & Day, K. P. 2000 Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* **17**, 1467-1482.

André, J. B. & Gandon, S. 2006 Vaccination, within-host dynamics, and virulence evolution. *Evolution* **60**, 13-23.

Antia, R., Yates, A. & De Roode, J. C. 2007 The dynamics of acute malaria infections. Effect of the parasite's red blood cell preference *Proc R Soc Lond B Biol Sci* **275**, 1449-1458.

Antonovics, J., Abbate, J. L., Baker, C. H., Daley, D., Hood, M. E., Jenkins, C. E., Johnson, L. J., Murray, J. J., Panjeti, V., Rudolf, V. H. W., Sloan, D. & Vondrasek, J. 2007 Evolution by any other name: Antibiotic resistance and avoidance of the E-word. *PLoS Biology* **5**, e30.

Arnot, D. 1998 Clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. *Trans R Soc Trop Med Hyg* **92**, 580-585.

Ash, C. 1996 Antibiotic resistance: The new apocalypse? *Trends Microbiol* **4**, 371-372.

Atkinson, W., Wolfe, C., Humiston, S. & Nelson, R 2000 *Epidemiology and Prevention of Vaccine-Preventable Disease* 6th Ed. Centers for Disease Control and Prevention, Atlanta.

Awadalla, P., Walliker, D., Babiker, H. & Mackinnon, M. 2001 The question of *Plasmodium falciparum* population structure. *Trends Parasitol* **17**, 351-353.

Babiker, H. A., Creasey, A. M., Fenton, B., Bayoumi, R. A. L., Arnot, D. E. & Walliker, D. 1991 Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. Diversity of enzymes, 2D-PAGE proteins and antigens. *Trans R Soc Trop Med Hyg* **85**, 572-577.

Babiker, H. A., Ranford-Cartwright, L. C. & Walliker, D. 1999 Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. *Trans R Soc Trop Med Hyg* **93**, 11-14.

Bai, T., Becker, M., Gupta, A., Strike, P., Murphy, V. J., Anders, R. F. & Batchelor, A. H. 2005 Structure of AMA1 from *Plasmodium falciparum* reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket. *Proc Natl Acad Sci U S A* **102**, 12736-12741.

Baird, J. K. 1998 Age-dependent characteristics of protection versus susceptibility to *Plasmodium falciparum*. *Ann Trop Med Parasitol* **92**, 367-90.

Ballou, W. R., Arevalo-Herrera, M., Carucci, D., Richie, T. L., Corradin, G., Diggs, C., Druilhe, P., Giersing, B. K., Saul, A., Heppner, D. G., Kester, K. E.,

Lanar, D. E., Lyon, J., Hill, A. V. S., Pan, W. & Cohen, J. D. 2004 Update on the clinical development of candidate malaria vaccines. *Am J Trop Med Hyg* **71**, 239-247.

Barclay, V. C., Råberg, L., Chan, B. H. K., Brown, S., Gray, D. & Read, A. F. 2008a CD4+T cells do not mediate within-host competition between genetically diverse malaria parasites. *Proc R Soc Lond B Biol Sci* **275**, 1171-1179.

Barclay, V. C., Chan, B. H. K., Anders, R. F. & Read, A. F. 2008b Mixed allele malaria vaccines: host protection and within-host selection. *Vaccine* **26**, 6099-6107.

Barnwell, J. W., Howard, R. J. & Miller, L. H. 1983 Influence of the spleen on the expression of surface antigens on parasitized erythrocytes. In: Evered D, Whelan, J, editors. *CIBA Foundation symposium on malaria and the red cell*. London: Pitman

Beale, G. H., Walliker, D. & Carter, R. 1978 Genetics. In: Killick-Kendrick R & Peters W editors. Rodent malaria. London: Academic Press, 1978. p213-45.

Bell, A. S., Roode, J. C., Sim, D. & Read, A. F. 2006 Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**, 1358-71.

Benedict, C. A., Norris, P. S. & Ware, C. F. 2002 To kill or be killed: viral evasion of apoptosis. *Nat Immunol* **3**, 1013-1018.

Bernard, H. U., Chan, S. Y., Manos, M. M., Ong, C. K., Villa, L. L., Delius, H., Peyton, C. L., Bauer, H. M. & Wheeler, C. M. 1994 Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* **170**, 1077-85.

Biggs, B. A., Gooza, L., Wycherley, K., Wollish, W., Southwell, B., Leech, J. H. & Brown, G. V. 1991 Antigenic variation in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* **88**, 9171-9174.

Blaxter, M. L., Page, A. P., Rudin, W. & Maizels, R. M. 1992 Nematode surface coats: Actively evading immunity. *Parasitol Today* **8**, 243-247.

Bodescot, M., Silvie, O., Siau, A., Refour, P., Pino, P., Franetich, J.-F., Hannoun, L., Sauerwein, R. & Mazier, D. 2004 Transcription status of vaccine candidate

genes of *Plasmodium falciparum* during the hepatic phase of its life cycle. *Parasitol Res* **92**, 449-452.

Bojang, K. A., Milligan, P. J. M., Pinder, M., Vigneron, L., Alloueche, A., Kester, K. E., Ballou, W. R., Conway, D. J., Reece, W. H. H., Gothard, P., Yamuah, L., Delchambre, M., Voss, G., Greenwood, B. M., Hill, A., McAdam, K. P. W. J., Tornieporth, N., Cohen, J. D. & Doherty, T. 2001 Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial. *The Lancet* **358**, 1927-1934.

Bolad, A. & Berzins, K. 2000 Antigenic diversity of *Plasmodium falciparum* and antibody-mediated parasite neutralization. *Scand J Immunol* **52**, 233-239.

Boni, M. F. 2008 Vaccination and antigenic drift in influenza. *Vaccine* **26**, C8-C14.

Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R., Shan, K. V. & International Biological Study on Cervical Cancer Study, G. 1995 Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* **87**, 796-802.

Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F. & Druilhe, P. 1995 Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *J Exp Med* **182**, 409-418.

Bremermann, H. J. & Pickering, J. 1983 A game-theoretical model of parasite virulence. *J Theor Biol* **100**, 411-426.

Brown, K. N. & Brown, I. N. 1965 Immunity to malaria.Antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature* **208**, 1286-1288.

Bruce, M. C., Donnelly, C. A., Alpers, M. P., Galinski, M. R., Barnwell, J. W., Walliker, D. & Day, K. P. 2000 Cross-species interactions between malaria parasites in humans. *Science* **287**, 845-848.

Buckling, A. & Read, A. F. 2001 The effect of partial host immunity on the transmission of malaria parasites. *Proc R Soc Lond B Biol Sci* **268**, 2325-2330.

Bull, J. J. 1994 Perspective: Virulence. *Evolution* **48**, 1423-1437.

Bull, P. C. & Marsh, K. 2002 The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. *Trends Microbiol* **10**, 55-58.

Bull, P. C., Pain, A., Ndungu, F. M., Kinyanjui, S. M., Roberts, D. J., Newbold, C. I. & Marsh, K. 2005 *Plasmodium falciparum* antigenic variation: relationships between in vivo selection, acquired antibody response, and disease severity. *J Infect Dis* **192**, 1119-1126.

Burns, J. J. M., Flaherty, P. R., Romero, M. M. & Weidanz, W. P. 2003 Immunization against *Plasmodium chabaudi* malaria using combined formulations of apical membrane antigen-1 and merozoite surface protein-1. *Vaccine* **21**, 1843-1852.

Burns, J. M., Jr., Flaherty, P. R., Nanavati, P. & Weidanz, W. P. 2004 Protection against *Plasmodium chabaudi* malaria induced by immunization with Apical Membrane Antigen 1 and Merozoite Surface Protein 1 in the absence of gamma interferon or interleukin-4. *Infect Immun* **72**, 5605-5612.

Cardigan, F. C. & Chaicumpa, V. 1969 *Plasmodium falciparum* in the white-handed gibbon: protection afforded by previuos infection with homologous and heterologous starins obtained in Thailand. *Military Medicine* **134**, 1135-1139.

Carlton, J., Mackinnon, M. & Walliker, D. 1998 A chloroquine resistance locus in the rodent malaria parasite *Plasmodium chabaudi*. *Mol Bio Para* **93**, 57-72.

Carlton, J. M., Angiuoli, S. V., Suh, B. B., Kooij, T. W., Pertea, M., Silva, J. C., Ermolaeva, M. D., Allen, J. E., Selengut, J. D., Koo, H. L., Peterson, J. D., Pop, M., Kosack, D. S., Shumway, M. F., Bidwell, S. L., Shallom, S. J., van Aken, S. E., Riedmuller, S. B., Feldblyum, T. V., Cho, J. K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L. M., Florens, L., Yates, J. R., Raine, J. D., Sinden, R. E., Harris, M. A., Cunningham, D. A., Preiser, P. R., Bergman, L. W., Vaidya, A. B., van Lin, L. H., Janse, C. J., Waters, A. P., Smith, H. O., White, O. R., Salzberg, S. L., Venter, J. C., Fraser, C. M., Hoffman, S. L., Gardner, M. J. & Carucci, D. J. 2002 Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii. *Nature* **419**, 512-519.

Carlton, J. M. R., Hayton, K., Cravo, P. V. L. & Walliker, D. 2001 Of mice and malaria mutants: unravelling the genetics of drug resistance using rodent malaria models. *Trends Parasitol* **17**, 236-242.

Carman, W. F., Karayiannis, P., Waters, J., Thomas, H. C., Zanetti, A. R., Manzillo, G. & Zuckerman, A. J. 1990 Vaccine-induced escape mutant of hepatitis B virus. *Lancet* **336**, 325-329.

Carter, R. & Walliker, D. 1975 New observations on the malaria parasites of the Central African Republic: *Plasmodium vinckei petteri* subsp. nov. and *Plasmodium chabaudi* Landau. *Ann Trop Med Parasitol* **69**, 187-196.

Carter, R. & Walliker, D. 1976 Malaria parasites of rodents of the Congo (Brazzaville). *Ann Parasitol Hum Comp* **51**, 637-646.

Carter, R. 1978. Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei*, *P. yoelli*, *P. vinckei* and *P. chabaudi* by starch gel electrophoresis. *Parasitology* **76**, 241-267

Carvalho, L. J. M., Daniel-Ribeiro, C. T. & Goto, H. 2002 Malaria vaccine: candidate antigens, mechanisms, constraints and prospects. *Scand J Immunol* **56**, 327-343.

Centres for Disease Control and Prevention 2006 Press Release: CDC's Advisory committee recommends human papillomavirus virus vaccination. Available at: www.cdc.gov/od/oc/media/pressrel/r060629.htm.

Chang, K.-H. & Stevenson, M. M. 2004 Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int J Parasitol* **34**, 1501-1516.

Chatterjee, S., Perignon, J., Van Marck, E. & Druilhe, P. 2006 How reliable are models for malaria vaccine development? Lessons from irradiated sporozoite immunizations *J Postgrad Med* **52**, 321-4.

Cheesman, S., Raza, A. & Carter, R. 2006 Mixed strain infections and strain-specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. *Infect Immun* **74**, 2996-3001.

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S. & Wahlgren, M. 1998 Identification of *Plasmodium*

falciparum Erythrocyte Membrane Protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. *J Exp Med* **187**, 15-23.

Cheng, Q. & Saul, A. 1994 Sequence analysis of the apical membrane antigen I (AMA-1) of *Plasmodium vivax*. *Mol Biochem Parasitol* **65**, 183-187.

Chotivanich, K., Udomsangpetch, R., Simpson, J. A., Newton, P., Pukrittayakamee, S., Looareesuwan, S. & White, N. J. 2000 Parasite multiplication potential and the severity of *falciparum* malaria. *J Infect Dis* **181**, 1206-1209.

Clough, B., Atilola, F. A. & Pasvol, G. 1998 The role of rosetting in the multiplication of *Plasmodium falciparum*: rosette formation neither enhances nor targets parasite invasion into uninfected red cells. *Br J Haematol* **100**, 99-104.

Coatney, G. R., Elder, H. A., Contacos, P. G., Getz, M. E. & Greenland, R. e. a. 1961 Transmission of the M strain of *Plasmodium cynomolgi* to man. *Am J Trop Med Hyg* **10**, 673-678.

Coleman, P. 2006 Detecting hepatits B surface antigen mutants. *Emerg Infect Dis* **12**, 198-203.

Coley, A. M., Gupta, A., Murphy, V. J., Bai, T., Kim, H., Anders, R. F., Foley, M. & Batchelor, A. H. 2007 Structure of the malaria antigen AMA1 in complex with a growth-inhibitory antibody. *PLoS Pathogens* **3**, e138.

Collins, W. E., Pye, D., Crewther, P. E., Vandenberg, K. L., Galland, G. G., Sulzer, A. J., Kemp, D. J., Edwards, S. J., Coppel, R. L., Sullivan, J. S., Morris, C. L. & Anders, R. F. 1994 Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of *Plasmodium fragile*. *Am J Trop Med Hyg* **51**, 711-719.

Collins, W. E. & Jeffery, G. M. 1999 A retrospective examination of sporozoite- and trophozoite-induced infections with *Plasmodium falciparum*: development of parasitologic and clinical immunity during primary infection. *Am J Trop Med Hyg* **61**, 4-19.

Collins, C. R., Withers-Martinez, C., Bentley, G. A., Batchelor, A. H., Thomas, A. W. & Blackman, M. J. 2007 Fine mapping of an epitope recognized by an invasion-inhibitory monoclonal antibody on the malaria vaccine candidate Apical Membrane Antigen 1. *J Biol Chem* **282**, 7431-7441.

Conway, D. J., Greenwood, B. M. & McBride, J. S. 1991 The epidemiology of multiple-clone *Plasmodium falciparum* infections in Gambian patients. *Parasitology* **103**, 1-6.

Corley, L. S. & Strand, M. R. 2003 Evasion of encapsulation by the polyembryonic parasitoid Copidosoma floridanum is mediated by a polar body-derived extraembryonic membrane. *J Invertebr Pathol* **83**, 86-89.

Cortes, A., Mellombo, M., Mueller, I., Benet, A., Reeder, J. C. & Anders, R. F. 2003 Geographical structure of diversity and differences between symptomatic and asymptomatic infections for *Plasmodium falciparum* vaccine candidate AMA1. *Infect Immun* **71**, 1416-1426.

Cortes, A., Mellombo, M., Benet, A., Lorry, K., Rare, L. & Reeder, J. C. 2004 *Plasmodium falciparum*: distribution of msp2 genotypes among symptomatic and asymptomatic individuals from the Wosera region of Papua New Guinea. *Exp Parasitol* **106**, 22 - 29.

Cortes, A., Mellombo, M., Masciantonio, R., Murphy, V. J., Reeder, J. C. & Anders, R. F. 2005 Allele specificity of naturally acquired antibody responses against *Plasmodium falciparum* apical membrane antigen 1. *Infect Immun* **73**, 422-430.

Cowman, A. F. & Crabb, B. S. 2006 Invasion of red blood cells by malaria parasites. *Cell* **124**, 755-766.

Cox-Singh, J., Davis, T. M. E., Lee, K.-S., Shamsul, S. S. G., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. & Singh, B. 2008 *Plasmodium knowlesi* Malaria in humans Is widely distributed and potentially life threatening. *Clin Infect Dis* **46**, 165-171.

Crewther, P. E., Matthew, M. L., Flegg, R. H. & Anders, R. F. 1996 Protective immune responses to apical membrane antigen 1 of *Plasmodium chabaudi* involve recognition of strain-specific epitopes. *Infect Immun* **64**, 3310-3317.

Darko, C. A., Angov, E., Collins, W. E., Bergmann-Leitner, E. S., Girouard, A. S., Hitt, S. L., McBride, J. S., Diggs, C. L., Holder, A. A., Long, C. A., Barnwell, J. W. & Lyon, J. A. 2005 The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of Plasmodium falciparum strain

FVO expressed in Escherichia coli protects Aotus nancymai against challenge with homologous erythrocytic-stage parasites. *Infect Immun* **73**, 287-297.

Daubersies, P., Sallenave-Sales, S., Magne, S., Trape, J.-F., Contamin, H., Fandeur, T., Rogier, C., Mercereau-Puijalon, O. & Druilhe, P. 1996 Rapid turnover of *Plasmodium falciparum* populations in asymptomatic individuals living in a high transmission area. *Am J Trop Med Hyg* **54**, 18-26.

Davidson, F. & Nair, L. 2004 *Marek's Disease an Evolving Problem*. London, Elsevier Academic Press.

Day, K. P. & Marsh, K. 1991 Naturally acquired immunity to *Plasmodium falciparum*. *Immunol Today* **12**, A68-71.

Day, T., Galvani, A., Struchiner, C. & Gumel, A. 2008 The evolutionary consequences of vaccination. *Vaccine* **26**, C1-C3.

de Roode, J. C., Read, A. F., Chan, H. K. & Mackinnon, M. J. 2003 Rodent malaria parasites suffer from the presence of con-specific clones in three-clone *Plasmodium chabaudi* infections. *Parasitology* **127**, 411-418.

de Roode, J. C., Culleton, R., Bell, A. S. & Read, A. F. 2004a Competitive release of drug resistance following drug treatment of mixed *Plasmodium chabaudi* infections. *Malaria Journal* **3**, 33.

de Roode, J. C., Culleton, R., Cheesman, S. J., Carter, R. & Read, A. F. 2004b Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. *Proc R Soc Lond B Biol Sci* **271**, 1073-1080.

de Roode, J. C., Helinski, M. E. H., Anwar, M. A. & Read, A. F. 2005a Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am Nat* **166**, 531-542.

de Roode, J. C., Pansini, R., Cheesman, S. J., Helinski, M. E. H., Huijben, S., Wargo, A. R., Bell, A. S., Chan, B. H. K., Walliker, D. & Read, A. F. 2005b Virulence and competitive ability in genetically diverse malaria infections. *Proc Natl Acad Sci U S A* **102**, 7624-7628.

Deans, A.-M., Lyke, K. E., Thera, M. A., Plowe, C. V., Kone, A., Doumbo, O. K., Kai, O., Marsh, K., Mackinnon, M. J., Raza, A. & Rowe, J. A. 2006 Low multiplication rates of African *Plasmodium falciparum* isolates and lack of

association of multiplication rates and red blood cell sensitivity with malaria virulence *Am J Trop Med Hyg* **74**, 554-563.

Deans, J. A., Knight, A. M., Jean, W. C., Waters, A. P., Cohen, S. & Mitchell, G. H. 1988 Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66kD merozoite antigen. *Parasite Immunol* **10**, 535-552.

Dieckman, U., Metz, H., Sabelis, M. W. & Sigmund, K., editors. 2002 The Adaptive Dynamics of Pathogen-Host Interactions. Cambridge: Cambridge University Press. 532 p.

Doolan, D., Sedegah, M., Hedstrom, R. C., Hobart, P., Charoenvit, Y. & Hoffman, S. L. 1996 Circumventing genetic restriction of protection against malaria with muligene DNA immunization: CD8+ cell-interferon gamma-, and nitric oxide-dependent immunity. *J Exp Med* **183**, 1739-1746.

Doolan, D. L. & Hoffman, S. L. 2001 DNA-based vaccines against malaria: status and promise of the multi-Stage malaria DNA vaccine operation. *Int J Parasitol* **31**, 753-762.

Doolan, D. L., Aguiar, J. C., Weiss, W. R., Seete, A., Lelgner, P. L., Regis, D. P., Quinones-Casas, P., Yates 3rd, J. R., Blair, P. L., L, R. T., Hoffman, S. L. & Carucci, D. L. 2003 Utilization of genomic sequence information to develop malaria vaccines. *Exp Biol* **206**, 3789-3802.

Duan, J., Mu, J., Thera, M. A., Joy, D., Kosakovsky Pond, S. L., Diemert, D., Long, C., Zhou, H., Miura, K., Ouattara, A., Dolo, A., Doumbo, O., Su, X.-z. & Miller, L. 2008 Population structure of the genes encoding the polymorphic *Plasmodium falciparum* apical membrane antigen 1: Implications for vaccine design. *Proc Natl Acad Sci USA* **105**, 7857-7862.

Dutta, S., Lee, S. Y., Batchelor, A. H. & Lanar, D. E. 2007 Structural basis of antigenic escape of a malaria vaccine candidate. *Proc Natl Acad Sci USA* **104**, 12488-12493.

Dzikowski, R. & Deitsch, K. 2006 Antigenic variation by protozoan parasites: insights from *Babesia bovis*. *Mol Microbiol* **59**, 364-366.

Dzikowski, R., Templeton, T. J. & Deitsch, K. 2006 Variant antigen gene expression in malaria. *Cell Microbiol* **8**, 1371-1381.

Ebert, D. & Herre, E. A. 1996 The evolution of parasitic diseases. *Parasitol Today* **12**, 96-101.

Ebert, D. 1998 Experimental evolution of parasites. *Science* **282**, 1432-1436.

Eisenhut, M. 2007 Immunity to blood stages of *Plasmodium falciparum* is dependent on a specific pattern of immunoglobulin subclass responses to multiple blood stage antigens. *Med Hypotheses* **69**, 804-808.

Elomaa, A., Advani, A., Donnelly, D., Antila, M., Mertsola, J., Hallander, H. & He, Q. 2005 Strain variation among *Bordetella pertussis* isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 Years. *J Clin Microbiol* **43**, 3681-3687.

Engelbrecht, F., Felger, I., Genton, B., Alpers, M. & Beck, H. P. 1995 *Plasmodium falciparum*: malaria morbidity is associated with specific merozoite surface antigen 2 genotypes. *Exp Parasitol* **81**, 90-96.

Escalante, A. A., Lal, A. A. & Ayala, F. J. 1998 Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. *Genetics* **149**, 189-202.

Ewald, P. W. 1994 *Evolution of Infectious Diseases*. Oxford, Oxford University Press.

Ewald, P. W. 1996 Vaccines as evolutionary tools: the virulecne-antigen strategy. In: Kaufmann SH E, editor. *Concepts in Vaccine Development*. Berlin, de Gruyter & Co.

Ewald, P. W. 2002 Virulence management in humans. In: Dieckmann U, Metz JAJ, Sabelis MW, Sigmundand K, editors. *Adaptive Dynamics of Infectious Diseases. In Pursuit of Virulence Management*. Cambridge, Cambridge University Press

Faber, B. W., Remarque, E. J., Morgan, W. D., Kocken, C. H. M., Holder, A. A. & Thomas, A. W. 2007 Malaria vaccine-related benefits of a single protein comprising *Plasmodium falciparum* Apical Membrane Antigen 1 domains I and II fused to a modified form of the 19-Kilodalton C-terminal fragment of Merozoite Surface Protein 1. *Infect Immun* **75**, 5947-5955.

FDA. 2006 US Food and Drug Administration. FDA licenses new vaccine for prevention of cervical cancer and other diseases in females caused by human papillomavirus:www.cdc.gov/od/oc/media/pressrel/r060629.htm.

Feng, Z.-P., Keizer, D. W., Stevenson, R. A., Yao, S., Babon, J. J., Murphy, V. J., Anders, R. F. & Norton, R. S. 2005 Structure and inter-domain interactions of domain II from the blood-stage malarial protein, apical membrane antigen 1. *J Mol Biol* **350**, 641-656.

Fenner, F. & Ratcliffe, F. N. 1965 *Myxomatosis*, Cambridge University Press.

Fenner, F. & Myers, K. 1978 Myxoma virus and myxomatosis in retrospect: the first quarter century of a new disease. In: Kurstak E, Maramorosch K, editors. *Viruses and Environment*. New York, Academic.

Fenner, F. 1983 Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B Biol Sci*.

Fenner, F. & Kerr, P. J. 1994 Evolution of poxviruses, including the coevoluiton of virus and host in myxomatosis. In: Morse SS, editor. The Evolutionary Biology of Viruses. New York, Raven Press.

Ferguson, H. M. & Read, A. F. 2002 Genetic and environmental determinants of malaria parasite virulence in mosquitoes. *Proc R Soc Lond B Biol Sci* **269**, 1217-1224.

Ferguson, N. M., Galvani, A. P. & Bush, R. M. 2003 Ecological and immunological determinants of influenza evolution. *Nature* **422**, 428-433.

Ferreira, M. U., da Silva Nunes, M. & Wunderlich, G. 2004 Antigenic diversity and immune evasion by malaria parasites. *Clin Diagn Lab Immunol* **11**, 987-995.

Finlay, B. B. & McFadden, G. 2006 Anti-immunology: Evasion of the host immune system by bacterial and viral pathogens. *Cell* **124**, 767-782.

FitzSimons, D., Francois, G., Hall, A., McMahon, B., Meheus, A., Zanetti, A., Duval, B., Jilg, W., Bocher, W. O., Lu, S.-N., Akarca, U., Lavanchy, D., Goldstein, S., Banatvala, J. & Damme, P. V. 2005 Long-term efficacy of hepatitis B vaccine, booster policy, and impact of hepatitis B virus mutants. *Vaccine* **23**, 4158-4166.

Flannery, B., Heffernan, R. T., Harrison, L. H., Ray, S. M., Reingold, A. L., Hadler, J., Schaffner, W., Lynfield, R., Thomas, A. R., Li, J., Campsmith, M.,

Whitney, C. G. & Schuchat, A. 2006 Changes in invasive pneumococcal disease among HIV-infected adults living in the era of childhood pneumococcal immunization. *Ann Intern Med* **144**, 1-9.

Florens, L., Washburn, M. P., Raine, J. D., Anthony, R. M., Grainger, M., Haynes, J. D., Moch, J. K., Muster, N., Sacci, J. B., Tabb, D. L., Witney, A. A., Wolters, D., Wu, Y., Gardner, M. J., Holder, A. A., Sinden, R. E., Yates, J. R. & Carucci, D. J. 2002 A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* **419**, 520-526.

Fluck, C., Smith, T., Beck, H.-P., Irion, A., Betuela, I., Alpers, M. P., Anders, R., Saul, A., Genton, B. & Felger, I. 2004 Strain-specific humoral response to a polymorphic malaria vaccine. *Infect Immun* **72**, 6300-6305.

Fluck, C., Schopflin, S., Smith, T., Genton, B., Alpers, M. P., Beck, H.-P. & Felger, I. 2007 Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. *Infect Genet Evol* **7**, 44-51.

Francis, S. E., Malkov, V. A., Oleinikov, A. V., Rossnagle, E., Wendler, J. P., Mutabingwa, T. K., Fried, M. & Duffy, P. E. 2007 Six genes are preferentially transcribed by the circulating and sequestered forms of *Plasmodium falciparum* parasites that infect pregnant women. *Infect Immun* **75**, 4838-4850.

Francois, G., Kew, M., Van Damme, P., Mphahlele, M. J. & Meheus, A. 2001 Mutant hepatitis B viruses: a matter of academic interest only or a problem with far-reaching implications? *Vaccine* **19**, 3799-3815.

Frank, S. A. 1996 Models of parasite virulence. *Q Rev Biol* **71**, 37-78.

Frank, S. A. & Schmid-Hempel, P. 2008 Mechanisms of pathogenesis and the evolution of parasite virulence. *J Evol Biol* **21**, 396-404.

Fraser, T. S., Kappe, S. H. I., Narum, D. L., VanBuskirk, K. M. & Adams, J. H. 2001 Erythrocyte-binding activity of *Plasmodium yoelii* apical membrane antigen-1 expressed on the surface of transfected COS-7 cells. *Mol Bio Para* **117**, 49-59.

Gandon, S., Mackinnon, M. J., Nee, S. & Read, A. F. 2001 Imperfect vaccines and the evolution of pathogen virulence. *Nature* **414**, 751-756.

Gandon, S., Mackinnon, M. J., Nee, S. & Read, A. F. 2002 Microbial evolution (Communication arising): Antitoxin vaccines and pathogen virulence. *Nature* **417**, 610-610.

Gandon, S. & Day, T. 2003 Understanding and managing pathogen evolution: a way forward. *Trends Microbiol* **11**, 206-207.

Gandon, S. & Day, T. 2007 The evolutionary epidemiology of vaccination. *J Roy Soc Int* **4**, 803-817.

Ganusov, V. V. & Antia, R. 2006 Imperfect vaccines and the evolution of pathogens causing acute infections in invertebrates. *Evolution* **60**, 957-969.

Garamszegi, L. Z. 2006 The evolution of virulence and host specialization in malaria parasites of primates. *Ecol Lett* **9**, 933-940.

Garcia, J. E., Puentes, A. & Patarroyo, M. E. 2006 Developmental biology of sporozoite-host interactions in *Plasmodium falciparum* malaria: Implications for vaccine design. *Clin Microbiol Rev* **19**, 686-707.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. & Barrell, B. 2002 Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**, 498-511.

Garnham, P. C. C. 1966 Malaria parasites and other haemosporidia. Oxford, Blackwell Scientific Publications.

Genton, B., Betuela, I., Felger, I., Al-Yaman, F., Anders, R. F., Saul, A., Rare, L., Baisor, M., Lorry, K., Brown, G. V., Pye, D., Irving, D. O., Smith, T. A., Beck, H.-P. & Alpers, M. P. 2002 A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis* **185**, 820-7.

Genton, B., Anders, R. F., Alpers, M. P. & Reeder, J. C. 2003 The malaria vaccine development program in Papua New Guinea. *Trends Parasitol* **19**, 264-270.

Genton, B. & Reed, Z. H. 2007 Asexual blood-stage malaria vaccine development: facing the challenges. *Curr Opin Infect Dis* **20**, 467-475.

Ghany, M. G., Ayola, B., Villamil, F. G., Gish, R. G., Rojter, S., Vierling, J. M. & Lok, A. S. 1998 Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. *Hepatology* **27**, 213-222.

Gibb, R., Nimmo, G., O'Loughlin, P. & Lowe, D. 2007 Detection of HBsAg mutants in a population with a low prevalence of hepatitis B virus infection. *J Med Virol* **79**, 351-355.

Gog, J. R. 2008 The impact of evolutionary constraints on influenza dynamics. *Vaccine* **26**, C15-C24.

Good, M. F. & Doolan, D. L. 1999 Immune effector mechanisms in malaria. *Curr Opin Immunol* **11**, 412-419.

Gravenor, M. B., Mclean, A. R. & Kwiatkowski, D. 1995 The regulation of malaria parasitemia-parameter estimates for a population-model. *Parasitology* **110**, 115-122.

Gray, J. C., Corran, P. H., Mangia, E., Gaunt, M. W., Li, Q., Tetteh, K. K. A., Polley, S. D., Conway, D. J., Holder, A. A., Bacarese-Hamilton, T., Riley, E. M. & Crisanti, A. 2007 Profiling the antibody immune response against blood stage malaria vaccine candidates. *Clin Chem* **53**, 1244-1253.

Grech, K., Watt, K. & Read, A. F. 2006 Host-parasite interactions for virulence and resistance in a malaria model system. *J Evol Biol* **19**, 1620-1630.

Grech, K., Chan, B. H. K., Anders, R. F. & Read, A. F. 2008 The impact of immunization on competition within *Plasmodium* infections *Evolution* **62**, 2359-2371.

Grech, K., Anders, R. F., Chan, B. H. K., Cheesman, S. & Read, A. F. 2008 *in prep* The impact of virulence and genetic diversity in *Plasmodium chabaudi* on vaccination with AMA-1 protein

Greenwood, B. 2005 Malaria vaccines: Evaluation and implementation. *Acta Trop* **95**, 298-304.

Gurarie, D., Zimmerman, P. A. & King, C. H. 2006 Dynamic regulation of single- and mixed-species malaria infection: Insights to specific and non-specific mechanisms of control. *J Theor Biol* **240**, 185-199.

Hall, N., Karras, M., Raine, J. D., Carlton, J. M., Kooij, T. W. A., Berriman, M., Florens, L., Janssen, C. S., Pain, A., Christophides, G. K., James, K., Rutherford, K., Harris, B., Harris, D., Churcher, C., Quail, M. A., Ormond, D., Doggett, J., Trueman, H. E., Mendoza, J., Bidwell, S. L., Rajandream, M.-A., Carucci, D. J., Yates, J. R., III, Kafatos, F. C., Janse, C. J., Barrell, B., Turner, C. M. R., Waters, A. P. & Sinden, R. E. 2005 A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science* **307**, 82-86.

Hart, A. R. & Cloyd, M. W. 1990 Interference patterns of human immunodeficiency viruses HIV-1 and HIV-2. *Virology* **177**, 1-10.

Hastings, I. M. 1997 A model for the origins and spread of drug-resistant malaria *Parasitology* **115**, 133-41.

Hastings, I. M. & D'Alessandro, U. 2000 Modelling a predictable disaster: The rise and spread of drug-resistant malaria. *Parasitol Today* **16**, 340-347.

Hastings, I. M. 2003 Malaria control and the evolution of drug resistance: an intriguing link. *Trends Parasitol* **19**, 70-73.

Hastings, I. M. 2006 Complex dynamics and stability of resistance to antimalarial drugs. *Parasitology* **132** 615-624.

Healer, J., Murphy, V., Hodder, A. N., Masciantonio, R., Gemmill, A. W., Anders, R. F., Cowman, A. F. & Batchelor, A. 2004 Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. *Mol Microbiol* **52**, 159-168.

Hehl, A. B., Lekutis, C., Grigg, M. E., Bradley, P. J., Dubremetz, J.-F., Ortega-Barria, E. & Boothroyd, J. C. 2000 *Toxoplasma gondii* homologue of *Plasmodium* apical membrane antigen 1 is involved in invasion of host cells. *Infect Immun* **68**, 7078-7086.

Hellriegel, B. 1992 Modelling the immune response to malaria with ecological concepts: Short-term behaviour against long-term equilibrium. *Proceedings of the Royal Society Biological Sciences Series B* **250**, 249-256.

Hentschel, C. C. 2002 The medicines for malaria venture. *Nature* **415**, 715-715.

Heppner, J. D. G., Kester, K. E., Ockenhouse, C. F., Tornieporth, N., Ofori, O., Lyon, J. A., Stewart, V. A., Dubois, P., Lanar, D. E., Krzych, U., Moris, P., Angov, E., Cummings, J. F., Leach, A., Hall, B. T., Dutta, S., Schwenk, R., Hillier, C., Barbosa, A., Ware, L. A., Nair, L., Darko, C. A., Withers, M. R., Ogutu, B., Polhemus, M. E., Fukuda, M., Pichyangkul, S., Gettyacamin, M., Diggs, C., Soisson, L., Milman, J., Dubois, M.-C., Garcon, N., Tucker, K., Wittes, J., Plowe, C. V., Thera, M. A., Duombo, O. K., Pau, M. G., Goudsmit, J., Ballou, W. R. & Cohen, J. 2005 Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine* **23**, 2243-2250.

Hetzel, C. & Anderson, R. 1996 The within-host cellular dynamics of blood-stage malaria: theoretical and experimental studies. *Parasitology* **113**, 25-38.

Hisaeda, H., Yasutomo, K. & Himeno, K. 2005 Malaria: immune evasion by parasites. *Int J Biochem Cell Biol* **37**, 700-706.

Hodder, A. N., Crewther, P. E., Matthew, M. L. S. M., Reid, G. E., Moritz, R. L., Simpson, R. J. & Anders, R. F. 1996 The disulfide bond structure of *Plasmodium* apical membrane antigen-1. *J Biol Chem* **271**, 29446-29452.

Hodder, A. N., Crewther, P. E. & Anders, R. F. 2001 Specificity of the protective antibody response to apical membrane antigen 1. *Infect Immun* **69**, 3286-3294.

Hoffmann, S. L. & Miller, L. H. 1996 Malaria Vaccine Development: a multi-immune response approach. In: Hoffman, SL, editor. American Society for Microbiology. Washington, DC

Holder, A. A. 1999 Malaria vaccines. *Proc Natl Acad Sci USA* **96**, 1167-1169.

Holt, R. D. 1977 Predation, apparent competition, and the structure of prey communities. *Theor Popul Biol* **12**, 197-229.

Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M. C., Wides, R., Salzberg, S. L., Loftus, B., Yandell, M., Majoros, W. H., Rusch, D. B., Lai, Z., Kraft, C. L., Abril, J. F., Anthouard, V., Arensburger, P., Atkinson, P. W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R.,

Boscus, D., Barnstead, M., Cai, S., Center, A., Chatuverdi, K., Christophides, G. K., Chrystal, M. A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C. A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M. E., Hladun, S. L., Hogan, J. R., Hong, Y. S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.-J., Lobo, N. F., Lopez, J. R., Malek, J. A., McIntosh, T. C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S. D., O'Brochta, D. A., Pfannkoch, C., Qi, R., Regier, M. A., Remington, K., Shao, H., Sharakhova, M. V., Sitter, C. D., Shetty, J., Smith, T. J., Strong, R., Sun, J., Thomasova, D., Ton, L. Q., Topalis, P., Tu, Z., Unger, M. F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K. J., Wortman, J. R., Wu, M., Yao, A., Zdobnov, E. M., Zhang, H., Zhao, Q., et al. 2002 The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* **298**, 129-149.

Hornef, M. W., Wick, M. J., Rhen, M. & Normark, S. 2002 Bacterial strategies for overcoming host innate and adaptive immune responses. *Nature Immunol* **3**, 1033-1040.

Howell, S. A., Hackett, F., Jongco, A. M., Withers-Martinez, C., Kim, K., Carruthers, V. B. & Blackman, M. J. 2005 Distinct mechanisms govern proteolytic shedding of a key invasion protein in apicomplexan pathogens. *Mol Microbiol* **57**, 1342-1356.

Hu, J., Chen, Z., Gu, J., Wan, M., Shen, Q., Kieny, M.-P., He, J., Li, Z., Zhang, Q., Reed, Z. H., Zhu, Y., Li, W., Cao, Y., Qu, L., Cao, Z., Wang, Q., Liu, H., Pan, X., Huang, X., Zhang, D., Xue, X. & Pan, W. 2008 Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. *PLoS ONE* **3**, e1952.

Huang, S. S., Platt, R., Rifas-Shiman, S. L., Pelton, S. I., Goldmann, D. & Finkelstein, J. A. 2005 Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* **116**, e408-413.

Hviid, L., Kurtzhals, J. A., Goka, B. Q., Oliver-Commey, J. O., Nkrumah, F. K. & Theander, T. G. 1997 Rapid reemergence of T cells into peripheral circulation following treatment of severe and uncomplicated Plasmodium falciparum malaria. *Infect Immun* **65**, 4090-4093.

Hyde, J. E. 2005 Drug-resistant malaria. *Trends Parasitol* **21**, 494-498.

Jacobs, R. L. 1964 Role of p-aminobenzoic acid in Plasmodium berghei infection in the mouse. *Exp Parasitol* **15**, 213-225.

Jafari, S., Le Bras, J., Bouchaud, O. & Durand, R. 2004 *Plasmodium falciparum* clonal population dynamics during malaria treatment. *J Infect Dis* **189**, 195-203.

Jakeman, G., Saul, A., Hogarth, W. & Collins, W. 1999 Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology* **119**, 127-33.

James, S. P., Nicol, W. D. & Shute, P. G. 1936 Clinical and parasitological observations on induced malaria. *Proc R Soc Med* **29**, 879-894.

Jarra, W., Brown, K. N. 1985 Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. The effectiveness and inter-and intra-species specificity of immunity induced by infection. *Parasite Immunol* **7**, 595-606.

Jarra, W. & Brown, K. N. 1989 Invasion of mature and immature erythrocytes of CBA/ca mice by a cloned line of *Plasmodium chabaudi chabaudi*. *Parasitology* **99**, 157-163.

Jeffery, G. M. 1966 Epidemiological significance of repeated infections with homologous and heterologous starins and species of *Plasmodium*. *WHO Bulletin* **35**, 873-882.

Johnson, A. H., Leke, R. G. F., Mendell, N. R., Shon, D., Suh, Y. J., Bomba-Nkolo, D., Tchinda, V., Kouontchou, S., Thuita, L. W., van der Wel, A. M., Thomas, A., Stowers, A., Saul, A., Zhou, A., Taylor, D. W. & Quakyi, I. A. 2004 Human leukocyte antigen class II alleles influence levels of antibodies to the *Plasmodium falciparum* asexual-stage apical membrane antigen 1 but not to merozoite surface antigen 2 and merozoite surface protein 1. *Infect Immun* **72**, 2762-2771.

Jones, T. R., Obaldia, N. r., Gramzinski, R. A. & Hoffman, S. L. 2000 Repeated exposure of *Aotus* monkeys with *Plasmodium falciparum* induces protection against subsuquent challenge with homologous and hetrologous strains of parasite. *Am J Trop Med Hyg* **62**, 675-80.

Kappe, S. H. I. & Adams, J. H. 1996 Sequence analysis of the apical membrane antigen-1 genes (ama-1) of *Plasmodium yoelii yoelii* and *Plasmodium berghei*. *Mol Bio Para* **78**, 279-283.

Kennedy, M. C., Wang, J., Zhang, Y., Miles, A. P., Chitsaz, F., Saul, A., Long, C. A., Miller, L. H. & Stowers, A. W. 2002 In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): Production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun* **70**, 6948-6960.

Kester, K. E., McKinney, D. A., Tornieporth, N., Ockenhouse, C. F., Heppner, D. G., Hall, T., Krzych, U., Delchambre, M., Voss, G., Dowler, M. G., Palensky, J., Wittes, J., Cohen, J. & Ballou, W. R. 2001 Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental *Plasmodium falciparum* Malaria. *J Infect Dis* **183**, 640-647.

Knowles, D. & Walliker, D. 1980 Variable expression of virulence in the rodent malaria parasite *Plasmodium yoeli yoeli*. *Parasitology* **81**, 211-219.

Kocken, C. H. M., Withers-Martinez, C., Dubbeld, M. A., van der Wel, A., Hackett, F., Blackman, M. J. & Thomas, A. W. 2002a High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that Inhibit erythrocyte Invasion. *Infect Immun* **70**, 4471-4476.

Kocken, C. H. M., Withers-Martinez, C., Dubbeld, M. A., van der Wel, A., Hackett, F., Blackman, M. J. & Thomas, A. W. 2002b High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and nduction of antibodies that inhibit erythrocyte invasion. *Infect Immun* **70**, 4471-4476.

Krzych, U., Lyon, J. A., Jareed, T., Schneider, I., Hollingdale, M. R., Gordon, D. M. & Ballou, W. R. 1995 T lymphocytes from volunteers immunized with

irradiated *Plasmodium falciparum* sporozoites recognize liver and blood stage malaria antigens. *J Immunol* **155**, 4072-4077.

Lalitha, P. V., Biswas, S., Pillai, C. R. & Saxena, R. K. 2008 Immunogenicity of a recombinant malaria vaccine candidate, domain I + II of AMA-1 ectodomain, from Indian *P. falciparum* alleles. *Vaccine* **26**, 4526-4535.

Lamb, T. J., Brown, D. E., Potocnik, A. J. & Langhorne, J. 2006 Insights into the immunopathogenesis of malaria using mouse models *Exp Rev Mol Med* **8**, 1-22.

Landau, I. & Boulard, Y. 1978 Life cycles and morphology. In: Rodent Malaria Killick-Kendrick R, Peters W, editors. Academic Press Inc, London.

Lee, H.-S., Ulrich, P. P. & Vyas, G. N. 1991 Mutations in the S-gene affecting the immunologic determinants of the envelope protein of hepatitis B virus. *J Hepatol* **13**, S97-S101.

Levy, S. B. & Marshall, B. 2004 Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* **10**, S122-S129.

Li, C., Seixas, E. & Langhorne, J. 2001 Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocyte stages of the parasite. *Med Microbiol Immunol* **189**, 115-126.

Lipsitch, M. & Samore, M. H. 2002 Antimicrobial use and antimicrobial resistance: a population perspective. *Emerg Infect Dis* **8**, 347-354.

MacGregor, P. & Matthews, K. R. 2008 Modelling trypanosome chronicity: VSG dynasties and parasite density. *Trends Parasitol* **24**, 1-4.

Mackinnon, M. J. & Hastings, I. M. 1998 The evolution of multiple drug resistance in malaria parasites. *Trans R Soc Trop Med Hyg* **92**, 188-95.

Mackinnon, M. J. & Read, A. F. 1999a Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. *Proc R Soc Lond B Biol Sci* **266**, 741-748.

Mackinnon, M. J. & Read, A. F. 1999b Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi Evolution* **53**, 689-703.

Mackinnon, M. J., Gaffney, D. J. & Read, A. F. 2002 Virulence in rodent malaria: host genotype by parasite genotype interactions. *Infect Genet Evol* **1**, 287-296.

Mackinnon, M. & Read, A. F. 2003 The effects of host immunity on virulence transmission relationships in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* **126**, 103-112.

Mackinnon, M. & Read, A. F. 2004a Immunity promotes virulence evolution in a malaria model. *PLoS Biology* **2**, 1-7.

Mackinnon, M. & Read, A. F. 2004b Virulence in malaria: an evolutionary viewpoint. *Philos Trans R Soc Lond B Biol Sci* **359**, 965-986.

Mackinnon, M. J. 2005a Drug resistance models for malaria. *Acta Trop* **94**, 207-217.

Mackinnon, M. J., Bell, A. & Read, A. F. 2005b The effects of mosquito transmission and population bottlenecking on virulence, multiplication rate and rosetting in rodent malaria. *Int J Parasitol* **35**, 145-153.

Mackinnon, M. J., Gandon, S. & Read, A. F. 2008 Virulence evolution in response to vaccination: The case of malaria. *Vaccine* **26**, C42-C52.

Magesa, S. M., Mdira, K. Y., Babiker, H. A., Alifrangis, M., Färnert, A., Simonsen, P. E., Bygbjerg, I. C., Walliker, D. & Jakobsen, P. H. 2002 Diversity of *Plasmodium falciparum* clones infecting children living in a holoendemic area in north-eastern Tanzania. *Acta Trop* **84**, 83-92.

Mahajan, R. C., Faroog, U., Dubey, M. L. & Malla, N. 2005 Genetic polymorphism in *Plasmodium falciparum* vaccine candidate antigens. *Indian J Pathol Micro* **48**, 429-38.

Maher, B. A. 2002 The hopes and realities of the Plasmodium falciparum genome. *The Scientist* **16**, 24-7.

Maher, B. 2008 The end of the beginning. *Nature* **451**, 1042-1046.

Malkin, E. M., Diemert, D. J., McArthur, J. H., Perreault, J. R., Miles, A. P., Giersing, B. K., Mullen, G. E., Orcutt, A., Muratova, O., Awkal, M., Zhou, H., Wang, J., Stowers, A., Long, C. A., Mahanty, S., Miller, L. H., Saul, A. & Durbin, A. P. 2005 Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *Infect Immun* **73**, 3677-3685.

Marshall, V. M., Peterson, M. G., Lew, A. M. & Kemp, D. J. 1989 Structure of the apical membrane antigen I (AMA-1) of *Plasmodium chabaudi*. *Mol Bio Para* **37**, 281-283.

Marshall, V. M., Coppel, R. L., Martin, R. K., Oduola, A. M. J., Anders, R. F. & Kemp, D. J. 1991 A *Plasmodium falciparum* MSA-2 gene apparently generated by intragenic recombination between the two allelic families. *Mol Bio Para* **45**, 349-351.

Marshall, V. M., Zhang, L., Anders, R. F. & Coppel, R. L. 1996 Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum*. *Mol Bio Para* **77**, 109-113.

Martinelli, A., Cheesman, S., Hunt, P., Culleton, P., Raza, A., Mackinnon, M. & Carter, R. 2005 A genetic approach to the de novo identification of targets of strain specific immunity in malaria parasites. *Proc Natl Acad Sci U S A* **102**, 814-819.

Mason, D. P. & McKenzie, F. E. 1999 Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax-Plasmodium falciparum* infections. *Am J Trop Med Hyg* **61**, 367-374.

Maynard Smith, J. 1998 *Evolutionary Genetics* 2nd Ed. Oxford, Oxford University Press..

McEllistrem, M. C., Adams, J., Mason, E. & Wald, E. 2003 Epidemiology of acute otitis media caused by *Streptococcus pneumoniae* before and after licensure of the 7 valent pneumococcal protein conjugate vaccine. *J Infect Dis* **188**, 1679-1684.

McGregor, I. A., Gilles, H. M., Walters, J. H., Davies, A. H. & Pearson, F. A. 1956 Effects of heavy and repeated malaria infections on Gambian infants and children; effects of erythrocytic parasitization. *British Medical Journal* **2**, 686-92.

McGregor, I. A. 1974 Mechanisms of acquired immunity and epidemiological patterns of antibody response in malaria in man. *Bull WHO* **50**, 259-66.

McIntosh, R. S., Shi, J., Jennings, R. M., Chappel, J. C., de Koning-Ward, T. F., Smith, T., Green, J., van Egmond, M., Leusen, J. H. W., Lazarou, M., de Winkel, J. v., Jones, T. S., Crabb, B. S., Holder, A. A. & Pleass, R. J. 2007

The importance of human FcyRI in mediating protection to malaria. *PLoS Pathogens* **3**, e72.

McIntyre, P., Gidding, H., Gilmour, R., Lawrence, G., Hull, B., Horby, P., Wang, H., Andrews, R. & Burgess, M. 2002 Vaccine preventable diseases and vaccine coverage in Australia, 1999 to 2000. Commonwealth Dept. of Health and Ageing, Canberaa, Austalia.

McKenzie, F. E. & Bossert, W. H. 1997 The dynamics of *Plasmodium falciparum* blood-stage infection. *J Theor Biol* **188**, 127-40.

McKenzie, F. E., Smith, D. L., O'Meara, W. P. & Riley, E. M. 2008 Strain theory of malaria: the first 50 years. *Adv Parasitol* **66**, 1-46.

McLean, A. R. 1995 Vaccination, evolution and changes in the efficacy of vaccines: a theoretical framework. *Proc R Soc Lond B Biol Sci* **261**, 389-393.

McLean, A. R. 1998 Vaccines and their impact on the control of disease. *Br Med Bull* **54**, 545-556.

McQueen, P. G., McKenzie, F. E. & Singer, B. H. 2004 Age-structured red blood cell susceptibility and the dynamics of malaria infections. *Proc Natl Acad Sci U S A* **101**, 9161-9166.

Mendis, K. N., David, P. H. & Carter, R. 1991 Anigenic polymorphism in malaria: is it an important mechanism for immune evasion? *Immunol Today* **12**, A34-7.

Mercereau-Puijalon, O. 1996 Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans. *Parasite Immunol* **18**, 173-180.

Messenger, S. L., Molineux, I. J. & Bull, J. J. 1999 Virulence evolution in a virus obeys a trade off. *Proc R Soc Lond B Biol Sci* **266**, 397-404.

Mideo, N., Day, T. & Read, A. F. 2008a Modelling malaria pathogenesis. *Cell Microbiol* **10**, 1947-1955.

Mideo, N., Barclay, V. C., Chan, B. H., Savill, N. J., Read, A. F. & Day, T. 2008b *in press* Understanding and predicting strain-specific patterns of pathogenesis in the rodent malaria, *Plasmodium chabaudi*. *Am Nat* **172**, 214-238.

Miller, L. H., Roberts, T., Shahabuddin, M. & McCutchan, T. F. 1993 Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). *Mol Bio Para* **59**, 1-14.

Miller, L. H., Baruch, D. I., Marsh, K. & Doumbo, O. K. 2002 The pathogenic basis of malaria. *Nature* **415**, 673-679.

Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R. & Bannister, L. H. 2004 Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of ivasive merozoites to host red blood cells. *Infect Immun* **72**, 154-158.

Miura, K., Zhou, H., Muratova, O. V., Orcutt, A. C., Giersing, B., Miller, L. H. & Long, C. A. 2007 In immunization with *Plasmodium falciparum* apical membrane antigen 1, the specificity of antibodies depends on the species immunized. *Infect Immun* **75**, 5827-5836.

Mons, B. & Sinden, R. E. 1990 Laboratory models for research in vivo and in vitro on malaria parasites of mammals: Current status. *Parasitol Today* **6**, 3-7.

Mooi, F. R., van Oirschot, H., Heuvelman, K., van der Heide, H. G. J., Gaastra, W. & Willems, R. J. L. 1998 Polymorphism in the Bordetella pertussis virulence factors P.69/pertactin and pertussis toxin in The Netherlands: Temporal trends and evidence for vaccine-driven evolution. *Infect Immun* **66**, 670-675.

Moorthy, V. S., Good, M. F. & Hill, A. V. S. 2004 Malaria vaccine developments. *The Lancet* **363**, 150-156.

Mota, M. M., Brown, K. N., Holder, A. A. & Jarra, W. 1998 Acute Plasmodium chabaudi chabaudi malaria infection induces antibodies which bind to the surfaces of parasitized erythrocytes and promote their phagocytosis by macrophages In vitro. *Infect Immun* **66**, 4080-4086.

Mueller, M. S., Renard, A., Boato, F., Vogel, D., Naegeli, M., Zurbriggen, R., Robinson, J. A. & Pluschke, G. 2003 Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of *Plasmodium falciparum* Apical Membrane Antigen 1. *Infect Immun* **71**, 4749-4758.

Nair, M., Hinds, M. G., Coley, A. M., Hodder, A. N., Foley, M., Anders, R. F. & Norton, R. S. 2002 Structure of domain III of the blood-stage malaria vaccine candidate, *Plasmodium falciparum* apical membrane antigen 1 (AMA1). *J Mol Biol* **322**, 741-753.

Narum, D. L. & Thomas, A. W. 1994 Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. *Mol Bio Para* **67**, 59-68.

Narum, D. L., Ogun, S. A., Thomas, A. W. & Holder, A. A. 2000 Immunization with parasite-derived apical membrane antigen 1 or passive immunization with a specific monoclonal antibody protects BALB/c mice against lethal *Plasmodium yoelii yoelii* YM blood-stage infection. *Infect Immun* **68**, 2899-2906.

Narum, D. L., Ogun, S. A., Batchelor, A. H. & Holder, A. A. 2006 Passive immunization with a multicomponent vaccine against conserved domains of Apical Membrane Antigen 1 and 235-Kilodalton rhoptry proteins protects mice against *Plasmodium yoelii* blood-stage challenge infection. *Infect Immun* **74**, 5529-5536.

Nash, T., E. . 2002 Surface antigenic variation in *Giardia lamblia*. *Mol Microbiol* **45**, 585-590.

Nesse, R. M., Stearns, S. C. & Omenn, G. S. 2006 Medicine needs evolution. *Science* **311**, 1071.

Nouen, C. L., Rivallan, G., Toquin, D., Darlu, P., Morin, Y., Beven, V., de Boisseson, C., Cazaban, C., Comte, S., Gardin, Y. & Eterradossi, N. 2006 Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment-B-reassorted isolate. *J Gen Virol* **87**, 209-216.

Ntezayabo, B., Serres, G. & Duval, B. 2003 Pertussis resurgence in Canada largely caused by a cohort effect. *Pediatr Infect Dis J* **22**, 22-27.

Ntoumi, F., Contamin, H., Rogier, C., Bonnefoy, S., Trape, J. F. & Mercereau-Puijalon, O. 1995 Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am J Trop Med Hyg* **52**, 81 - 88.

O'Donnell, R. A., Saul, A., Cowman, A. F. & Crabb, B. S. 2000 Functional conservation of the malaria vaccine antigen MSP-119across distantly related Plasmodium species. *Nat Med* **6**, 91-95.

Ofosu-Okyere, A., Mackinnon, M. J., Sowa, M. P. K., Koram, K. A., Nkrumah, F., Osei, Y. D., Hill, W. G., Wilson, M. D. & Arnot, D. E. 2001 Novel

Plasmodium falciparum clones and rising clone multiplicities are associated with the increase in malaria morbidity in Ghanaian children during the transition into the high transmission season. *Parasitology* **123**, 113-123.

Oliveira, D. A., Udhayakumar, V., Bloland, P., Shi, Y. P., Nahlen, B. L., Oloo, A. J., Hawley, W. E. & Lal, A. A. 1996 Genetic conservation of the *Plasmodium falciparum* apical membrane antigen-1 (AMA-1). *Mol Bio Para* **76**, 333-336.

Orange, J. S., Fassett, M. S., Koopman, L. A., Boyson, J. E. & Strominger, J. L. 2002 Viral evasion of natural killer cells. *Nat Immunol* **3**, 1006-1012.

Osier, F. H. A., Fegan, G., Polley, S. D., Murungi, L., Verra, F., Tetteh, K. K. A., Lowe, B., Mwangi, T., Bull, P. C., Thomas, A. W., Cavanagh, D. R., McBride, J. S., Lanar, D. E., Mackinnon, M. J., Conway, D. J. & Marsh, K. 2008 Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect Immun* **76**, 2240-2248.

Pan, W., Huang, D., Zhang, Q., Qu, L., Zhang, D., Zhang, X., Xue, X. & Qian, F. 2004 Fusion of two malaria vaccine candidate antigens enhances product yield, immunogenicity, and antibody-mediated inhibition of parasite growth in vitro. *J Immunol* **172**, 6167-6174.

Pappenheimer, A. M. 1982 Diptheria: Studies on the biology of infectious disease. *Harvey Lect* **76**, 45-73.

Partridge, J. M. & Koutsky, L. A. 2006 Genital human papillomavirus infection in men. *Lancet Infec Dis* **6**, 21-31.

Peters, W. 1987 Chemotherapy and drug resistance in malaria. *Volume 1. London: Academic Press. 542p.*

Peterson, M. G., Marshall, V. M., Smythe, J. A., Crewther, P. E., Lew, A., Silva, A., Anders, R. F. & Kemp, D. J. 1989 Integral membrane protein located in the apical complex of *Plasmodium falciparum*. *Mol Cell Biochem* **9**, 3151-3154.

Peterson, M. G., Nguyen-Dinh, P., Marshall, V. M., Elliott, J. F., Collins, W. E., Anders, R. F. & Kemp, D. J. 1990 Apical membrane antigen of *Plasmodium fragile*. *Mol Bio Para* **39**, 279-283.

Phillips, R. S., Brannan, L. R., Balmer, P. & Neuville, P. 1997 Antigenic variation during malaria infection: the contribution from the murine parasite *Plasmodium chabaudi*. *Parasite Immunol* **19**, 427-434.

Phillips, R. S. 2001 Current status of malaria and potential for control. *Clin Microbiol Rev* **14**, 208-226.

Pizarro, J. C., Normand, B. V.-L., Chesne-Seck, M.-L., Collins, C. R., Withers-Martinez, C., Hackett, F., Blackman, M. J., Faber, B. W., Remarque, E. J., Kocken, C. H. M., Thomas, A. W. & Bentley, G. A. 2005 Crystal structure of the malaria vaccine candidate Apical Membrane Antigen 1. *Science* **308**, 408-411.

Plebanski, M., Proudfoot, O., Pouniotis, D., Coppel, R. L., Apostolopoulos, V. & Flannery, G. 2002 Immunogenetics and the design of *Plasmodium falciparum* vaccines for use in malaria-endemic populations. *J Clin Investig* **110**, 295-301.

Polhemus, M. E., Magill, A. J., Cummings, J. F., Kester, K. E., Ockenhouse, C. F., Lanar, D. E., Dutta, S., Barbosa, A., Soisson, L., Diggs, C. L., Robinson, S. A., Haynes, J. D., Stewart, V. A., Ware, L. A., Brando, C., Krzych, U., Bowden, R. A., Cohen, J. D., Dubois, M.-C., Ofori-Anyinam, O., De-Kock, E., Ballou, W. R. & Heppner, J. D. G. 2007 Phase I dose escalation safety and immunogenicity trial of *Plasmodium falciparum* apical membrane protein (AMA-1) FMP2.1, adjuvanted with AS02A, in malaria-naive adults at the Walter Reed Army Institute of Research. *Vaccine* **25**, 4203-4212.

Polley, S., Tetteh, K. A., Lloyd, J., Akpogheneta, O., Greenwood, B., Bojang, K. & Conway, D. 2007 *Plasmodium falciparum* merozoite surface protein 3 is a target of allele specific immunity and alleles are maintained by natural selection. *J Infect Dis* **195**, 279-287.

Polley, S. D. & Conway, D. J. 2001 Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1. *Genetics* **158**, 1505-1512.

Polley, S. D., Chokejindachai, W. & Conway, D. J. 2003 Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. *Genetics* **165**, 555-561.

Polley, S. D., Mwangi, T., Kocken, C. H. M., Thomas, A. W., Dutta, S., Lanar, D. E., Remarque, E., Ross, A., Williams, T. N., Mwambingu, G., Lowe, B., Conway, D. J. & Marsh, K. 2004 Human antibodies to recombinant protein constructs of *Plasmodium falciparum* apical membrane antigen 1 (AMA1) and their associations with protection from malaria. *Vaccine* **23**, 718-728.

Pombo, D. J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K., Mahakunkijcharoen, Y., Martin, L. B. & Wilson, D. 2002 Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *The Lancet* **360**, 610-617.

Pond, S. L. K., Posada, D., Gravenor, M. B., Woelk, C. H. & Frost, S. D. W. 2006 Automated phylogenetic detection of recombination using a genetic algorithm. *Mol Biol Evol* **23**, 1891-1901.

Poolman, E. M., Elbasha, E. H. & Galvani, A. P. 2008 Vaccination and the evolutionary ecology of human papillomavirus. *Vaccine* **26**, C25-C30.

Posada, D. & Crandall, K. A. 1998 Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Powell, R. D. J., McNamara, J. V. & Rieckman, K. H. 1972 Clinical aspects of acquisition of immunity to Falciparum malaria *Proceedings of the helminthology society* **39**, 51-68.

Protzer-Knolle, U., Naumann, U., Bartenschlager, R., Berg, T., Hopf, U., Meyer zum, K. H., Büschenfelde, P. & Gerken, N. G. 1998 Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* **27**, 254-263.

Råberg, L., de Roode, J. C., Bell, A. S., Stamou, P., Gray, D. & Read, A. F. 2006 The role of immune-mediated apparent competition in genetically diverse malaria infections. *Am Nat* **168**, 41-53.

Rautenschlein, S. & Haase, C. 2005 Differences in the immunopathogenesis of infectious bursal disease virus (IBDV) following in ovo and post-hatch vaccination of chickens. *Vet Immunol Immunopathol* **106**, 139-150.

Read, A. F. & Taylor, L. H. 2001 The ecology of genetically diverse infections. *Science* **292**, 1099-1102.

Read, A. F., Gandon, S., Nee, S. & Mackinnon, M. J. 2004 The evolution of pathogen virulence in response to animal and public health interventions. In: Dronamraju KR, editors. *Infectious Disease and Host-Pathogen Evolution*.

Read, A. F. & Mackinnon, M. J. 2008 Pathogen evolution in a vaccinated world. In: Stearns SC, Koella, JC editors. *Evolution in Health and Disease*. 2nd Ed. Oxford, Oxford University Press.

Reece, S. E., Drew, D. R. & Gardner, A. 2008 Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**, 609-614.

Reed, Z. H., Friede, M. & Kieny, M. P. 2006 Malaria vaccine development: progress and challenges. *Cur Mol Med* **6**, 231-245.

Remarque, E. J., Faber, B. W., Kocken, C. H. M. & Thomas, A. W. 2008a Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol* **24**, 74-84.

Remarque, E. J., Faber, B. W., Kocken, C. H. M. & Thomas, A. W. 2008b A diversity-covering approach to immunization with *Plasmodium falciparum* apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits. *Infect Immun* **76**, 2660-2670.

Restif, G. & Grenfell, B. T. 2006 Integrating life-history and cross-immunity into the evolutionary dynamics of pathogens. *Proc R Soc Lond B Biol Sci* **273**, 409-416.

Richie, T. L. & Saul, A. 2002 Progress and challenges for malaria vaccines. *Nature* **415**, 694-701.

Riley, M. A. & Gordon, D. M. 1999 The ecological role of bacteriocins in bacterial competition. *Trends Microbiol* **7**, 129-133.

Robert, F., Ntoumi, F., Angel, G., Candito, D., Rogier, C., Fandeur, T., Sarthou, J. L. & Mercereau-Puijalon, O. 1996 Extensive genetic diversity of *Plasmodium falciparum* isolates collected from patients with severe malaria in Dakar, Senegal. *Trans R Soc Trop Med Hyg* **90**, 704-711.

Roberts, D. J., Craig, Berendt, A. R., Pinches, R., Nash, G., Marsh, K. & Newbold, C. I. 1992 Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* **357**, 689-692.

Roberts, D. J., Pain, A., Kai, O., Kortok, M. & Marsh, K. 2000 Autoagglutination of malaria-infected red blood cells and malaria severity. *The Lancet* **355**, 1427-1428.

Rodrigues, M. H. C., Rodrigues, K. M., Oliveira, T. R., Cômodo, A. N., Rodrigues, M. M., Kocken, C. H. M., Thomas, A. W. & Soares, I. S. 2005 Antibody response of naturally infected individuals to recombinant *Plasmodium vivax* apical membrane antigen-1. *Int J Parasitol* **35**, 185-192.

Rowe, A., Obeiro, J., Newbold, C. I. & Marsh, K. 1995 *Plasmodium falciparum* rosetting is associated with malaria severity in Kenya. *Infect Immun* **63**, 2323-2326.

Sacks, D. & Sher, A. 2002 Evasion of innate immunity by parasitic protozoa. *Nat Immunol* **3**, 1041-1047.

Salanti, A., Dahlback, M., Turner, L., Nielsen, M. A., Barfod, L., Magistrado, P., Jensen, A. T. R., Lavstsen, T., Ofori, M. F., Marsh, K., Hviid, L. & Theander, T. G. 2004 Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J Exp Med* **200**, 1197-1203.

Saul, A., Lawrence, G., Smillie, A., Rzepczyk, C. M., Reed, C., Taylor, D., Anderson, K., Stowers, A., Kemp, R., Allworth, A., Anders, R. F., Brown, G. V., Pye, D., Schoofs, P., Irving, D. O., Dyer, S. L., Woodrow, G. C., Briggs, W. R. S., Reber, R. & Stürchler, D. 1999 Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. *Vaccine* **17**, 3145-3159.

Saul, A. 2007 Malaria vaccines based on the *Plasmodium falciparum* merozoite surface protein 3-should we avoid amino acid sequence polymorphisms or embrace them? *J Infect Dis* **195**, 171-173.

Scherer, A. & McLean, A. 2002 Mathematical models of vaccination. *Br Med Bull* **62**, 187-199.

Schmid-Hempel, P. 2008 Parasite immune evasion: a momentous molecular war. *Trends Ecol Evol* **23**, 318-326.

Schneerson, R., Robbins, J. B., Taranger, J., Lagergard, T. & Trollfors, B. 1996 A toxoid vaccine for pertussis as well as diphtheria? Lessons to be relearned. *The Lancet* **348**, 1289-1292.

Schofield, L. & Grau, G. E. 2005 Immunological processes in malaria pathogenesis. *Nature Reviews Immunology* **5**, 722-735.

Scorza, T., Grubb, K., Cambos, M., Santamaria, C., Malu, D. T. & Spithill, T. W. 2008 Vaccination with a *Plasmodium chabaudi adami* multivalent DNA vaccine cross-protects A/J mice against challenge with *P. c. adami* DK and virulent *Plasmodium chabaudi chabaudi* AS parasites. *Int J Parasitol* **38**, 819-827.

Silvie, O., Franetich, J.-F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., van Gemert, G.-J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G. & Mazier, D. 2004 A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J Biol Chem* **279**, 9490-9496.

Smith, T., Lehmann, D., Montgomery, J., Gratten, M., Riley, I. D. & Alpers, M. P. 1993 Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children. *Epidemiol Infect* **111**, 27-39.

Smith, T., Felger, I., Kitua, A., Tanner, M. & Beck, H. P. 1999a Dynamics of multiple *Plasmodium falciparum* infections in infants in a highly endemic area of Tanzania. *Trans R Soc Trop Med Hyg* **93**, 35-39.

Smith, T., Felger, I., Tanner, M. & Beck, H. P. 1999b Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Trans R Soc Trop Med Hyg* **93**, 59-64.

Smythe, J. A., Peterson, M. G., Coppel, R. L., Saul, A. J., Kemp, D. J. & Anders, R. F. 1990 Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. *Mol Biochem Para* **39**, 227-234.

Snewin, V. A., Herrera, M., Sanchez, G., Scherf, A., Langsley, G. & Herrera, S. 1991 Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Mol Bio Para* **49**, 265-275.

Soubeyrand, B. & Plotkin, S. A. 2002 Microbial evolution (Communication arising): Antitoxin vaccines and pathogen virulence. *Nature* **417**, 609-610.

Stearns, S. C. & Hoekstra, R. F. 2005 *Evolution: An Introduction*. 2nd Ed. Oxford, Oxford University Press.

Stearns, S. C., Nesse, R. M. & Haig, D. 2008 Introducing evolutionary thinking for medicine. In: Stearns SC, Koella JC,editors. *Evolution in Health and Disease* 2nd Ed. Oxford, Oxford University Press.

Stephens, R. & Langhorne, J. 2006 Priming $CD4^+$ T cells and development of $CD4^+$ T cell memory; lessons for malaria. *Parasite Immunol* **28**, 25-30.

Stevenson, M. M. & Riley, E. M. 2004 Innate immunity to malaria. *Nature Reviews Immunology* **4**, 169-180.

Stoute, J. A., Slaoui, M., Heppner, D. G., Momin, P., Kester, K. E., Desmons, P., Wellde, B. T., Garcon, N., Krzych, U., Marchand, M., Ballou, W. R., Cohen, J. D. & The Rts, S. M. V. E. G. 1997 A Preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *N Engl J Med* **336**, 86-91.

Stoute, J. A., Kester, K. E., Krzych, U., Wellde, B. T., Hall, T., White, K., Glenn, G., Ockenhouse, C. F., Garcon, N., Schwenk, R., Lanar, D. E., Sun, P., Momin, P., Wirtz, R. A., Golenda, C., Slaoui, M., Wortmann, G., Holland, C., Dowler, M., Cohen, J. & Ballou, W. R. 1998 Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. *J Infect Dis* **178**, 1139-1144.

Stowers, A. W., Cioce, V., Shimp, R. L., Lawson, M., Hui, G., Muratova, O., Kaslow, D. C., Robinson, R., Long, C. A. & Miller, L. H. 2001 Efficacy of two alternate vaccines based on *Plasmodium falciparum* merozoite surface protein 1 in an Aotus challenge trial. *Infect Immun* **69**, 1536-1546.

Stowers, A. W., Kennedy, M. C., Keegan, B. P., Saul, A., Long, C. A. & Miller, L. H. 2002 Vaccination of monkeys with recombinant *Plasmodium falciparum* apical membrane antigen 1 confers protection against blood-stage malaria. *Infect Immun* **70**, 6961-6967.

Suzuki, M., Ohneda, K., Hosoya-Ohmura, S., Tsukamoto, S., Ohneda, O., Philipsen, S. & Yamamoto, M. 2006 Real-time monitoring of stress erythropoiesis in vivo using Gata1 and beta-globin LCR luciferase transgenic mice. *Blood* **108**, 726-733.

Swofford, D. L. 2003 PAUP*: Phylogenetic analysis using parsimony (*and other methods) version 4.0. Sunderland (Massachusetts): Sinauer Associates.

Talisuna, A. O., Erhart, A., Samarasinghe, S., Van Overmeir, C., Speybroeck, N. & D'Alessandro, U. 2006 Malaria transmission intensity and the rate of spread of chloroquine resistant *Plasmodium falciparum*: Why have theoretical models generated conflicting results? *Infect Genet Evol* **6**, 241-248.

Tanabe, K., Mackay, M., Gorman, M. & Scaife, J. G. 1987 Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum J Mol Biol* **195**, 273-87.

Tanabe, K., Sakihama, N., Rooth, I., Bjorkman, A. & Farnert, A. 2007 High frequency of recombination-driven allelic diversity and temporal variation of *Plasmodium falciparum* MSP1 in Tanzania *Am J Trop Med Hyg* **76**, 1037-1045.

Tanner, M., Beck, H. P., Felger, I. & Smith, T. 1999 The epidemiology of multiple *Plasmodium falciparum* infections. *Trans R Soc Trop Med Hyg* **93**, 1-2.

Taylor-Robinson, A. W. 1995 Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol Today* **11**, 334-342.

Taylor, L. H., Walliker, D. & Read, A. F. 1997 Mixed-genotype infections of malaria parasites: Within-host dynamics and transmission success of competing clones. *Proc R Soc Lond B Biol Sci* **264**, 927-935.

Taylor, L. H. & Read, A. F. 1998 Determinants of transmission success of individual clones from mixed-clone infections of the rodent malaria parasite, *Plasmodium chabaudi*. *Int J Parasitol* **28**, 719 - 725.

Thera, M. A., Doumbo, O. K., Coulibaly, D., Diallo, D. A., Kone, A. K., Guindo, A. B., Traore, K., Dicko, A., Sagara, I., Sissoko, M. S., Baby, M., Sissoko, M., Diarra, I., Niangaly, A., Dolo, A., Daou, M., Diawara, S. I., Heppner, D. G., Stewart, V. A., Angov, E., Bergmann-Leitner, E. S., Lanar, D. E., Dutta, S., Soisson, L., Diggs, C. L., Leach, A., Owusu, A., Dubois, M.-C., Cohen, J., Nixon, J. N., Gregson, A., Takala, S. L., Lyke, K. E. & Plowe, C. V. 2008 Safety and immunogenicity of an AMA-1 malaria vaccine in Malian adults: Results of a phase 1 randomized controlled trial. *PLoS ONE* **3**, e1465.

Thomas, A. W., Waters, A. P. & Carr, D. 1990 Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of *Plasmodium falciparum*. *Mol Bio Para* **42**, 285-287.

Thomas, A. W., Trape, J.-F., Rogier, C., Goncalves, A., Rosario, V. E. & Narum, D. L. 1994 High prevalence of natural antibodies against *Plasmodium falciparum* 83-kilodalton apical membrane antigen (PF83/AMA-1) as detected by capture-enzyme-linked immunosorbent assay using full-length baculovirus recombinant PF83/AMA-1. *Am J Trop Med Hyg* **51**, 730-740.

Thompson, J., Janse, C. J. & Waters, A. P. 2001 Comparative genomics in *Plasmodium*: a tool for the identification of genes and functional analysis. *Mol Bio Para* **118**, 147-154.

Timms, R., Colegrave, N., Chan, B. H. K. & Read, A. F. 2001 The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. *Parasitology* **123**, 1-11.

Torresi, J. 2002 The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* **25**, 97-106.

Triglia, T., Healer, J., Caruana, S. R., Hodder, A. N., Anders, R. F., Crabb, B. S. & Cowman, A. F. 2000 Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. *Mol Microbiol* **38**, 706-718.

Trottier, H. & Franco, E. L. 2006 The epidemiology of genital human papillomavirus infection. *Vaccine* **24**, S4-S15.

Urban, B. C., Ing, R. & Stevenson, M. M. 2005 Early interactions between blood-stage plasmodium parasites and the immune system. *Curr Top Microbiol Immunol*, 25-70.

van Baalan, M. & Sabelis, M. W. 1995 The dynamics of multiple infection and the evolution of virulence. *Am Nat* **146**, 881-910.

van Boven, M., Mooi, F., Schellekens, J., de Melker, H. & Kretzschmar, M. 2005 Pathogen adaptation under imperfect vaccination: implications for pertussis. *Proc R Soc Lond B Biol Sci* **272**, 1617-1624.

van den Berg, T. P., Eterradossi, N., Toqui, D. & Meulemans, G. 2000 Infectious bursal disease (Gumboro disease). *Rev Sci Technol* **19**, 509-43.

van Loo, I. H. M., van der Heide, H. G. J., Nagelkerke, N. J. D., Verhoef, J. & Mooi, F. R. 1999 Temporal trends in the population structure of *Bordetella pertussis* during 1949-1996 in a highly vaccinated population. *J Infect Dis* **179**, 915-923.

Walliker, D., Sanderson, A., Yoeli, M., Harrant, J. & Hargreaves, B. 1976 A genetic investigation of virulence in a rodent malaria parasite *Parasitology* **72**, 183-194.

Walliker, D., Hunt, P. & Babiker, H. 2005 Fitness of drug-resistane malaria parasites. *Acta Trop* **94**, 251-259.

Wargo, A. R., Huijben, S., de Roode, J. C., Shepherd, J. & Read, A. F. 2007 Competitive release and facilitation of drug-resistant parasites after therapeutic chemotherapy in a rodent malaria model. *Proc Natl Acad Sci USA* **104**, 19914-19919.

Waters, A. P., Thomas, A. W., Deans, J. A., Mitchell, G. H., Hudson, D. E., Miller, L. H., McCutchan, T. F. & Cohen, S. 1990 A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium*. *J Biol Chem* **265**, 17974-17979.

WHO. 2001 Pertussis surveillence: a global meeting, Geneva, 16-18 October 2000 http://www.who.int/vaccines-documents/DocsPDF01/www605.pdf

Wickramarachchi, T., Premaratne, P. H., Perera, K. L. R. L., Bandara, S., Kocken, C. H. M., Thomas, A. W., Handunnetti, S. M. & Udagama-Randeniya, P. V. 2006 Natural human antibody responses to *Plasmodium vivax* Apical Membrane Antigen 1 under low transmission and unstable malaria conditions in Sri Lanka. *Infect Immun* **74**, 798-801.

Williams, P. D. & Day, T. 2008 Epidemiological and evolutionary consequences of targeted vaccination. *Mol Ecol* **17**, 485-499.

Wilson, J. N., Nokes, D. J. & Carman, W. F. 1998 Current status of HBV vaccine escape variants - a mathematical model of their epidemiology. *J Vir Hep* **5**, 25-30.

Wilson, J. N., Nokes, D. J. & Carman, W. F. 1999 The predicted pattern of emergence of vaccine-resistant hepatitis B: a cause for concern? *Vaccine* **17**, 973-978.

Wipasa, J., Elliot, S., Xu, H. & Good, M. F. 2002a Immunity to asexual blood-stage malaria vaccine approaches. *Immunol Cell Biol* **80**, 401-414.

Wipasa, J., Hirunpetcharat, C., Mahakunkijcharoen, Y., Xu, H., Elliott, S. & Good, M. F. 2002b Identification of T cell epitopes on the 33-kDa fragment of *Plasmodium yoelii* merozoite surface protein 1 and their antibody-independent protective role in immunity to blood stage malaria. *J Immunol* **169**, 944-951.

Witter, R. L. 1997 Avian tumour viruses: persistent and evolving pathogens. *Acta Veterinaria Hungarica* **45**, 251-266.

Witter, R. L. 2001 Protective efficacy of Marek's disease vaccines. *Curr Top Microbiol Immunol*.

Yokosuka, O. & Arai, M. 2006 Molecular biology of hepatitis B virus: effect of nucleotide substitutions on the clinical features of chronic hepatitis B. *Medical Molecular Morphology* **39**, 113-120.

Zanetti, A., Tanzi, E. & Manzillo, G. 1988 Hepatitis B variant in Europe. *The Lancet* **2**, 1132-3.

10. Thesis Appendix

Scientific publications

Appendix 1

Barclay, V.C., Chan, B.H.K., Anders, R.F and Read, A. F. (2008)

Mixed allele malaria vaccines: Host protection and within-host selection. *Vaccine* 26, 6099-6107

(Based on Chapter 4)

Appendix 2

Barclay, V.C., Råberg, L., Chan, B.H.K., Brown, S., Gray, D and Read, A.F. (2008)

$CD4^+$ T cells do not mediate within host competition between genetically diverse malaria parasites. *Proc R Soc Lond B Biol Sci* **275,** 1171-1179.

(*Based on Chapter 6*)

Appendix 3

Mideo, N., Barclay, V.C., Chan, B.H.K., Savill, N. J., Read, A.F., Day, T. (2008)

Understanding and predicting strain-specific patterns of pathogenesis in the rodent malaria, *Plasmodium chabaudi*. *Am Nat* **172**

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Mixed allele malaria vaccines: Host protection and within-host selection

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ABSTRACT

Malaria parasites are frequently polymorphic at the antigenic targets of many candidate vaccines, presumably as a consequence of selection pressure from protective immune responses. Conventional wisdom is therefore that vaccines directed against a single variant could select for non-target variants, rendering the vaccine useless. Many people have argued that a solution is to develop vaccines containing the products of more than one variant of the target. However, we are unaware of any evidence that multi-allele vaccines better protect hosts against parasites or morbidity. Moreover, selection of antigen-variants is not the only evolution that could occur in response to vaccination. Increased virulence could also be favored if more aggressive strains are less well controlled by vaccine-induced immunity. Virulence and antigenic identity have been confounded in all studies so far, and so we do not know formally from any animal or human studies whether vaccine failure has been due to evasion of protective responses by variants at target epitopes, or whether vaccines are just less good at protecting against more aggressive strains.

Using the rodent malaria model *Plasmodium chabaudi* and recombinant apical membrane antigen-1 (AMA-1), we tested whether a bi-allelic vaccine afforded greater protection from parasite infection and morbidity than did vaccination with the component alleles alone. We also tested the effect of mono- and bi-allelic vaccination on within-host selection of mixed *P. chabaudi* infections, and whether parasite virulence mediates pathogen titres in immunized hosts. We found that vaccination with the bi-allelic AMA-1 formulation did not afford the host greater protection from parasite infection or morbidity than did mono-allelic AMA-1 immunization. Mono-allelic immunization increased the frequency of heterologous clones in mixed clone infections. There was no evidence that any type of immunization regime favored virulence. A single AMA-1 variant is a component of candidate malaria vaccines current in human trials; our results suggest that adding extra AMA-1 alleles to these vaccines would not confer clinical benefits, but that that mono-allelic vaccines could alter AMA-1 allele frequencies in natural populations.

1. Introduction

Malaria parasite antigens which are the targets of protective immune responses are frequently polymorphic, with antigen-coding genes having multiple allelic forms [1]. Polymorphisms likely arise as a consequence of immune-mediated selection because host responses can be more effective against parasites of the immunising strain than against different strains (strain-specific immunity) [2–6]. Sequence polymorphisms have been directly implicated in antigenic escape [7–9], and in malaria endemic areas, immunity is acquired slowly, probably because repeated exposure is required to generate an effective response against a repertoire of strains [10–12]. The existence of antigenic polymorphism is therefore of considerable concern to malaria vaccine developers because it implies that single antigen vaccines will have trouble inducing protective immunity against polymorphic targets [13–16].

One approach to minimizing vaccine-induced strain-specificity has been to design vaccines which combine more than one allele of an antigen [1,17–19]. However, the inclusion of more than one allelic form of an antigen may not be sufficient to overcome substantial polymorphisms [9], and there is little experimental evidence that multi-allele vaccines actually afford the host more protection from morbidity than do single antigen vaccines.

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Furthermore, selection of antigen-variants is not the only evolution that could occur in response to widespread vaccination. Theoretically, vaccination has the potential to cause evolutionary change in parasite virulence (parasite-induced host damage) by altering the way natural selection acts on parasite populations [20–27]. In experimental evolution experiments, the rodent malaria *Plasmodium chabaudi* became virulent more rapidly if serially passaged through mice previously immunized with live parasites [28]. The most likely explanation for this is that more aggressive variants are less well controlled by immunity.

To date, we still do not fully understand how vaccines will alter gene frequencies in malaria parasite populations. Evidence for selection in the field comes from a small phase 1-2b trial of the "Combination B" blood-stage malaria vaccine [29]. This vaccine contained a single antigen from each of three polymorphic loci of *P. falciparum*. One of these loci, merozoite surface protein-2 (MSP-2), is dimorphic, with each parasite having an allele from one of two allelic families (labelled 3D7 and FC27). The MSP-2 allele in the Combination B vaccine came from the 3D7 family. Among parasites subsequently acquired by vaccines, 3D7-type alleles were rarer than in people given a placebo. Vaccination thus selected against the variant contained in the vaccine. Interestingly, the FC27 allelic family is associated with more virulent infections [30]. Therefore, it is not clear whether the vaccine-imposed selection was due to immune specificity [15,31] or whether the vaccine was less good at controlling more virulent infections.

Many candidate vaccines against malaria are directed against the asexual blood stage, with the principal target being the merozoite. Apical merozoite antigen-1 (AMA-1) is a promising vaccine candidate as it possesses fewer polymorphisms than other merozoite antigens [2,13]. AMA-1 is thought to play a major role during erythrocyte re-modelling and invasion [32]. Immunization with AMA-1 confers protection against parasite challenge in a number of animal models, probably by inducing antibodies which inhibit invasion [2,7,33–36]. Furthermore, humans and other species immunized with single allele AMA-1 vaccines raise antibodies which inhibit erythrocyte invasion in vitro [13,37]. In endemic populations, naturally acquired antibody to *P. falciparum* AMA-1 (PfAMA-1) is associated with protection from falciparum malaria [38–42]. At least six different vaccines based on the AMA-1 allele from the *P. falciparum* 3D7 strain are currently in efficacy trials in humans [43–45].

However, there are more than 60 polymorphic sites in the AMA-1 protein, and most of these are non-randomly dispersed point mutations on domain I [46–50]. These point mutations may be of immunological importance. Protection in mice is strain-specific, and growth and invasion inhibition assays (GIA) and ELISA show that antibodies from animals and human field sera inhibit growth in a strain-specific manner [2,13,34,38,43,47]. Allelic replacement experiments have directly implicated sequence polymorphism in antigenic escape [7], and cross-strain inhibition assays suggest that the extent of escape correlates with sequence distance between the vaccine and the target strain [8]. In an attempt to overcome strain-specificity, vaccine researchers are beginning to combine allelic variants of AMA-1. For example, one group immunized rhesus monkeys with a mixture of two allelic forms of PfAMA-1 (designated AMA-1-C1) or the component alleles and measured responses in vitro using GIA and ELISA [8,51,52]. The resulting antibodies were similarly effective regardless of whether immunization was with a single variant or AMA-1-C1. Another group immunized mice and rabbits with two allelic variants of domain I and II of AMA-1 ectodomain from *P. falciparum* isolates. The anti-AMA-1 antibodies obtained with both proteins were active in an in vitro parasite growth invasion/inhibition assay, but to no greater extent than with either of the variants alone [53]. Together these results have raised questions about the necessity of using multi-allele vaccines.

Here we use the rodent malaria *P. chabaudi* and two alleles of the blood-stage malaria vaccine candidate AMA-1 to investigate (i) whether immunization with a single or bi-allelic AMA-1 variant formulation afforded the host the greatest protection from morbidity and parasite infection, (ii) how these different vaccination regimes can alter clonal frequencies in mixed infections, and (iii) whether more virulent clones are better at evading heterologous vaccine-induced protective responses.

2. Materials and methods

2.1. Parasites and hosts

P. chabaudi adami clones were originally derived from wild-caught thicket rats (*Thamnomys rutilans*) in the Congo and stored as frozen stabilites in liquid nitrogen with subscript codes used to identify their position in clonal history [54,55]. In this experiment we used clones DS_{500} and DK_{122} originally cloned from isolates 408XZ and 556KA, respectively. The nucleotide sequences of the DK and DS *P. chabaudi* AMA-1 (PcAMA-1) gene differ at 79 sites [34,56]. Hosts were inbred female C57BL/6 mice aged 6–8 weeks (Harlan, England) maintained as described previously [57]. Studies by others [34] and our own pilot studies showed that these clones differ in virulence during the infection of C57BL/6 female mice, with clone DS generating substantially more parasites and inducing greater weight and red blood cell loss relative to DK.

2.2. Immunizations and isotype ELISA

Here we used an immunization protocol adapted from Anders et al. [2]. Prior to immunization, mice were randomized into four groups of eighteen (Table 1). Immunization was with the highly immunogenic ectodomian of the full AMA-1 protein termed AMA-1B. For mono-allelic immunizations (hereafter referred to as DS AMA-1 or DK AMA-1), groups of mice were injected intraperitoneally with 10 μg of the appropriate protein emulsified in 100 μl of the adjuvant Montanide ISA720 (Seppic, France). For bi-allelic immunizations, mice were injected with a mixture of 5 μg of both DS and DK AMA-1, giving the same total dose of antigen as for the single antigen immunizations, again emulsified in Montanide ISA720. Control mice were injected with 100 μl emulsion of PBS in Montanide ISA720. Mice were given a single booster immunization with the same amount of antigen emulsified in Montanide ISA720 4 weeks after the primary immunization.

To ensure that antigen immunization successfully generated antibody responses, and to determine whether there was any cross-reactivity between the antibodies generated to the different immunizing antigens, we first carried out a pilot experiment. A total of 11 mice were immunized with DS AMA-1, 11 with DK AMA-1, and 10 were sham-immunized. We estimated the quantity of IgG2b antigen-specific antibodies in all mice sera 11 days after the booster immunization by ELISA using wells coated with DS AMA-1 or DK AMA-1. Thus the sera from 32 mice were tested in 64 wells. We used IgG2b as previous work in our laboratory showed that C57BL/6 produce this isotype in response to *P. chabaudi* infection (K. Grocock, A. Graham, unpublished). Protection induced by immunization with recombinant AMA-1 is isotype independent [58b]. Given the lack of cross-reactivity we observed in this pilot experiment (see Section 3), in the main experiment, we measured IgG2b isotype antibodies to each antigen separately only from the sera of mice immunized with a mixture of DS and DK AMA-1 and in sham-immunized control mice.

Table 1
Experimental design

	Number of mice per immunization	Infecting clone	Number of mice per parasite infection	Number of deaths	Number of euthanized
Sham-immun		DS	6	3	2
Sham-immun	18	DK	6		
Sham-immun		DS + DK	6		
DK AMA-1		DS	6		
DK AMA-1	18	DK	6		
DK AMA-1		DS + DK	6		
DS AMA-1		DS	6	1	
DS AMA-1	18	DK	6		
DSAMA-1		DS + DK	6	1	
Bi-allelic		DS	6	2	1
Bi-allelic	18	DK	6		
Bi-allelic		DS + DK	6		
Total	72		72	7	3

Immunization was either with DK AMA-1, DS AMA-1, a formulation containing an equal mix of both forms of AMA-1 (bi-allelic), or immunization with adjuvant only ('sham-immunization'). Groups of 18 mice were immunized with one of the four treatments before being separated into groups of 6. Infection was with parasites of clone DK alone, clone DS alone or a mixture of both. During the experiment 7 mice were found dead and 3 had to been euthanized due to severe morbidity. Euthanization was at predetermined levels of morbidity prescribed by animal care protocols.

In both the pilot and main experiments, sera fractions were separated by centrifugation from 20 μl of blood taken from a tail snip and were stored at −80 °C. High binding 96 well ELISA Maxisorb immunoplates (Nunc) were coated with either DS AMA-1 or DK AMA-1 at a concentration of 1 μg/ml in 0.06 M carbonate buffer (0.04 M $NaHCO_3$, 0.02 M $NaCO_3$, pH 9.6) in a final volume of 50 μl per well. Plates were stored at 4 °C overnight to allow the antigen to bind. Non-specific binding was blocked by incubating wells with 5% BSA: carbonate buffer (200 μl per well) for 2 h at 37 °C. Wells were then washed three times in Tris buffered saline with 0.01% Tween 20 (TBST). We used end-point dilution methods to detect IgG2b titres: serum samples were detected in a serial dilution 1/100-1/204800 using TBST as a diluent, in a final volume of 50 μl per well and incubated for 2 h at 37 °C. Wells were washed three times in TBST. HRP conjugated goat anti-mouse IgG2b detection antibody (Southern Biotech 1100-05) was diluted 1/4000 in TBST to a final volume of 50 μl per well. Plates were incubated for 1 h at 37 °C. Wells were washed three times in TBST followed by a final wash in distilled water. ABTS peroxide substrate (Insight Biotechnology) was added at 100 μl per well and allowed to develop at room temperature for 20 min. Optical density was read at 405 nm using a spectrophotometer. IgG2b isotype antibody titres were calculated as the reciprocal of the greatest dilution at which optical density (O.D.) was greater than the mean (plus 2 standard deviations) O.D. values observed for naïve mouse sera assayed against both DS and DK AMA-1 at 1/100.

2.3. Parasite challenge and monitoring of within-host dynamics

Two weeks after the boost immunization, groups of immunized mice (18 per group) were further randomized into groups of six and challenged with 10^5 parasites of either clone DS alone, clone DK alone or a mixture of clone DS and DK (Table 1). Thus, mice infected with both clones received twice as many parasites as those infected with one clone. A two-fold difference in infective dose has negligible effects on the population dynamics of the parasite [58a]. During the course of infection, we measured body weights and took blood samples from the tail to (i) make Giemsa-stained blood smears, (ii) estimate red blood cell density by flow cytometry (Beckman Coulter), and (iii) for genotype-specific real-time quantitative PCR (qPCR) assays as described previously [59]. For amplification of the DK genotype, we used primers previously designed to amplify AS/AJ genotypes as described elsewhere [59]. DS genotype-specific primers were as follows: DS forward 5′-GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA-3′; DS reverse 5′-CAG GAG AAA TGT TTA CAT CTG CTT T-3′.

2.4. Trait definition and statistical analyses

Since *P. chabaudi* has a 24-h replication cycle, the total number of parasites present in any period can be estimated by summing the daily parasite counts. Data were analysed using General Linear Models (GLMs) in MINITAB. To meet normality and homogeneity of variance assumptions, data on antibodies, weight and red blood cell density were log transformed while all parasite densities and proportions were square root transformed. GLMs were used to test whether the magnitude of protection differed between the three antigen immunizations (DK AMA-1, DS AMA-1, or the bi-allelic form); that is whether there was a statistical interaction between infecting clone and immunizing treatments. Maximal models (response variable = infecting clone + immunizing treatment + infecting clone × immunization treatment) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. For analyses of within-host selection, we asked for mixed clone infections, whether the frequency of clone DS in the parasite population differed between the sham-immunized controls and the antigen immunizations.

3. Results

Table 1 gives details of the immunization treatments, infecting clone and sample size of the experiment. Some mice died; these were included in the calculation of daily densities until death, and in the analyses of peak parasite densities since death always occurred as initial parasiteamias were declining.

3.1. Pre-challenge anti-AMA-1 IgG2b antibodies

Fig. 1 illustrates the data from a pilot experiment where IgG2b antigen-specific antibodies were measured to each of the immunizing antigens and the cross-reactivity between them. All antigen immunization treatments generated antibody titres that were higher than those present in sham-immunized controls (sham-immunized versus antigen immunized: $F_{1,62} = 8.92$, $p = 0.004$). IgG2b antibodies were specific for the antigen they had been exposed to during immunization (immunizing treatment × ELISA antigen: $F_{1,40} = 9.99$, $p = 0.003$). For example, anti-DS AMA-1 IgG2b

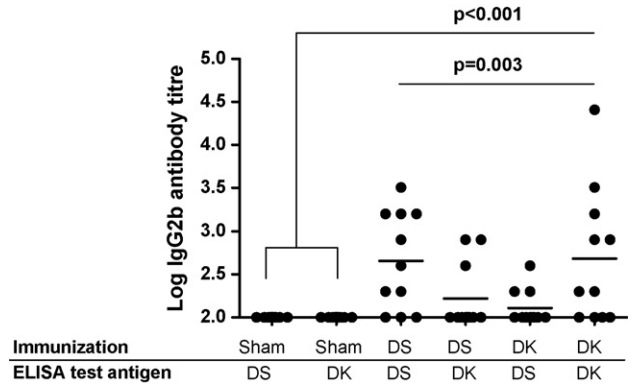

Fig. 1. IGg2b antibody levels from the serum of mice in the pilot experiment. Mice were either sham-immunized or immunised with one of the two atingens (DS AMA-1, DK AMA-1). Each of the treatments used to immunize mice and the AMA-1 test antigen used to coat ELISA plates are shown on the *x*-axis. Dots represent the antibody titre against a particular immunizing antigen for a single mouse. Horizontal lines indicate mean antibody levels. Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls ($p < 0.001$) and, among the immunized mice, the levels induced between the antigen immunized groups differed (immunizing treatment × ELISA antigen: $p = 0.003$) with higher titres against the homologous antigen. Neither of the immunising antigens induced higher titres ($p > 0.05$).

antibody tires were higher when assayed against the homologous DS antigen than the heterologous DK antigen and vice versa. Thus, neither antigen elicited a stronger response overall.

Fig. 2 illustrates the IgG2b antibody titres in mice from the main experiment 3 days prior to parasite infection. All antigen immunization treatments induced antibody titres that were higher than those present in sham-immunized mice (sham-immunized versus immunized: $F_{1,106} = 58.89$, $p < 0.001$). Among antigen-immunized groups, titres did not differ ($F_{2,69} = 2.56$, $p = 0.085$). In those mice which had been immunized with the bi-allelic form, antibodies were not more specifically recognising either component antigen ($F_{1,35} = 0.61$, $p = 0.44$). These data show that immunization successfully elicited antibody responses, and that, at least as measured by IgG2b titres, these responses were of equal magnitude in all immunized groups. Among antigen-immunized mice, antibody titres prior to challenge did not predict subsequent parasite intensities, weight loss or anaemia (all correlations, $p > 0.2$).

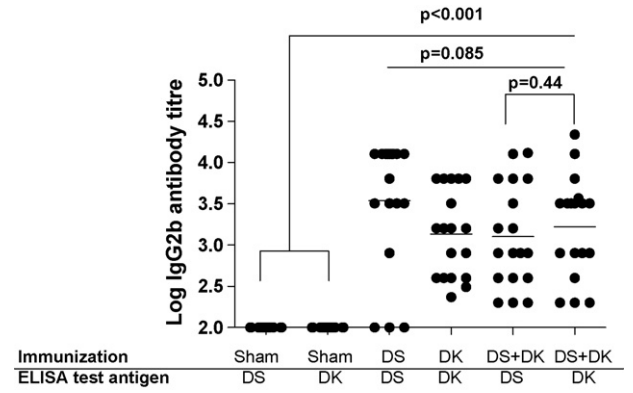

Fig. 2. IgG2b antibody levels from the serum of mice in the main experiment. Mice were either sham-immunized, or immunized with one of the antigen immunization treatments (DS AMA-1, DK AMA-1 or the bi-allelic formulation). Each of the treatments used to immunize mice and the AMA-1 test antigen used to coat ELISA plates are shown on the *x*-axis. Dots represent the antibody titre for individual mice against a particular antigen. Mice that were sham-immunized or immunized with the bi-allelic formulation were assayed for antibody responses against both DS and DK AMA-1 antigens. Horizontal lines indicate mean antibody levels. Antibody levels in antigen immunized groups of mice were higher than in sham-immunized controls ($p < 0.001$), and among the antigen immunized mice, antibody titres did not differ ($p = 0.085$). The antibody titres in animals immunized with both antigens were not dominated by responses to either one ($p = 0.44$).

3.2. Bi-allelic immunization did not generate a greater anti-morbidity response than did mono-allelic immunization

Red blood cell density and weight kinetics following parasite challenge for each of the immunization treatments are illustrated in Fig. 3A–F, and the minimum red blood cell density and minimum weight reached are illustrated in Fig. 3G–H. In sham-immunized control mice, clone DK was less virulent than clone DS, induced less anaemia and less weight loss (Fig. 3A–H; anaemia: $F_{2,14} = 6.29$, $p = 0.011$; weight loss: $F_{2,14} = 9.97$, $p = 0.002$).

Immunization protected mice against anaemia induced by infection with any of the clones (Fig. 3A–C, G; sham-immunized versus immunized: $F_{1,69} = 16.94$, $p < 0.001$). Bi-allelic immunization reduced anaemia no more than did immunization with either of the alleles alone (Fig. 3G; immunizing treatment × infecting clone: $F_{4,44} = 0.71$, $p = 0.59$). All pairwise immunization comparisons were non-significant ($p > 0.5$ in all cases).

As infection with clone DK did not induce any weight loss in sham-immunized controls (Fig. 3D) the protective effects of immunization were analysed only for infections that contained clone DS (Fig. 3E–F). We found that all immunizations protected mice against weight loss due to DS infections (Fig. 3E–F, H; sham-immunized versus immunized: $F_{1,45} = 11.13$, $p = 0.002$). Similar to the anaemia data, we found that immunization with either the bi-allelic form or either of the alleles alone afforded similar levels of protection against weight loss (Fig. 3H; immunizing treatment × infecting clone: $F_{2,29} = 2.43$, $p = 0.11$). All pairwise immunization comparisons were non-significant ($p > 0.5$ in all cases).

Together, these results show that immunization with the bi-allelic vaccine does not afford the host greater protection from morbidity, as measured by anaemia and weight loss. Immunization with either of the variants alone provided protection which was as effective as that induced by the two variants together.

3.3. Bi-allelic immunization did not generate greater anti-parasite response than did mono-allelic immunization

Parasite dynamics under each of the treatments are illustrated in Fig. 4. Clone DS achieved higher parasite density in sham-immunized control mice than did clone DK (infecting clone: $F_{1,10} = 7.03 = 0.024$).

All three immunizations reduced peak parasite densities relative to those which had received a sham inoculation (Fig. 4D; sham-immunized versus immunized: $F_{1,69} = 11.55$, $p = 0.001$). The extent of anti-parasite protection depended on the identity of the immunising antigen and the identity of the challenge clone (Fig. 4D; immunizing treatment × infecting clone: $F_{4,44} = 8.71$, $p < 0.001$). We found that protection was clone-specific: immunization with DS AMA-1 antigen reduced DS parasite densities more than it reduced the densities of clone DK, and vice versa (among single antigen immunized groups, immunizing treatment × infecting clone: $F_{1,19} = 36.26$, $p < 0.001$).

When we compared the extent of anti-parasite protection between the immunized groups we found that under no circumstances did the bi-allelic immunization afford greater protection than did immunization with a single allele. For example, immunization with DS AMA-1 reduced the peak density of DK infections and infections with both clones together, but the bi-allelic immunization did not protect against DS alone (Fig. 4D; immunizing treatment × infecting clone: $F_{2,30} = 9.84$, $p = 0.001$). Although the bi-allelic immunization reduced the densities of clone DK,

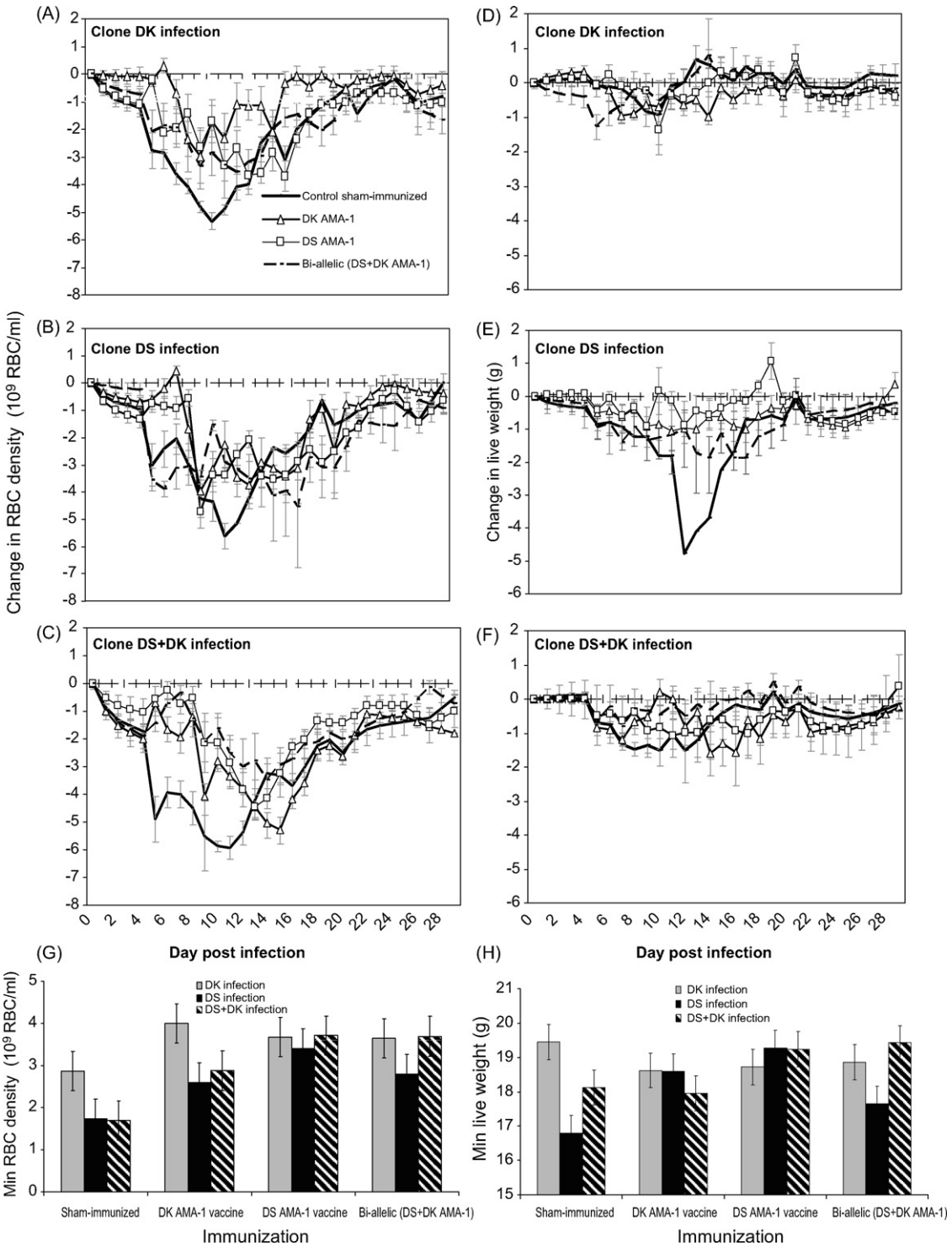

Fig. 3. Effect of *Plasmodium chabaudi* infection (clone DK alone, DS alone or DS + DK) and immunization (sham-immunized control, DK AMA-1, DS AMA-1, or bi-allelic form) on the kinetics of minimum red blood cell density (left panels) and minimum weight (right panels). In A–F lines represent the change in RBC density (left panels) and weight (right panels) over time. Each line represents the mean of up to 6 mice (±1 S.E.M.) that were infected with DK alone (A and D), DS alone (B and E) or a mixed clone (C and F) infection during immunization with either a sham-inoculation control (solid thick black line), DK AMA-1 (open triangle), DS AMA-1 (open squares), or the bi-allelic mixture (dotted black line). In G–H bars represent the minimum red blood cell density (left panel) and minimum weight (right panel) reached during infection with clone DK alone (grey bars), DS alone (black bars) or a mixture of both clones (black and white bars) under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice (±1 S.E.M.).

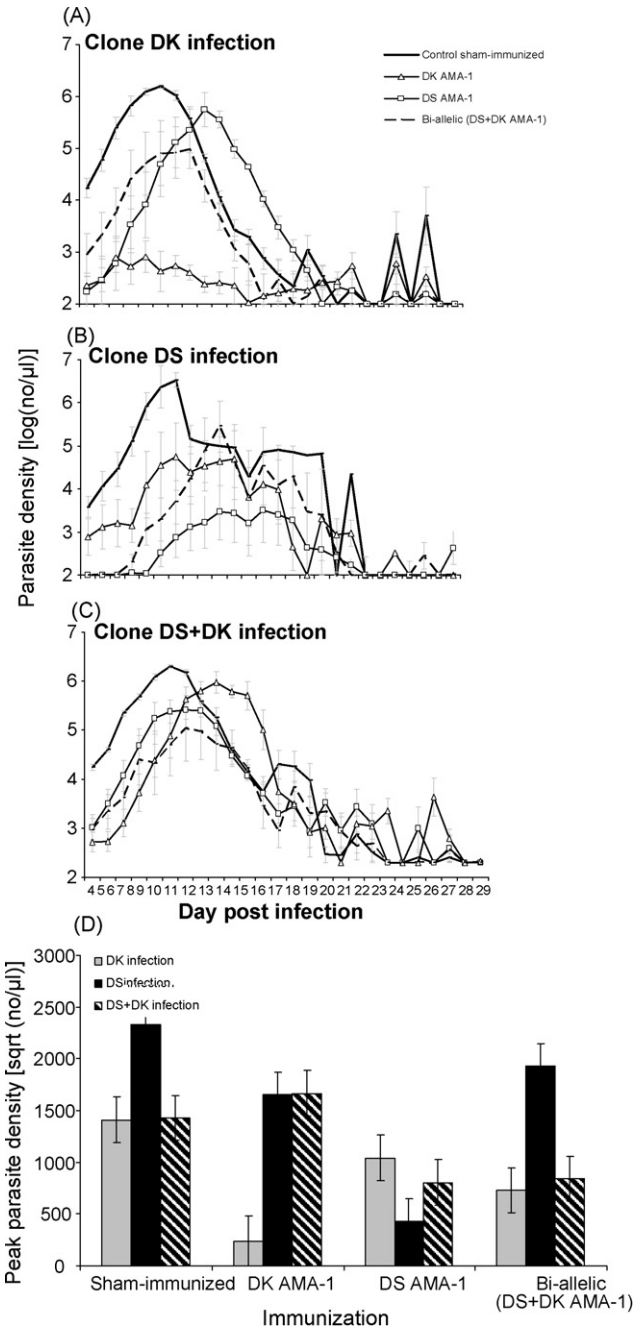

Fig. 4. Kinetics of *P. chabaudi* infections (clones DK alone, DS alone or both together) following immunization (DK AMA-1, DS AMA-1, or bi-allelic formulation or sham-immunized control). In A–C, lines represent the change in parasite density over time. Each line represents the mean of up to 6 mice (±1 S.E.M.) that were infected with DK alone (A), DS alone (B) or a mixed clone (C) infection during immunization with either a sham-inoculation control (solid thick black line), DK AMA-1 (open triangle), DS AMA-1 (open squares), or the bi-allelic mixture (dotted black line). In (D), bars represent peak parasite densities reached during infection with clone DK alone (grey bars), DS alone (black bars) or a mixture of both clones (black and white diagonal) under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice (±1 S.E.M.).

reduction was no greater than with a single DK AMA-1 immunization (Fig. 4D; immunizing treatment × infecting clone $F_{2,29} = 4.09$, $p = 0.027$).

Together these results show that bi-allelic immunization did not afford the host greater anti-parasite protection than did mono-allelic immunization. Unlike morbidity, where protection

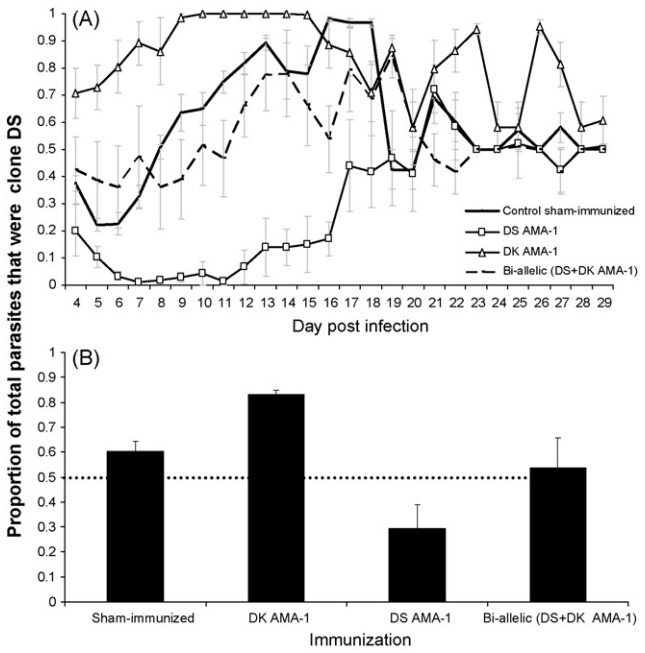

Fig. 5. Proportion of clone DS in mixed DS and DK infections following immunization with DK AMA-1, DS AMA-1, the bi-allelic formulation, or in sham-immunized controls. (A) Lines represents the proportion of clone DS through time in control (solid black line), DK AMA-1 (open triangles), DS AMA-1 (open diamonds) or bi-allelic (dotted black line) immunized mice. Each line represents the mean of up to 6 mice (±1 S.E.M.). (B) Bar graphs represent the proportion of total parasites in a mixed infection that were DS under each of the immunization treatments. Each bar represents the least squares mean of up to 6 mice with 95% confidence intervals. The black horizontal dotted line represents the proportion DS present in the inoculum.

was induced regardless of the antigen used in immunization, we found that immunization with a single allele achieved better protection against the homologous clone, and bi-allelic immunization never did as well. Indeed, we found just one of the variants (DS AMA-1) to be the most effective at reducing parasite densities.

3.4. Vaccine-induced anti-parasite protection was clone specific in mixed infections and independent of clone virulence

To examine how the antigenic composition of the immunizing formulation affects within-host selection (relative frequency) in mixed clone infections, and whether heterologous immunity less effectively controlled the virulent clone, we compared the frequency of clone DS in mixed infections (Fig. 5).

We found that antigen immunization altered clone frequencies. In sham-immunized mice, and those immunized with the bi-allelic formulation, DS made up about 60% of all the parasites present in the infections. Thus, immunization with a mixture of DS and DK AMA-1 had negligible effect on clone frequency and thus within-host selection (Fig. 5A; sham-immunized versus bi-allelic immunization: $F_{1,10} = 2.02$, $p = 0.19$). In contrast, immunization with a single antigen reduced parasites in a clone-specific manner, facilitating the heterologous clone (Fig. 5A and B; immunizing treatment $F_{1,10} = 105.54$, $p < 0.001$). Immunization with DK AMA-1 increased the frequency of clone DS, while DS AMA-1 immunization increased the frequency of DK. These effects were essentially symmetrical. Thus, there was no evidence that the more virulent clone, DS, was less affected by heterologous immunization than was the less virulent clone DK.

4. Discussion

In this study, we investigated (i) whether immunization with a single or bi-allelic AMA-1 formulation afforded the host the greatest protection from morbidity and parasite infection, (ii) how these different vaccination regimes altered clonal compositions in mixed infections, and (iii) whether a more virulent clone was less successfully controlled by vaccine-induced protective responses. Addressing each of these in turn, we found the following. (i) Bi-allelic immunization did not generate better anti-morbidity or anti-parasite protection than did single allele immunization. Rather, immunization with one of the two variants alone (DS) provided the best protection. (ii) Both single variant immunizations reduced the frequency of homologous clones in mixed infections; bi-allelic immunization had no impact on within-host selection. (iii) There was no evidence that the more virulent clone (DS) was better at evading vaccine-induced immunity than was the less virulent clone.

Rightly, protecting individual hosts from morbidity is one of the goals of malaria vaccines directed against the blood-stage of infection. If infection densities are positively correlated with host morbidity (virulence) [27] multi-allele vaccines could potentially improve the health of the host by suppressing more of the parasite population and reducing strain-specific responses. Subject to the usual cautions about generalising from animal models (reviewed in this context by Råberg et al. and Wargo et al. [60,61]), the results presented here argue against that, and suggest that protective efficacy may not be increased by including alternative variants of AMA-1. Our in vivo observations are consistent with previous results showing that immunization of rhesus monkeys with only one of two PfAMA-1 variants is sufficient to induce cross-protective antibody responses as measured by GIA and ELISA assays in vitro [52]. Our results are also consistent with another study which demonstrated that mice and rabbits immunized with two allelic variants of domain I and II of the full length AMA-1 ectodomain from Indian *P. falciparum* isolates were able to inhibit in vitro parasite growth, but to no greater extent than with either of the allelic variants alone [53].

Our results also demonstrate strain-specific anti-parasite responses (Fig. 4D) need not result in strain-specific protection against disease (Fig. 3G and H). The observation that there are two different types of anti-malarial responses – immunity against the parasite itself and immunity against disease – is poorly understood on a molecular basis although the distinction is widely appreciated [62]. An explanation for the two different responses observed here could be that the specificity of the anti-AMA-1 antibody response lies with the generation of inhibitory antibodies which may target the hypervariable region located around a conserved hydrophobic pocket on domain I [63]. The presence of such antibodies could determine the observed parasitaemias. For bi-allelic immunizations there may exist a dominant epitope in one allelic form of AMA-1. Thus, high titres of cross-reactive antibodies may be sufficient to lessen morbidity (hence the similar effects for mono-and-bi-allelic vaccination on morbidity) but the inhibitory antibodies are more effective at controlling parasite numbers by inhibiting invasion. In our pilot studies we did not observe a disproportional IgG2b antibody response to one of the immunizing antigens (Fig. 1). However, since immunization with AMA-1 is likely to induce a repertoire of IgG isotypes [58a,64–66] some of the other isotypes may be sufficiently cross-reactive. An implication of this may be that while strain-specific immunization may alter allele frequencies in parasite populations, this need not have clinical consequences in a vaccinated host. Changes in allele frequencies without public health consequences have been seen in some other diseases, such as pertussis (reviewed in [26]).

The 'Combination B' malaria vaccine, one of the few to reach field trials, demonstrated strain-specific anti-parasite effects despite being comprised of an allele of each of 3 asexual blood stage proteins, MSP-1, MSP-2 and RESA (ring-infected erythrocyte surface antigen) [29]. Of particular interest was that vaccination increased the frequency of parasites with an MSP-2 genotype belonging to the FC27 allelic family. No representatives of this allelic family, which had been found previously to be associated with severe morbidity, were included in the vaccine [16,29,30]. Selection for the FC27 form of MSP-2 could have been because of strain-specific protection [15,31], or because the vaccine was less effective at protecting against more virulent strains [26–28]. In the study we report here, we looked at the relative proportion of the more virulent clone in a mixed infection under the different immunization compositions. In sham-immunized control mice and those which received the bi-allelic immunization, the more virulent clone (DS) was proportionally the most dominant. Thus, bi-allelic immunization did not alter within-host selection. On the other hand, immunization with a single AMA-1 variant did facilitate evasion of the heterologous clone in mixed infections. In our experiments, this effect was symmetrical (Fig. 5), so that immunization with AMA-1 appears to induce protective responses that are strain-specific and evasion is independent of parasite virulence.

Nevertheless, selection for virulence could be an inadvertent consequence of including just one allele from a given locus in a vaccine, as apparently happened in the Combination B trial. As far as we are aware, there are no reports that variants of AMA-1 have different intrinsic virulence, so that the strain-specific immunity against this locus we report here and that has been seen by others [7,34], should not directly alter virulence. But caution is necessary for all antigens involved in processes like cell invasion which are associated with pathogenesis. Population-level association studies for disease severity should be performed for all antigens included in candidate vaccines. Should associations like that for MSP-2 be found [30], we suggest on the basis of our results that there would be a strong case for including all known variants at that locus in the vaccine. This would not confer short-term clinical advantage, but it would be the safest way to avoid inadvertent selection for virulent variants, which would put unvaccinated people at greater risk.

More generally, though, we still have some way to go to understand the potential for vaccine-driven virulence evolution, even in the *P. chabaudi* model. One experimental study demonstrated that parasites from a single *P. chabaudi* clone serially passaged through whole-parasite immunized mice evolved to be more virulent than those evolved in naive hosts [28]. That study was the first to show under controlled conditions that immunization can favour the evolution of more virulent parasites. The implication was that more virulent variants had a selective advantage in immunized hosts. In the study we report here, which did not involve serial passage, we saw no signs of such an advantage. DS, the more virulent clone, dominated in mice immunized with the bi-allelic form, but to the same extent as in non-immunized mice. In single antigen immunized mice, strain-specific immunity dominated with symmetrical effects for both clones. Competition experiments with other *P. chabaudi* clones also failed to find an increased advantage to virulence in immunized hosts [67]. It may be that the accelerated evolution of virulence seen during serial passage in immunized hosts [28,68] is a feature of selection of virulence variants on an antigenically identical background. In future experiments, we will serially passage single *P. chabaudi* clones through AMA-1 immunized and naïve mice to determine whether vaccination can evolve virulence to be greater when measured in naïve hosts.

Our experiments concerned antigenic polymorphism at a single target antigen. Considerably more work has focused on vaccines combining single variants from multiple antigenic loci [1,69,70]. For

example, animal and human phase I trails have shown safety, tolerability and immunogenecity of formulations containing AMA-1 and MSP-1 [1,71–73]. Moreover, such 'multi-valent' vaccines have been shown to reduce parasitaemias in mice of distinct MHC haplotypes [74] and against infections with different parasite strains as well as subspecies of different virulence [75]. Thus, multi-valency may be required to induce antibody responses against a repertoire of polymorphic parasite antigens [64,66,76–81] in the human outbred population exposed to multiple parasite genotypes [29,78,82–84]. We suspect that multi-valent vaccines will prove to be a more efficient means of generating protection against the widest range of parasite genotypes. Certainly, we found no evidence that the anti-morbidity and anti-parasitic potency of a malaria vaccine would be enhanced by increasing the number of variants of a particular antigen.

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References

[1] Richie TL, Saul A. Progress and challenges for malaria vaccines. Nature 2002;415:694–701.
[2] Anders RF, Crewther PE, Edwards S, Margetts M, Matthew MLSM, Pollock B, et al. Immunisation with recombinant AMA-1 protects mice against infection with *Plasmodium chabaudi*. Vaccine 1998;16:240–7.
[3] Martinelli A, Cheesman S, Hunt P, Culleton P, Raza A, Mackinnon M, et al. A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites. Proc Natl Acad Sci USA 2005;102:814–9.
[4] Cheesman S, Raza A, Carter R. Mixed strain infections and strain-specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. Infect Immun 2006;74:2996–3001.
[5] Mendis KN, David PH, Carter R. Anigenic polymorphism in malaria: is it an important mechanism for immune evasion? Immunol Today 1991;12: A34–7.
[6] Jeffery GM. Epidemiological significance of repeated infections with homologous and heterologous starins and species of *Plasmodium*. WHO Bull 1966;35:873–82.
[7] Healer J, Murphy V, Hodder AN, Masciantonio R, Gemmill AW, Anders RF, et al. Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. Mol Microbiol 2004;52:159–68.
[8] Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, et al. In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infect Immun 2002;70:6948–60.
[9] Saul A. Malaria vaccines based on the *Plasmodium falciparum* merozoite surface protein 3-should we avoid amino acid sequence polymorphisms or embrace them? J Infect Dis 2007;195:171–3.
[10] McGregor IA. Mechanisms of acquired immunity and epidemiological patterns of antibody response in malaria in man. Bull WHO 1974;50:259–66.
[11] McGregor IA, Gilles HM, Walters JH, Davies AH, Pearson FA. Effects of heavy and repeated malaria infections on Gambian infants and children; effects of erythrocytic parasitization. Br Med J 1956;2:686–92.
[12] Day KP, Marsh K. Naturally acquired immunity to *Plasmodium falciparum*. Immunol Today 1991;12:A68–71.
[13] Hodder AN, Crewther PE, Anders RF. Specificity of the protective antibody response to apical membrane antigen 1. Infect Immun 2001;69: 3286–94.
[14] Genton B, Reed ZH. Asexual blood-stage malaria vaccine development: facing the challenges. Curr Opin Infect Dis 2007;20:467–75.
[15] Fluck C, Smith T, Beck H-P, Irion A, Betuela I, Alpers MP, et al. Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun 2004;72:6300–5.
[16] Genton B, Anders RF, Alpers MP, Reeder JC. The malaria vaccine development program in Papua New Guinea. Trends Parasitol 2003;19:264–70.
[17] Bolad A, Berzins K. Antigenic diversity of *Plasmodium falciparum* and antibody-mediated parasite neutralization. Scand J Immunol 2000;52:233–9.
[18] Hoffmann SL, Miller LH. In: Hoffman SL, editor. Malaria Vaccine Development: a multi-immune response approach. Washington, DC: American Society for Microbiology; 1996. p. 35–76.
[19] Polley S, Tetteh KA, Lloyd J, Akpogheneta O, Greenwood B, Bojang K, et al. *Plasmodium falciparum* merozoite surface protein 3 is a target of allele specific immunity and alleles are maintained by natural selection. J Infect Dis 2007;195:279–87.
[20] Gandon S, Mackinnon MJ, Nee S, Read AF. Imperfect vaccines and the evolution of pathogen virulence. Nature 2001;414:751–6.
[21] Gandon S, Day T. Understanding and managing pathogen evolution: a way forward. Trends Microbiol 2003;11:206–7.
[22] Restif G, Grenfell BT. Integrating life-history and cross-immunity into the evolutionary dynamics of pathogens. Proc R Soc Lond B Biol Sci 2006;273:409–16.
[23] Gandon S, Day T. The evolutionary epidemiology of vaccination. J R Soc Int 2007;4:803–17.
[24] André JB, Gandon S. Vaccination, within-host dynamics, and virulence evolution. Evolution 2006;60:13–23.
[25] Williams PD, Day T. Epidemiological and evolutionary consequences of targeted vaccination. Mol Ecol 2008;17:485–99.
[26] Read AF, Mackinnon MJ. Pathogen evolution in a vaccinated world. In: Stearns SC, Koella JC, editors. Evolution in health and disease. 2nd ed. Oxford University Press; 2008. p. 139–52.
[27] Mackinnon MJ, Gandon S, Read AF. Virulence evolution in response to vaccination: the case of malaria. Vaccine 2008;26:C42–52.
[28] Mackinnon M, Read AF. Immunity promotes virulence evolution in a malaria model. PLoS Biol 2004;2:1–7.
[29] Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, et al. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J Infect Dis 2002;185:820–7.
[30] Engelbrecht F, Felger I, Genton B, Alpers M, Beck HP. *Plasmodium falciparum*: malaria morbidity is associated with specific merozoite surface antigen 2 genotypes. Exp Parasitol 1995;81:90–6.
[31] Fluck C, Schopflin S, Smith T, Genton B, Alpers MP, Beck H-P, et al. Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. Infect Genet Evol 2007;7:44–51.
[32] Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006;124:755–66.
[33] Collins WE, Pye D, Crewther PE, Vandenberg KL, Galland GG, Sulzer AJ, et al. Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of *Plasmodium fragile*. Am J Trop Med Hyg 1994;51:711–9.
[34] Crewther PE, Matthew ML, Flegg RH, Anders RF. Protective immune responses to apical membrane antigen 1 of *Plasmodium chabaudi* involve recognition of strain-specific epitopes. Infect Immun 1996;64:3310–7.
[35] Deans JA, Knight AM, Jean WC, Waters AP, Cohen S, Mitchell GH. Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. Parasite Immunol 1988;10:535–52.
[36] Stowers AW, Kennedy MC, Keegan BP, Saul A, Long CA, Miller LH. Vaccination of monkeys with recombinant *Plasmodium falciparum* apical membrane antigen 1 confers protection against blood-stage malaria. Infect Immun 2002;70:6961–7.
[37] Kocken CHM, Withers-Martinez C, Dubbeld MA, van der Wel A, Hackett F, Blackman MJ, et al. High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that Inhibit erythrocyte Invasion. Infect Immun 2002;70:4471–6.
[38] Cortes A, Mellombo M, Masciantonio R, Murphy VJ, Reeder JC, Anders RF. Allele specificity of naturally acquired antibody responses against *Plasmodium falciparum* apical membrane antigen 1. Infect Immun 2005;73:422–30.
[39] Johnson AH, Leke RGF, Mendell NR, Shon D, Suh YJ, Bomba-Nkolo D, et al. Human leukocyte antigen class II alleles influence levels of antibodies to the *Plasmodium falciparum* asexual-stage apical membrane antigen 1 but not to merozoite surface antigen 2 and merozoite surface protein 1. Infect Immun 2004;72:2762–71.
[40] Thomas AW, Trape J-F, Rogier C, Goncalves A, Rosario VE, Narum DL. High prevalence of natural antibodies against *Plasmodium falciparum* 83-kilodalton apical membrane antigen (PF83/AMA-1) as detected by capture-enzyme-linked immunosorbent assay using full-length baculovirus recombinant PF83/AMA-1. Am J Trop Med Hyg 1994;51:730–40.
[41] Polley SD, Mwangi T, Kocken CHM, Thomas AW, Dutta S, Lanar DE, et al. Human antibodies to recombinant protein constructs of *Plasmodium falciparum* apical membrane antigen 1 (AMA1) and their associations with protection from malaria. Vaccine 2004;23:718–28.
[42] Rodrigues MHC, Rodrigues KM, Oliveira TR, Cômodo AN, Rodrigues MM, Kocken CHM, et al. Antibody response of naturally infected individuals to recombinant *Plasmodium vivax* apical membrane antigen-1. Int J Parasitol 2005;35:185–92.
[43] Polhemus ME, Magill AJ, Cummings JF, Kester KE, Ockenhouse CF, Lanar DE, et al. Phase I dose escalation safety and immunogenicity trial of *Plasmodium falciparum* apical membrane protein (AMA-1) FMP2.1, adjuvanted with AS02A, in malaria-naive adults at the Walter Reed Army Institute of Research. Vaccine 2007;25:4203–12.
[44] Thera MA, Doumbo OK, Coulibaly D, Diallo DA, Kone AK, Guindo AB, et al. Safety and immunogenicity of an AMA-1 malaria vaccine in Malian adults: results of a phase 1 randomized controlled trial. PLoS ONE 2008;3:e1465.
[45] Maher B. The end of the beginning. Nature 2008;451:1042–6.
[46] Dutta S, Lee SY, Batchelor AH, Lanar DE. Structural basis of antigenic escape of a malaria vaccine candidate. Proc Natl Acad Sci USA 2007;104:12488–93.
[47] Polley SD, Chokejindachai W, Conway DJ. Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. Genetics 2003;165:555–61.

[48] Polley SD, Conway DJ. Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1. Genetics 2001;158: 1505–12.
[49] Peterson MG, Marshall VM, Smythe JA, Crewther PE, Lew A, Silva A, et al. Integral membrane protein located in the apical complex of *Plasmodium falciparum*. Mol Cell Biol 1989;9:3151–4.
[50] Marshall VM, Zhang L, Anders RF, Coppel RL. Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum*. Mol Biochem Parasitol 1996;77:109–13.
[51] Malkin EM, Diemert DJ, McArthur JH, Perreault JR, Miles AP, Giersing BK, et al. Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. Infect Immun 2005;73:3677–85.
[52] Miura K, Zhou H, Muratova OV, Orcutt AC, Giersing B, Miller LH, et al. *Plasmodium falciparum* apical membrane antigen 1(AMA1) immunization: fine specificity of antibodies depends on species immunized. Infect Immun 2007;75:5827–36.
[53] Lalitha PV, Biswas S, Pillai CR, Saxena RK. Immunogenicity of a recombinant malaria vaccine candidate, domain I + II of AMA-1 ectodomain, from Indian *P. falciparum* alleles. Vaccine 2008;26:4526–35.
[54] Beale GH, Walliker D, Carter R. Genetics. In: Killick-Kendrick R, Peters W, editors. Rodent malaria. London: Academic Press; 1978. p. 213–45.
[55] Mackinnon MJ, Read AF. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Evolution 1999;53:689–703.
[56] Marshall VM, Peterson MG, Lew AM, Kemp DJ. Structure of the apical membrane antigen I (AMA-1) of *Plasmodium chabaudi*. Mol Biol Parasitol 1989;37:281–3.
[57] de Roode JC, Culleton R, Bell AS, Read AF. Competitive release of drug resistance following drug treatment of mixed *Plasmodium chabaudi* infections. Mala J 2004;3:33.
[58] (a) Timms R, Colegrave N, Chan BHK, Read AF. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitol 2001;123:1–11;
(b) Burns Jr JM, Flaherty PR, Nanavati P, Weidanz WP. Protection against *Plasmodium chabaudi* malaria induced by immunization with apical membrane antigen 1 and merozoite surface protein 1 in the absence of gamma interferon or interleukin-4. Infect Immun 2004;72:5605–12.
[59] Bell AS, Roode JC, Sim D, Read AF. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution 2006;60:1358–71.
[60] Råberg L, de Roode JC, Bell AS, Stamou P, Gray D, Read AF. The role of immune-mediated apparent competition in genetically diverse malaria infections. Am Nat 2006;168:41–53.
[61] Wargo AR, Huijben S, de Roode JC, Shepherd J, Read AF. Competitive release and facilitation of drug-resistant parasites after therapeutic chemotherapy in a rodent malaria model. Proc Natl Acad Sci USA 2007;104:19914–9.
[62] Schofield L, Grau GE. Immunological processes in malaria pathogenesis. Nat Rev Immunol 2005;5:722–35.
[63] Coley AM, Gupta A, Murphy VJ, Bai T, Kim H, Anders RF, et al. Structure of the malaria antigen AMA1 in complex with a growth-inhibitory antibody. PLoS Pathogens 2007;3:e138.
[64] Eisenhut M. Immunity to blood stages of *Plasmodium falciparum* is dependent on a specific pattern of immunoglobulin subclass responses to multiple blood stage antigens. Med Hypotheses 2007;69:804–8.
[65] Gray JC, Corran PH, Mangia E, Gaunt MW, Li Q, Tetteh KKA, et al. Profiling the antibody immune response against blood stage malaria vaccine candidates. Clin Chem 2007;53:1244–53.
[66] Osier FHA, Fegan G, Polley SD, Murungi L, Verra F, Tetteh KKA, et al. Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. Infect Immun 2008;76:2240–8.
[67] Grech K, Chan BHK, Anders RF, Read AF. The impact of immunization on competition within *Plasmodium* infections. Evolution 2008;62:2359–71.
[68] Mackinnon M, Read AF. Virulence in malaria: an evolutionary viewpoint. Phil Trans R Soc Lond B 2004;359:965–86.
[69] Garcia JE, Puentes A, Patarroyo ME. Developmental biology of sporozoite-host interactions in *Plasmodium falciparum* malaria: Implications for vaccine design. Clin Microbiol Rev 2006;19:686–707.
[70] Heppner JDG, Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, et al. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. Vaccine 2005;23:2243–50.
[71] Hu J, Chen Z, Gu J, Wan M, Shen Q, Kieny M-P, et al. Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. PLoS ONE 2008;3:e1952.
[72] Pan W, Huang D, Zhang Q, Qu L, Zhang D, Zhang X, et al. Fusion of two malaria vaccine candidate antigens enhances product yield, immunogenicity, and antibody-mediated inhibition of parasite growth in vitro. J Immunol 2004;172:6167–74.
[73] Burns JJM, Flaherty PR, Romero MM, Weidanz WP. Immunization against *Plasmodium chabaudi* malaria using combined formulations of apical membrane antigen-1 and merozoite surface protein-1. Vaccine 2003;21:1843–52.
[74] Doolan D, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL. Circumventing genetic restriction of protection against malaria with muligene DNA immunization: CD8+ cell-interferon gamma-, and nitric oxide-dependent immunity. J Exp Med 1996;183:1739–46.
[75] Scorza T, Grubb K, Cambos M, Santamaria C, Malu DT, Spithill TW. Vaccination with a *Plasmodium chabaudi adami* multivalent DNA vaccine cross-protects A/J mice against challenge with *P. c. adami* DK and virulent *Plasmodium chabaudi chabaudi* AS parasites. Int J Parasitol 2008;38:819–27.
[76] Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. J Exp Med 1995;182:409–18.
[77] Bull PC, Marsh K. The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. Trends Microbiol 2002;10:55–8.
[78] Doolan DL, Aguiar JC, Weiss WR, Seete A, Lelgner PL, Regis DP, et al. Utilization of genomic sequence information to develop malaria vaccines. Exp Biol (Berl) 2003;206:3789–802.
[79] Moorthy VS, Good MF, Hill AVS. Malaria vaccine developments. The Lancet 2004;363:150–6.
[80] Wipasa J, Elliot S, Xu H, Good MF. Immunity to asexual blood-stage malaria vaccine approaches. Immunol Cell Biol 2002;80:401–14.
[81] Wipasa J, Hirunpetcharat C, Mahakunkijcharoen Y, Xu H, Elliott S, Good MF. Identification of T cell epitopes on the 33-kDa fragment of *Plasmodium yoelii* merozoite surface protein 1 and their antibody-independent protective role in immunity to blood stage malaria. J Immunol 2002;169:944–51.
[82] Doolan DL, Hoffman SL. DNA-based vaccines against malaria: status and promise of the multi-stage malaria DNA vaccine operation. Int J Parasitol 2001;31:753–62.
[83] Plebanski M, Proudfoot O, Pouniotis D, Coppel RL, Apostolopoulos V, Flannery G. Immunogenetics and the design of *Plasmodium falciparum* vaccines for use in malaria-endemic populations. J Clin Investig 2002;110: 295–301.
[84] Cortes A, Mellombo M, Mueller I, Benet A, Reeder JC, Anders RF. Geographical structure of diversity and differences between symptomatic and asymptomatic infections for *Plasmodium falciparum* vaccine candidate AMA1. Infect Immun 2003;71:1416–26.

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CD4$^+$T cells do not mediate within-host competition between genetically diverse malaria parasites

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Ecological interactions between microparasite populations in the same host are an important source of selection on pathogen traits such as virulence and drug resistance. In the rodent malaria model *Plasmodium chabaudi* in laboratory mice, parasites that are more virulent can competitively suppress less virulent parasites in mixed infections. There is evidence that some of this suppression is due to immune-mediated apparent competition, where an immune response elicited by one parasite population suppress the population density of another. This raises the question whether enhanced immunity following vaccination would intensify competitive interactions, thus strengthening selection for virulence in *Plasmodium* populations. Using the *P. chabaudi* model, we studied mixed infections of virulent and avirulent genotypes in CD4$^+$T cell-depleted mice. Enhanced efficacy of CD4$^+$T cell-dependent responses is the aim of several candidate malaria vaccines. We hypothesized that if immune-mediated interactions were involved in competition, removal of the CD4$^+$T cells would alleviate competitive suppression of the avirulent parasite. Instead, we found no alleviation of competition in the acute phase, and significant enhancement of competitive suppression after parasite densities had peaked. Thus, the host immune response may actually be alleviating other forms of competition, such as that over red blood cells. Our results suggest that the CD4$^+$-dependent immune response, and mechanisms that act to enhance it such as vaccination, may not have the undesirable affect of exacerbating within-host competition and hence the strength of this source of selection for virulence.

Keywords: malaria; CD4$^+$T cells; competition

1. INTRODUCTION

Parasitic infections are often genetically diverse, with hosts concurrently infected by more than one genotype. Crowding, where pathogen populations within a host are suppressed by the presence of competitor strains, could affect the health and infectiousness of individual hosts as well as the evolution of medically relevant traits such as virulence and drug resistance (Read & Taylor 2001). For example, selection for increased virulence is expected when a slower growing parasite is outcompeted by a faster growing, more virulent parasite (Bremermann & Pickering 1983; van Baalan & Sabelis 1995; Frank 1996; Gandon *et al.* 2001; Alder & Losada 2002). Similarly, the relative fitness of drug-resistant strains, and hence their rate of spread in a population, could be substantially enhanced when co-infecting drug-sensitive competitors are removed by chemotherapy (e.g. Hastings 1997, 2003, 2006; Mackinnon & Hastings 1998; Hastings & D'Alessandro 2000; Mackinnon 2005). Analogous evolutionary processes could affect the rate of evolution of epitope variants in response to strain-specific vaccination (Lipsitch & Samore 2002; Read & Mackinnon 2008).

Infections with the human malaria parasite *Plasmodium falciparum* frequently consist of more than one genotype (Anderson *et al.* 2000; Awadalla *et al.* 2001; Jafari *et al.* 2004; Walliker *et al.* 2005), and a variety of epidemiological evidence is consistent with crowding (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith *et al.* 1999; Bruce *et al.* 2000; Hastings 2003; Talisuna *et al.* 2006). In the rodent malaria model *Plasmodium chabaudi* in laboratory mice, there is a strong relationship between parasite virulence and crowding such that more virulent strains have a competitive advantage (de Roode *et al.* 2003, 2005*a*,*b*; Bell *et al.* 2006).

A number of biological mechanisms may underlie competition between strains within hosts (Read & Taylor 2001). One of these is immune-mediated apparent competition (Holt 1977), where increasing densities of one pathogen population elicits a host response that suppresses the population of another. T cell-dependent immune-mediated competition has been demonstrated in *P. chabaudi* (Råberg *et al.* 2006): in nude mice, which cannot produce mature T cells, competition was less severe than in nude mice reconstituted with T cells. Because many malaria vaccines currently under trial are aimed at inducing T cell-dependent responses, that experiment raised the question of whether vaccination

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might exacerbate in-host competition and thus affect pathogen evolution, for instance by strengthening selection for competitive ability and hence virulence.

The effects of immunity on in-host competition are unlikely to be simple. The immune response to *Plasmodium* infection has both pathogen genotype-transcending (non-specific) and genotype-specific components. Protection is generally thought to become more specific during later stages of infection (Jarra & Brown 1989; Buckling & Read 2001; Mackinnon & Read 2003; Stevenson & Riley 2004; Martinelli *et al.* 2005; Cheesman *et al.* 2006). Thus, in contrast to non-specific immunity that could generate immune-mediated apparent competition, specific immunity could, in principle, act to alleviate competition (Råberg *et al.* 2006). Here, we extend the study by Råberg *et al.* (2006) by focusing on a specific subset of T cells, in order to further investigate the importance of immunity in determining competitive outcomes within hosts.

T cells can be divided into two major categories, CD4$^+$ and CD8$^+$ cells. It is well established from both experimental animal models and field studies in humans that the CD4$^+$T cells play a pivotal role in the development of blood stage immunity to *Plasmodium* infection (Good & Doolan 1999; Pombo *et al.* 2002). They are initially required to produce cytokines that amplify the phagocytic and parasitocidal response of the innate immune response and later on to dampen this response to limit immunopathology. As the response becomes more adaptive they are required to help B cells produce antibodies that are essential for parasite clearance (Urban *et al.* 2005; Stephens & Langhorne 2006).

Since the CD4$^+$T cells have been described as having such a crucial role in natural immunity to the blood stage of infection, and vaccine programmes strive to mimic and enhance this response (e.g. Stephens & Langhorne 2006), we have begun to investigate the specific role of these cells during competition in mixed infections of *P. chabaudi*. Specifically we looked at the acute phase of infection, where any interaction between the parasite and the host immune response could strongly influence host health (Urban *et al.* 2005). We chose two parasite genotypes that had been shown previously to differ in competitive ability and compared the extent of competition in immuno-competent and CD4$^+$T cell-depleted mice. We hypothesized two possible scenarios: (i) if T cell-dependent immunity induces a non-specific response, then a numerically subdominant clone would experience a stronger immune response in a mixed infection than when on its own. Thus, competition should be eased in CD4$^+$T cell-depleted mice. (ii) If the immune response is largely clone-specific and primarily elicited against the numerically dominant clone, then CD4$^+$T cell depletion may exacerbate other forms of competition, such as competition for limited resources such as red blood cells.

2. MATERIAL AND METHODS

(a) *Parasites and hosts*

Isolates of *P. chabaudi* were originally collected from *Thamnomys rutilans* in the Central African Republic (Beale *et al.* 1978). These isolates have been genotyped and are stored as frozen stabilates in liquid nitrogen with subscript codes used to identify their position in the clonal history (Mackinnon & Read 1999). Two genotypes, AS_{12062} and DK_{108}, were chosen based on their relative virulence and non-lethality. Pilot studies showed that clone DK achieved higher parasite densities when clone AS was absent than when AS was present. In contrast, clone AS was not competitively suppressed by DK. Hosts were inbred female C57BL/6JolaHsd mice aged six to eight weeks (Harlan England) maintained as described previously (de Roode *et al.* 2004).

(b) *Depletion of CD4$^+$T lymphocytes* in vivo

A rat monoclonal antibody, GK1.5, was used to deplete the CD4$^+$T cells. A non-depleting rat monoclonal antibody of the same isotype (IgG 14131, Sigma) was used as a control. Experimental mice were injected intraperitoneally with 500 μg of the appropriate purified antibody in phosphate-buffered saline (PBS) 5 days before parasite challenge, and then with 250 μg antibody 4 days and 1 day before parasite challenge and weekly after challenge.

A fluorescence-activated cell sorter (FACS) was used to confirm CD4$^+$T cell depletion. From the tail snip, 20 μl of blood was taken 1 day prior to injection with the appropriate antibodies. Single cell suspensions were made by removing red blood cells using Lympholyte according to the manufacturer's instructions (Cedarlane, Canada). Approximately 1×10^6 cells were then transferred to a round-bottomed plate and resuspended in FACS buffer (PBS with 2% FCS with 0.05% sodium azide) before incubation for 20 min at 4°C with Allophycocyanin (APC)-labelled anti-CD4$^+$ antibody (Pharmingen). The cells were washed three times in FACS buffer. Samples were collected on a FACS Calibre and 10 000 live events were collected for the majority of samples. FlowJo (TreeStar, CA) was used to analyse the data.

(c) *Experimental setup and sampling*

Groups of five mice were treated with: (i) control antibodies and challenged with 10^6 AS parasites, (ii) control antibodies and challenged with 10^6 DK parasites, (iii) control antibodies and challenged with 10^6 AS and 10^6 DK, (iv) anti-CD4 antibodies and challenged with 10^6 AS, (v) anti-CD4 antibodies and challenged with 10^6 DK, and (vi) anti-CD4 antibodies and challenged with 10^6 AS and 10^6 DK.

Parasites were delivered by intraperitoneal injection. We used the same dose of each genotype in single and mixed infections (rather than the same total dose in single and mixed infections) because the aim of the study was to compare the performance of a genotype when it is on its own, with its performance when it is in a mixed infection. A twofold difference in infective dose has negligible effects on the population dynamics of the parasite (Timms *et al.* 2001). In addition, we included two extra control groups, each of two mice that were not challenged with malaria, one group treated with anti-CD4 antibodies and another group with control antibodies. These mice were used to check whether CD4$^+$T cell depletion was continuous throughout the experiment, as the number of peripheral T cells was lower than normal during the acute stage of disease (Hviid *et al.* 1997).

During the course of infection, we measured body weights and took blood samples from the tail to make Giemsa-stained blood smears and to estimate RBC density (by flow cytometry; Beckman Coulter) and for genotype-specific real-time quantitative PCR (qPCR) assays.

One mouse died during the experiment (CD4$^+$T cell-depleted, mixed infection) and was included in the analyses only where possible. For unknown reasons, two infections

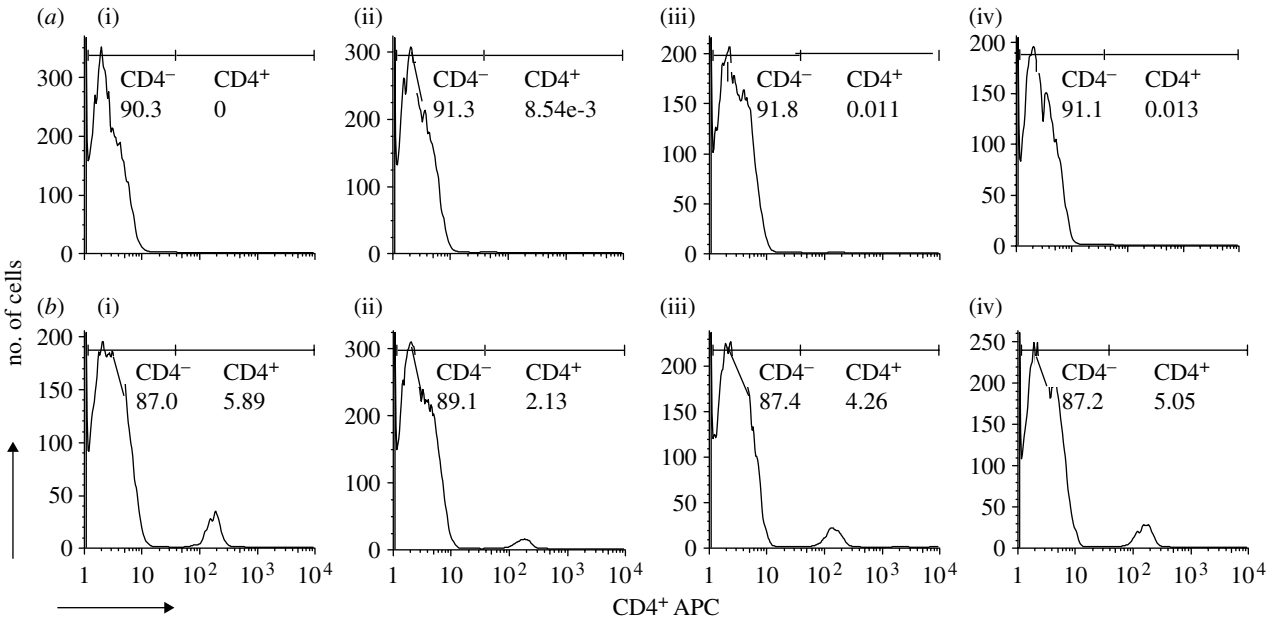

Figure 1. FACS plots of number of cells analysed and percentage of $CD4^+$T cells in (*a*) $CD4^+$T cell-depleted and (*b*) intact control mice. Percentage of the $CD4^+$T cells was analysed: (i) 1 day before parasite challenge and (ii–iv) once a week throughout the experiment. Each graph is a representative of one mouse from either the $CD4^+$T cell-depleted or immunocompetent control group.

(both non-depleted, a DK-only and an AS-only) achieved a peak parasite density two orders of magnitude lower than all others, and these were excluded from all the analyses.

(d) *Quantitative PCR*

Samples were taken in the morning as this is the stage when most parasites are in the ring or early trophozoite stage in the peripheral blood, when parasite ploidy is stable (de Roode *et al.* 2004). From each mouse, 5 µl of tail blood was taken and added to 100 µl of citrate saline on ice. Samples were subsequently pelleted by centrifugation and the citrate saline was removed. Blood was stored at −80°C until required. DNA extraction was performed using the BloodPrep kit (Applied Biosystems) on the ABI PRISM 6100 Nucleic Acid prep-station according to the manufacturer's instructions. DNA was eluted in a total volume of 200 µl and stored at −80°C until quantification. Genotype-specific qPCR was performed as described previously (Bell *et al.* 2006) with the addition of the DK-specific reverse primer: 5′-AGG CAT GTT TTG CAC ACA ATG A-3′.

(e) *Trait definition and statistical analyses*

We define competitive suppression to be a reduction of parasite numbers when another clone is present, which we tested for by comparing the performance of a clone in single and mixed infections. Performance was measured as the clonal density summed over a defined time period. *Plasmodium chabaudi* has a 24 hour replication cycle, so the total number of parasites present in any period can be estimated by summing the daily parasite counts. Thus, to test whether competitive suppression was $CD4^+$T cell mediated, we asked, for each clone, whether the magnitude of any competitive suppression differed between intact control and $CD4^+$T cell-depleted hosts; that is, whether there was a statistical interaction between immune treatment (intact control versus $CD4^+$T cell-depleted hosts) and infection type (single versus mixed).

The effects of competition and $CD4^+$ depletion on the performance of individual clone and red blood cell density were first examined by using general linear models (GLM) in the statistical package Minitab (release 14, Minitab, Inc.,). For GLM analysis, response variables included mean total parasite density and mean RBC density, with initial RBC as a covariate. Explanatory variables for GLM included $CD4^+$ depletion (depleted or intact control) and competition (clone alone or in mixed infection). Maximal models (response variable = $CD4^+$ depletion + competition + all higher order interactions) were tested in the first instance, and minimal models were obtained by dropping non-significant terms successively, beginning with highest order interactions, to obtain the significant minimal model. Second, we used repeated-measures analyses that take into account the importance of day post-infection. These analyses were performed as described by Råberg *et al.* (2006) using the statistical package SAS v. 9.1 (SAS Institute 1999, SAS OnlineDoc. v. 8. SAS Institute, Cary, NC). Briefly, the analyses were performed with PROC MIXED, using the REPEATED statement (subject=mouse), the Satterthwaite approximation of the denominator degrees of freedom, and the autoregressive covariance structure AR(1). Within each treatment group, the peak day varied ±2 days, presumably as a result of slight differences in inoculation dose. To control for this variation, we centred the peak day at the median peak day within each treatment group. All density data were transformed using [log (density +10)].

3. RESULTS

Mice treated with the anti-$CD4^+$T cell antibody were successfully depleted of $CD4^+$T cells, both prior to parasite challenge and during the whole course of the experiment (figure 1). The $CD4^+$T cell depletion resulted in more parasites of both clones (figure 2*a*,*b*; table 1).

(a) *Clone DK*

As found with other pairs of clones (de Roode *et al.* 2005*a*,*b*; Bell *et al.* 2006), here we found that the relatively

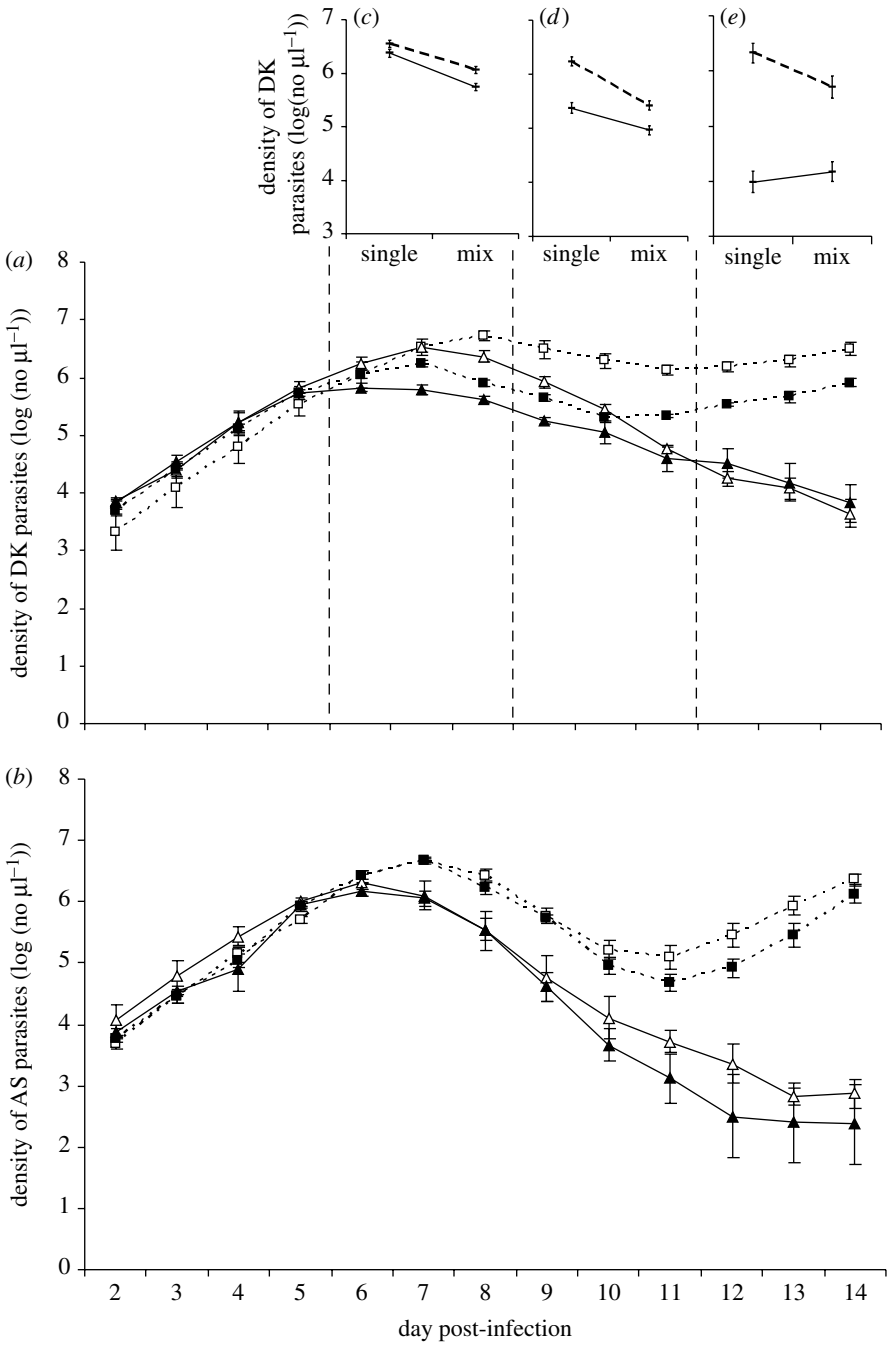

Figure 2. Parasite densities through time of (*a*) clone DK and (*b*) clone AS, and (*c–e*) average densities of DK during the periods denoted by the vertical dashed lines. Asexual density from qPCR of CD4$^+$T cell-depleted mice and intact control (normal) mice with single infections in CD4$^+$T cell-depleted mice and mixed infections in intact control (normal) mice. Mean densities ($\pm$1 s.e.m.) were calculated from all mice that were alive on the respective day of sampling (up to five). The interaction plots (*c–e*) show the total numbers of DK parasites when the competitor clone AS is absent (single infection) or present (mixed infection) in CD4$^+$-depleted mice (dashed lines) and normal animals (solid lines). Competition$\times$depletion interactions: (*c*) $F_{1,16}=1$, $p=0.33$; (*d*) $F_{1,17}=5.11$, $p=0.037$; and (*e*) $F_{1,14}=4.57$, $p=0.0499$ (open squares, CD4$^+$-depleted single; open triangles, intact single; filled squares, CD4$^+$-depleted mixed; filled triangles, intact mixed).

Table 1. GLM analyses of the effects of CD4$^+$T cell depletion, competition (presence/absence of co-infecting clone) and their interaction on the total number of parasites during the first two weeks of infection for parasite clone DK and AS.

	DK days 2–14			AS days 2–14		
effect	d.f.	*F*	*p*	d.f.	*F*	*p*
CD4$^+$ depletion	1,15	82.7	<0.001	1,15	93.36	<0.000
competition	1,15	84.2	<0.001	1,15	2.05	0.17
CD4$^+$ depletion$\times$competition	1,15	0.6	0.74	1,15	0.94	0.94

Table 2. Repeated-measures analyses of the effects of $CD4^+$ depletion, competition (presence/absence of co-infecting clone) and day post-infection on the daily densities of the two parasite clones DK and AS for days 6–14 post-infection.

	DK days 6–14			AS days 6–14		
effect	d.f.	F	p	d.f.	F	p
$CD4^+$ depletion	1,16	74.52	<0.0001	1,17	51.58	<0.0001
competition	1,16	17.92	0.0006	1,17	1.86	0.19
day	8,101	30.8	<0.0001	8,108	40.85	<0.0001
$CD4^+$ depletion × competition	1,16	2.65	0.12	1,17	0.02	0.88
$CD4^+$ depletion × day	8,101	22.35	<0.0001	8,108	15.67	<0.0001
competition × day	8,101	3.67	0.0009	8,108	0.91	0.51
$CD4^+$ depletion × competition × day	8,101	2.10	0.042	8,108	0.47	0.87

Table 3. Repeated-measures analyses of the effects of $CD4^+$ depletion, competition (presence/absence of co-infecting clone) and day post-infection on the daily parasite density of clone DK for days 6–8, 9–11 and 12–14 post-infection.

	days 6–8			days 9–11			days 12–14		
effect	d.f.	F	p	d.f.	F	p	d.f.	F	p
$CD4^+$ depletion	1,16	12.61	0.0026	1,17	54.79	<0.0001	1,14	105.95	<0.0001
competition	1,16	62.31	<0.001	1,17	47.53	<0.0001	1,14	1.33	0.26
day	2,29	15.32	<0.001	2,30	39.12	<0.0001	2,28	2.22	0.12
$CD4^+$ depletion × competition	1,16	1	0.33	1,17	5.11	0.037	1,14	4.57	0.049
$CD4^+$ depletion × day	2,29	2.88	0.071	2,30	11.87	0.0002	2,28	23.78	<0.0001
competition × day	2,29	12.34	0.0001	2,30	2.32	0.11	2,28	0.64	0.53
$CD4^+$ depletion × competition × day	2,29	3.78	0.034	2,30	2.3	0.11	2,28	0.17	0.84

Table 4. Repeated-measures analyses of the effects of $CD4^+$ depletion, competition (presence/absence of co-infecting clone) and day post-infection on the daily parasite density of clone AS for days 6–8, 9–11 and 12–14 post-infection.

	days 6–8			days 9–11			days 12–14		
effect	d.f.	F	p	d.f.	F	p	d.f.	F	p
$CD4^+$ depletion	1,14	17.58	0.0008	1,15	31.83	<0.001	1,14	58.71	<0.001
competition	1,14	0.3	0.58	1,15	2.08	0.16	1,14	1.6	0.22
day	2,28	39.82	<0.001	2,29	89.06	<0.001	2,27	6.52	0.0049
$CD4^+$ depletion × competition	1,14	0.01	0.91	1,15	0.11	0.74	1,14	0.03	0.86
$CD4^+$ depletion × day	2,28	12.17	0.0001	2,29	3.03	0.06	2,27	15.34	<0.001
competition × day	2,28	0.22	0.8	2,29	3.13	0.058	2,27	0.76	0.47
$CD4^+$ depletion × competition × day	2,28	1.20	0.31	2,29	0.08	0.92	2,27	0.70	0.50

avirulent clone was competitively suppressed by the more virulent clone, with DK achieving lower parasite densities when AS was present than when it was absent (figure 2*a*; table 1). However, the extent of competitive suppression of clone DK was similar regardless of $CD4^+$T cell depletion (table 1; depletion × competition interaction, n.s.). Thus, there was no evidence that the competitive suppression of the total number of DK parasites present in an infection was mediated by $CD4^+$T cell-dependent immunity.

However, repeated-measures analysis of the period where $CD4^+$T cell depletion affected parasite densities (day 6 onwards) showed a weak but significant three-way depletion × competition × day interaction (table 2). To investigate this further, and following Råberg *et al.* (2006), we divided the data into three parts, days 6–8, 9–11 and 12–14, and repeated the analyses with each of these (figure 2*a*,*c*–*e*; table 3). During each of these time periods, there were significant depletion × competition or depletion × competition × day interactions. Inspection of figure 2*a*,*c* shows that the three-way interaction in the first period is a very weak effect from which it is difficult to conclude much, given the rapid alterations in infection kinetics during that period caused by depletion. In the other two periods, there are significant competition × depletion interactions (figures 2*d*,*e*; table 3), with more severe competitive suppression in $CD4^+$T cell-depleted mice than in control mice. Thus, there was no evidence that competitive suppression is $CD4^+$T cell mediated: once the initial wave of parasitaemia began to subside, competitive suppression was exacerbated rather than alleviated in $CD4^+$-depleted mice.

(b) *Clone AS*

There was no evidence of competitive suppression of AS by DK, irrespective of the immune treatment (figure 2*b*; table 1). Repeated-measures analysis from day 6 onwards, when $CD4^+$ depletion had an effect, revealed no evidence of interactions between depletion and competition (table 2). However, for comparison with the analysis of

Table 5. Repeated-measures analyses of the effects of CD4$^+$ depletion and day post-infection on the mean red blood cell density in mixed infections during days 6–8, 9–11 and 12–14.

	days 6–8			days 9–11			days 12–14		
effect	d.f.	F	p	d.f.	F	p	d.f.	F	p
CD4$^+$ depletion	1,21	1.77	0.14	1,23	10.75	0.0033	1,19	4.57	0.045
day	2,36	42.18	<0.001	2,37	11.98	<0.0001	2,38	0.33	0.72
CD4$^+$ depletion$\times$ day	2,26	3.18	0.05	2,37	0.65	0.52	2,28	0.08	0.92

clone DK, we repeated the same analyses for AS on days 6–8, 9–11 and 12–14 (table 4). In none of these time periods was there any evidence of competitive suppression (in all cases, competition main effect and depletion$\times$ competition, $p>0.15$).

(c) *Red blood cells*

Red blood cell density over time for the different treatment groups are shown in figure 3. Uninfected red blood cells form an important resource for malaria parasites. To assess whether the potential for competition over this resource differed between CD4$^+$-depleted and intact control mice, we compared the red blood cell densities in mice with mixed infections. Repeated-measures analysis of days 6–14 revealed that CD4$^+$-depleted mice had significantly lower red blood cell densities during this time period ($F_{1,34.7}=4.35$, $p=0.045$; figure 3). There was also a significant depletion$\times$day interaction ($F_{8,94.1}=2.58$, $p=0.014$). Separate analyses of days 6–8, 9–11 and 12–14 showed that the difference in RBC density was most pronounced during days 9–14 (table 5).

4. DISCUSSION

We found no evidence that the CD4$^+$T cells enhanced competition during mixed genotype infections with *P. chabaudi* (figure 2*a*; table 1). Specifically, during the peak stages of acute infection (days 6–8) suppression was independent of the CD4$^+$T cells (figure 2*c*; table 3). After the peak of infection (day 9+), the CD4$^+$T cells acted to alleviate competition such that upon their removal competitive suppression was enhanced (figure 2*d*,*e*; table 3). In addition, the presence of CD4$^+$T cells did not cause suppression of the dominant genotype (figure 2*b*; table 4).

The immune response to *Plasmodium* infection has both pathogen genotype-transcending (non-specific) and genotype-specific components, with protection becoming more specific during later stages of infection (see §1). Here, we found that after the peak of acute infection (day 9 onwards), there was no competitive suppression of DK parasites in intact control mice; whereas in CD4$^+$T cell-depleted mice, there was still evidence of competition (figure 2*d*,*e*; table 3). Both clones did better in depleted mice, probably owing to an impaired early antibody production through lack of T cell help and possibly the reduced recruitment and activation of macrophages for the uptake of infected cells. Thus, in normal hosts, a largely clone-specific adaptive immune response towards a numerically dominant genotype may act to alleviate competition by regulating clonal populations and limiting other forms of competition, e.g. competition for red blood cells.

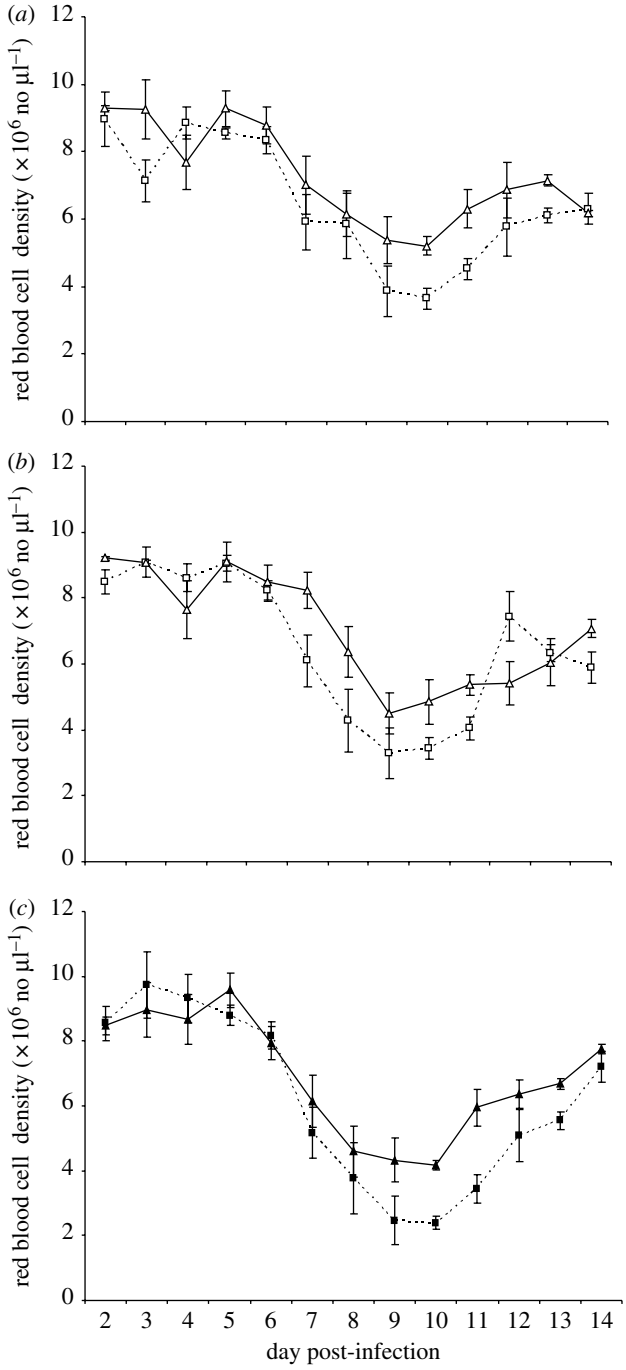

Figure 3. Mean red blood cell densities ($\pm$1 s.e.m) over time. (*a*) CD4$^+$T cell-depleted and intact mice infected with DK (squares, depleted single; triangles, intact single), (*b*) depleted and intact mice infected with AS (squares, depleted single; triangles, intact single), and (*c*) depleted and intact mice infected with DK and AS (squares, depleted mixed; triangles, intact mixed).

During the peak of infection (days 6–8), competition was $CD4^+$T cell independent so that the extent of competitive suppression of clone DK was similar in intact control and $CD4^+$T cell-depleted mice (figure 2*a*,*c*; table 3). A number of biological mechanisms could be the proximate cause of competitive suppression during the peak of infection. First, there may be direct interference between two infecting strains. This has not yet been demonstrated in any parasites, but pathogenic bacteria can produce allelopathic substances that actively suppress competitors (Riley & Gordon 1999), and competing viruses can produce interference molecules (Hart & Cloyd 1990). Second, the competition may be influenced by non-specific components of the innate immune response ($CD4^+$T cell independent). Third, there may be competition for resources as genotypes infecting mice simultaneously must divide the available red blood cells and other resources such as blood glucose between them (Hellriegel 1992; Hetzel & Anderson 1996; de Roode *et al.* 2005*a*; Gurarie *et al.* 2006). The $CD4^+$T cell-depleted mice were more anaemic than control mice (figure 3; table 5), so that if red cells are limiting, there is more potential for competition for that resource in depleted mice. Mathematical models have suggested that during mixed infection, the proximate cause of competitive advantage may be attributable to an earlier and wider red blood cell preference of dominant genotypes (Hellriegel 1992; Gravenor *et al.* 1995; McKenzie & Bossert 1997; Jakeman *et al.* 1999; Mason & McKenzie 1999; McQueen *et al.* 2004; Antia *et al.* submitted). Because these predictions are based on the data from the rodent malaria model, they could be tested directly by transferring red blood cells of different ages into a single mouse and determine their loss following infection, or indirectly by measuring competition in untreated mice and mice treated with erythropoietin (Suzuki *et al.* 2006).

Our conclusion that competition is not $CD4^+$T cell mediated apparently contradicts the recent finding of T cell-mediated apparent competition (Råberg *et al.* 2006). In that study, the authors looked at mixed infections with *P. chabaudi* in nude mice (which lack the ability to produce mature T cells) and compared the extent of competition with that in nude mice reconstituted with T cells. There was still pronounced competition in all animals, but there was some alleviation of competitive suppression in nude mice towards the end of the acute phase of infection, when the initial wave of parasitaemia was waning. This period corresponds roughly to days 9–14 in figure 2. A number of experimental differences could explain the contrasting results of Råberg *et al.* (2006) and the present study. First, different mouse strains were used in the two studies and host genotype has previously been shown to quantitatively affect the outcome of competition (de Roode *et al.* 2004). Second, different pairs of clones were used and *P. chabaudi* clone can induce different levels of strain-specific immunity (Cheesman *et al.* 2006). Third, there was a difference in the method used to modulate T cell-dependent immunity. Nude mice lack the ability to produce any mature T cells, including both $CD4^+$ and $CD8^+$T cells. The role of $CD8^+$T cells during malaria infection in mice is still unclear (Lamb *et al.* 2006), but it could be that they are involved in the relatively small component of competition that was shown to be immune-mediated competition in reconstituted nude mice (Råberg *et al.* 2006). In addition, the repertoire of serum antibodies (including both natural antibodies and antigen elicited antibodies) in the $CD4^+$T cell-depleted mice will be different from that in nude mice. Nude mice grow up producing only T cell-independent antibodies, while in the $CD4^+$T cell-depleted mice there will be both T cell-independent and persisting T cell-dependent antibodies (produced by existing plasma cells in the bone marrow) and these may cross react with the parasite.

Taken together, the present study and that of Råberg *et al.* (2006) show that the effect of T cell-dependent immunity on competition is relatively weak, and may be either positive or negative depending on specific details of host and parasite. Rather than further dissection of any immune mechanism-mediating competition, one could use this malaria model system to look at the strength of competition in hosts immunized by a variety of different candidate vaccines towards the blood stage of infection. Meanwhile, the result we report here suggest that vaccines that enhance $CD4^+$-dependent immunity will not increase the selection in favour of virulence arising from in-host competition.

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REFERENCES

Alder, F. R. & Losada, J. M. 2002 Super-and co-infection: filling the range. In *Adaptive dynamics of infectious diseases: in pursuit of virulence management* (eds U. Dieckmann, J. A. J. Metz, M. W. Sabelis & K. Sigmund), pp. 139–149. Cambridge, UK: Cambridge University Press.

Anderson, T. J. C. *et al.* 2000 Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol. Biol. Evol.* **17**, 1467–1482.

Antia, R., Yates, A. & De Roode, J. C. Submitted. Virulence and competition in malaria infections.

Arnot, D. 1998 Clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. *Trans. R. Soc. Trop. Med. Hyg.* **92**, 580–585. (doi:10.1016/S0035-9203(98)90773-8)

Awadalla, P., Walliker, D., Babiker, H. A. & Mackinnon, M. J. 2001 The question of *Plasmodium falciparum* population structure. *Trends Parasitol.* **17**, 351–353. (doi:10.1016/S1471-4922(01)02034-7)

Beale, G. H., Walliker, D. & Carter, R. 1978 In *Rodent malaria* (eds R. Killick-Kendrick & W. Peters), pp. 213–245. London: Academic Press.

Bell, A. S., de Roode, J. C., Sim, D. & Read, A. F. 2006 Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**, 1358–1371.

Bremermann, H. J. & Pickering, J. 1983 A game-theoretical model of parasite virulence. *J. Theor. Biol.* **100**, 411–426. (doi:10.1016/0022-5193(83)90438-1)

Bruce, M. C., Donnelly, C. A., Alpers, M. P., Galinski, M. R., Barnwell, J. W., Walliker, D. & Day, K. P. 2000 Cross-species interactions between malaria parasites in humans. *Science* **287**, 845–848. (doi:10.1126/science.287.5454.845)

Buckling, A. & Read, A. F. 2001 The effect of partial host immunity on the transmission of malaria parasites. *Proc. R. Soc. B* **268**, 2325–2330. (doi:10.1098/rspb.2001.1808)

Cheesman, S., Raza, A. & Carter, R. 2006 Mixed strain infections and strain-specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. *Infect. Immunol.* **74**, 2996–3001. (doi:10.1128/IAI.74.5.2996-3001.2006)

Daubersies, P., Sallenave-Sales, S., Magne, S., Trape, J.-F., Contamin, H., Fandeur, T., Rogier, C., Mercereau-Puijalon, O. & Druilhe, P. 1996 Rapid turnover of *Plasmodium falciparum* populations in asymptomatic individuals living in a high transmission area. *Am. J. Trop. Med. Hyg.* **54**, 18–26.

de Roode, J. C., Read, A. F., Chan, H. K. & Mackinnon, M. J. 2003 Rodent malaria parasites suffer from the presence of con-specific clones in three-clone *Plasmodium chabaudi* infections. *Parasitology* **127**, 411–418. (doi:10.1017/S0031182003004001)

de Roode, J. C., Culleton, R., Cheesman, S. J., Carter, R. & Read, A. F. 2004 Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. *Proc. R. Soc. B* **271**, 1073–1080. (doi:10.1098/rspb.2004.2695)

de Roode, J. C., Helinski, M. E. H., Anwar, M. A. & Read, A. F. 2005*a* Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am. Nat.* **166**, 531–542. (doi:10.1086/491659)

de Roode, J. C. *et al.* 2005*b* Virulence and competitive ability in genetically diverse malaria infections. *Proc. Natl Acad. Sci. USA* **102**, 7624–7628. (doi:10.1073/pnas.0500078102)

Frank, S. A. 1996 Models of parasite virulence. *Q. Rev. Biol.* **71**, 37–78. (doi:10.1086/419267)

Gandon, S., Mackinnon, M. J., Nee, S. & Read, A. F. 2001 Imperfect vaccines and the evolution of pathogen virulence. *Nature* **414**, 751–756. (doi:10.1038/414751a)

Good, M. F. & Doolan, D. L. 1999 Immune effector mechanisms in malaria. *Curr. Opin. Immunol.* **11**, 412–419. (doi:10.1016/S0952-7915(99)80069-7)

Gravenor, M. B., McLean, A. R. & Kwiatkowski, D. 1995 The regulation of malaria parasitemia–parameter estimates for a population-model. *Parasitology* **110**, 115–122.

Gurarie, D., Zimmerman, P. A. & King, C. H. 2006 Dynamic regulation of single- and mixed-species malaria infection: insights to specific and non-specific mechanisms of control. *J. Theor. Biol.* **240**, 185–199. (doi:10.1016/j.jtbi.2005.09.015)

Hart, A. R. & Cloyd, M. W. 1990 Interference patterns of human immunodeficiency viruses HIV-1 and HIV-2. *Virology* **177**, 1–10.

Hastings, I. M. 1997 A model for the origins and spread of drug-resistant malaria. *Parasitology* **115**, 133–141. (doi:10.1017/S0031182097001261)

Hastings, I. M. 2003 Malaria control and the evolution of drug resistance: an intriguing link. *Trends Parasitol.* **19**, 70–73. (doi:10.1016/S1471-4922(02)00017-X)

Hastings, I. M. 2006 Complex dynamics and stability of resistance to antimalarial drugs. *Parasitology* **132**, 615–624. (doi:10.1017/S0031182005009790)

Hastings, I. M. & D'Alessandro, U. 2000 Modelling a predictable disaster: the rise and spread of drug-resistant malaria. *Parasitol. Today* **16**, 340–347. (doi:10.1016/S0169-4758(00)01707-5)

Hellriegel, B. 1992 Modelling the immune response to malaria with ecological concepts: short-term behaviour against long-term equilibrium. *Proc. R. Soc. B* **250**, 249–256. (doi:10.1098/rspb.1992.0156)

Hetzel, C. & Anderson, R. 1996 The within-host cellular dynamics of blood-stage malaria: theoretical and experimental studies. *Parasitology* **113**, 25–38.

Holt, R. D. 1977 Predation, apparent competition, and the structure of prey communities. *Theor. Popul. Biol.* **12**, 197–229. (doi:10.1016/0040-5809(77)90042-9)

Hviid, L., Kurtzhals, J. A., Goka, B. Q., Oliver-Commey, J. O., Nkrumah, F. K. & Theander, T. G. 1997 Rapid reemergence of T cells into peripheral circulation following treatment of severe and uncomplicated *Plasmodium falciparum* malaria. *Infect. Immunol.* **65**, 4090–4093.

Jafari, S., Le Bras, J., Bouchaud, O. & Durand, R. 2004 *Plasmodium falciparum* clonal population dynamics during malaria treatment. *J. Infect. Dis.* **189**, 195–203. (doi:10.1086/380910)

Jakeman, G., Saul, A., Hogarth, W. & Collins, W. 1999 Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology* **119**, 127–133. (doi:10.1017/S0031182099004564)

Jarra, W. & Brown, K. N. 1989 Invasion of mature and immature erythrocytes of CBA/ca mice by a cloned line of *Plasmodium chabaudi chabaudi*. *Parasitology* **99**, 157–163.

Lamb, T. J., Brown, D. E., Potocnik, A. J. & Langhorne, J. 2006 Insights into the immunopathogenesis of malaria using mouse models. *Expert Rev. Mol. Med.* **8**, 1–22.

Lipsitch, M. & Samore, M. H. 2002 Antimicrobial use and antimicrobial resistance: a population perspective. *Emerg. Infect. Dis.* **8**, 347–354.

Mackinnon, M. J. 2005 Drug resistance models for malaria. *Acta Tropica* **94**, 207–217. (doi:10.1016/j.actatropica.2005.04.006)

Mackinnon, M. J. & Hastings, I. M. 1998 The evolution of multiple drug resistance in malaria parasites. *Trans. R. Soc. Trop. Med. Hyg.* **92**, 188–195. (doi:10.1016/S0035-9203(98)90745-3)

Mackinnon, M. J. & Read, A. F. 1999 Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* **53**, 689–703. (doi:10.2307/2640710)

Mackinnon, M. J. & Read, A. F. 2003 The effects of host immunity on virulence transmission relationships in the rodent malaria parasite *Plasmodium chabaudi*. *Parasitology* **126**, 103–112. (doi:10.1017/S003118200200272X)

Martinelli, A., Cheesman, S., Hunt, P., Culleton, R., Raza, A., Mackinnon, M. & Carter, R. 2005 A genetic approach to the de novo identification of targets of strain specific immunity in malaria parasites. *Proc. Natl Acad. Sci. USA* **102**, 814–819. (doi:10.1073/pnas.0405097102)

Mason, D. P. & McKenzie, F. E. 1999 Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax–Plasmodium falciparum* infections. *Am. J. Trop. Med. Hyg.* **61**, 367–374.

McKenzie, F. E. & Bossert, W. H. 1997 The dynamics of *Plasmodium falciparum* blood-stage infection. *J. Theor. Biol.* **188**, 127–140. (doi:10.1006/jtbi.1997.0478)

McQueen, P. G., McKenzie, F. E. & Singer, B. H. 2004 Age-structured red blood cell susceptibility and the dynamics of malaria infections. *Proc. Natl Acad. Sci. USA* **101**, 9161–9166. (doi:10.1073/pnas.0308256101)

Mercereau-Puijalon, O. 1996 Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans. *Parasite Immunol.* **18**, 173–180. (doi:10.1046/j.1365-3024.1996.d01-79.x)

Pombo, D. J. *et al.* 2002 Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *Lancet* **360**, 610–617. (doi:10.1016/S0140-6736(02)09784-2)

Råberg, L., de Roode, J. C., Bell, A. S., Stamou, P., Gray, D. & Read, A. F. 2006 The role of immune-mediated apparent competition in genetically diverse malaria infections. *Am. Nat.* **168**, 41–53. (doi:10.1086/505160)

Read, A. F. & Mackinnon, M. J. 2008 Pathogen evolution in a vaccinated world. In *Evolution in health and disease* (eds S. C. Stearns & J. Koella), 2nd edn. Oxford, UK: Oxford University Press.

Read, A. F. & Taylor, L. H. 2001 The ecology of genetically diverse infections. *Science* **292**, 1099–1102. (doi:10.1126/science.1059410)

Riley, M. A. & Gordon, D. M. 1999 The ecological role of bacteriocins in bacterial competition. *Trends Microbiol.* **7**, 129–133. (doi:10.1016/S0966-842X(99)01459-6)

Smith, T., Felger, I., Kitua, A., Tanner, M. & Beck, H. P. 1999 Dynamics of multiple *Plasmodium falciparum* infections in infants in a highly endemic area of Tanzania. *Trans. R. Soc. Trop. Med. Hyg.* **93**, 35–39. (doi:10.1016/S0035-9203(99)90325-5)

Stephens, R. & Langhorne, J. 2006 Priming CD4$^+$T cells and development of CD4$^+$T cell memory; lessons for malaria. *Parasite Immunol.* **28**, 25–30. (doi:10.1111/j.1365-3024.2006.00767.x)

Stevenson, M. M. & Riley, E. M. 2004 Innate immunity to malaria. *Nat. Rev. Immunol.* **4**, 169–180. (doi:10.1038/nri1311)

Suzuki, M., Ohneda, K., Hosoya-Ohmura, S., Tsukamoto, S., Ohneda, O., Philipsen, S. & Yamamoto, M. 2006 Real-time monitoring of stress erythropoiesis *in vivo* using Gata1 and beta-globin LCR luciferase transgenic mice. *Blood* **108**, 726–733. (doi:10.1182/blood-2005-10-4064)

Talisuna, A. O., Erhart, A., Samarasinghe, S., Van Overmeir, C., Speybroeck, N. & D'Alessandro, U. 2006 Malaria transmission intensity and the rate of spread of chloroquine resistant *Plasmodium falciparum*: why have theoretical models generated conflicting results? *Infect. Gen. Evol.* **6**, 241–248. (doi:10.1016.j.meegid.2005/06.003)

Timms, R., Colegrave, N., Chan, B. H. K. & Read, A. F. 2001 The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. *Parasitology* **123**, 1–11. (doi:10.1017/S0031182001008083)

Urban, B. C., Ing, R. & Stevenson, M. M. 2005 Early interactions between blood-stage plasmodium parasites and the immune system. *Curr. Top. Microbiol. Immunol.* **297**, 25–70.

van Baalan, M. & Sabelis, M. W. 1995 The dynamics of multiple infection and the evolution of virulece. *Am. Nat.* **146**, 881–910. (doi:10.1086/285830)

Walliker, D., Hunt, P. & Babiker, H. 2005 Fitness of drug-resistant malaria parasites. *Acta Tropica* **94**, 251–259. (doi:10.1016/j.actatropica.2005.04.005)

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E-Article

Understanding and Predicting Strain-Specific Patterns of Pathogenesis in the Rodent Malaria *Plasmodium chabaudi*

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abstract: Despite considerable success elucidating important immunological and resource-based mechanisms that control the dynamics of infection in some diseases, little is known about how differences in these mechanisms result in strain differences in patterns of pathogenesis. Using a combination of data and theory, we disentangle the role of ecological factors (e.g., resource abundance) in the dynamics of pathogenesis for the malaria species *Plasmodium chabaudi* in $CD4^+$ T cell–depleted mice. We build a series of nested models to systematically test a number of potential regulatory mechanisms and determine the "best" model using statistical techniques. The best-fit model is further tested using an independent data set from mixed-clone competition experiments. We find that parasites preferentially invade older red blood cells even when they are more fecund in younger reticulocytes and that inoculum size has a strong effect on burst size in reticulocytes. Importantly, the results suggest that strain-specific differences in virulence arise from differences in red blood cell age-specific invasion rates and burst sizes, since these are lower for the less virulent strain, as well as from differences in levels of erythopoesis induced by each strain. Our analyses highlight the importance of model selection and validation for revealing new biological insights.

Keywords: malaria, within-host dynamics, modeling, *Plasmodium chabaudi*, virulence.

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Malaria is one of the leading causes of death among infectious diseases in the world, killing more than one million people every year (WHO and UNICEF 2005). Despite this enormous burden and the large proportion of the world's population that is threatened by malaria, we know little about the relative importance of mechanisms that regulate malaria parasite growth within infected hosts or how differences in these mechanisms among strains give rise to different patterns of morbidity and mortality. A better understanding of these mechanisms is of interest in its own right and will likely yield important insights for improving control strategies and understanding the potential evolutionary consequences of these controls.

The rodent model system of malaria *Plasmodium chabaudi* provides an excellent opportunity to address this question, because controlled and replicated experimental manipulations are possible. In this article, we combine data from experimental manipulations with the development and testing of mathematical models. Our overriding goal is to provide a quantitative (mathematical) description of the main mechanisms governing the regulation of *P. chabaudi* growth within hosts and of how these differ among malaria strains. If a mathematical description cannot be made for mouse models of malaria, where host and parasite genotype together with a vast number of environmental variables can be carefully controlled, then there is little hope for a quantitative understanding of infection in humans.

Many factors regulating malaria infection dynamics have been identified. Among these, immune responses play a large role in the control of parasite densities and may take many forms with respect to the trigger and target of the response (i.e., free-living merozoites or infected red blood cells [RBCs]), the level of clone specificity of the response, and the significance of antigenic variation (for a review, see Stevenson and Riley 2004). Other nonimmunological mechanisms, however, are also likely to play a fundamental role. For example, simple resource abundance—such as the availability of RBCs—is likely a key regulator of parasite growth (Hellriegel 1992; Hetzel and Anderson 1996; Haydon et al. 2003) because it is primarily

within these cells that asexual replication occurs. Also, the age structure of available RBCs could be important (McQueen and McKenzie 2004; Cromer et al. 2006) because many malaria species preferentially invade either young RBCs (reticulocytes) or fully mature normocytes (Paul et al. 2003). Furthermore, the nature of the host's erythropoetic response to malaria-induced anemia will affect both the abundance and age structure of RBCs (Antia et al. 2008), as will temporal patterns of conversion to gametocytes during an infection (e.g., Eichner et al. 2001). Whereas some previous theoretical studies have explored the role of these factors in determining within-host dynamics of malaria, our approach is unique in that we compare multiple model variations that systematically test different combinations of these regulatory mechanisms. Furthermore, we allow for individual variation by fitting models to data sets from individual hosts and then performing a rigorous statistical analysis of the fit to experimental data.

To dissect the relative importance of these factors and how they differ among strains, a combination of experimental manipulation and mathematical modeling is required. Experimental manipulations can be used to remove some potential regulatory mechanisms so that we can examine the dynamics of parasite growth when only a few factors are in operation. In this simplified setting, one can build mathematical models to describe these dynamics. Rigorous tests of these models can then be conducted in this setting through a combination of statistical analyses and further experimental manipulations. Understanding, in a quantitative way, the strain-specific differences that occur in such "stripped-down" situations is an important step toward understanding the more complete picture in which all potential regulatory factors interact with one another.

The results presented here take this approach by focusing on *P. chabaudi* infections in mice that have depleted levels of $CD4^+$ T cells. $CD4^+$ T cells are essential for developing an effective immune response against malaria infections (Good and Doolan 1999; Pombo et al. 2002). They play a crucial role, both early in an infection by activating macrophages and initiating antiparasitic cell-mediated immune responses and in later stages of infection by helping B cells to produce antibodies and in regulating the adaptive immune responses (Langhorne et al. 1990; Urban et al. 2005; Stephens and Langhorne 2006). By removing this regulatory factor, we can then examine the extent to which the above-mentioned ecological factors can explain strain-specific differences in infections.

Material and Methods

Our approach and results have three main components. The first focuses on data from two clones of *Plasmodium chabaudi*, each singly infecting mice that have depleted levels of $CD4^+$ T cells. We build a suite of eight nested models of increasing complexity that represent eight potential descriptions of the within-host dynamics of infection. We then fitted each of these eight models to data using maximum likelihood techniques. Second, we use model selection criteria to choose the "best" model from the eight fitted models for each clone. After statistically determining that the best model does in fact provide a good fit, we use this model to infer the main mechanisms underlying the clone-specific differences in infection dynamics. Third, we take our best model for each clone and use it to derive predictions for the expected dynamics when both clones coinfect a single host. These predictions are compared with experimental data on coinfection as a further independent means of validating the model.

Model Development and Data Fitting

Previous Experimental Data. Experimental infections were generated using two genetically distinct *P. chabaudi* clones, denoted AS and DK, that were originally isolated from thicket rats *Thamnomys rutilans* in the Central African Republic (Beale et al. 1978). In mixed infections, DK is competitively suppressed by AS (Barclay et al. 2008), and previous studies have shown a strong relationship between competitive ability and virulence (de Roode et al. 2003, 2005*a*, 2005*b*; Bell et al. 2006).

Fifteen inbred female C57BL/6JolaHsd mice aged 6–8 weeks (Harlan, Bicester, United Kingdom) were depleted of their $CD4^+$ T cells using a rat monoclonal antibody GK1.5 (for details, see Barclay et al. 2008). Mice were infected via intraperitoneal injection. Five mice were inoculated with 10^5 AS parasites, five received 10^6 of this same clone, and five received 10^6 DK parasites. Mice were maintained as described previously (de Roode et al. 2004). Daily RBC densities were tracked using flow cytometry (Beckman Coulter, High Wycombe, United Kingdom), and parasite densities were measured using a genotype-specific, real-time quantitative polymerase chain reaction (qPCR). One mouse in each of the AS 10^5 and DK 10^6 treatment groups died prematurely; these were therefore excluded from our analysis.

The Model. Our aim is to understand what factors determine the clone-specific differences in dynamics of infection in $CD4^+$ T cell–depleted mice. We developed models that consider clone-specific effects on RBC age structure and cellular tropism, gametocytogenesis, and erythropoesis as possibilities. All of these factors were incorporated in a single large model, and the suite of eight nested models that are the objects of our analysis were then obtained as certain special cases of this single model.

Malaria parasites in most infections display a distinctly discrete replication cycle during an infection, with synchronous bursting of infected RBCs occurring every 24 h in *P. chabaudi* (Carter and Walliker 1975). Our basic model therefore tracks the infection dynamics in discrete time, with one time step corresponding to a single day (Molineaux and Dietz 1999). Immediately after bursting, merozoites begin infecting susceptible RBCs. This process occurs relatively quickly, with the majority of the events during the 24-h period between bursting events consisting of development within RBCs (fig. 1). For simplicity, in the model we census the populations of merozoites and RBCs immediately after bursting but prior to the infection of new RBCs. The basic model thus tracks the following events within a single day: (i) census, (ii) RBC invasion by merozoites, (iii) RBC turnover (production and natural death), and (iv) bursting of infected RBCs (fig. 1).

The discrete-time nature of the model requires that we define distinct classes of RBCs for exploring the importance of RBC age structure to the within-host dynamics. In rats, reticulocytes take 30–75 h to mature when released into the bloodstream, taking longer when the rat is anemic (Ganzoni et al. 1969; Wiczling and Krzyzanski 2007). This time frame is in agreement with the 2–3-day maturation time observed in C57 black mice (S. Reece, personal communication). Because the experimental mice have depressed RBC densities for a substantial duration of the experiment, we assume that the reticulocytes will, on average, take 3 days to mature. Thus, we define four age classes of RBCs: reticulocytes that have been newly introduced to the bloodstream ($R_{1,i}$), reticulocytes in their second day of being in the bloodstream ($R_{2,i}$), reticulocytes in their third day of being in the bloodstream ($R_{3,i}$), and fully mature normocytes (N_i). The subscript i denotes a value on day i after parasite inoculation. In addition to these age classes, there are four classes of infected RBCs corresponding to each type of blood cell ($I_{1,i}$, $I_{2,i}$, $I_{3,i}$, and $I_{N,i}$). Merozoites may infect reticulocytes and normocytes at different per capita rates (reflecting a particular cell age "preference") and may produce different numbers of daughter merozoites per infected cell on bursting. In the absence of evidence to the contrary, merozoites are assumed to respond to all reticulocytes the same way, regardless of how many days the RBC has been in the bloodstream. The production of RBCs varies according to the density of RBCs (i.e., production is increased when an individual becomes anemic), as has been well documented (e.g., Mackey 1997).

Incorporating all of these biological details in a single model gives the discrete-time dynamics system (see app. A for model derivation and table 1 for parameter descriptions):

$$P_{i+1} = \left[\omega_R\left(\sum_{j=1}^{3} R_{j,i}\left\{1 - \exp\left[-\frac{P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}\right) + \omega_N N_i\left\{1 - \exp\left[-\frac{P_i\beta_N}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}\right] \times (1 - d)(1 - g), \quad (1)$$

$$R_{1,i+1} = \theta[K - (R_{1,i-\tau} + R_{2,i-\tau} + R_{3,i-\tau} + N_{i-\tau})], \quad (2)$$

$$R_{2,i+1} = (1 - d) \times R_{1,i}\exp\left[-\frac{P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right], \quad (3)$$

$$R_{3,i+1} = (1 - d) \times R_{2,i}\exp\left[-\frac{P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right], \quad (4)$$

$$N_{i+1} = (1 - d)\left\{R_{3,i}\exp\left[-\frac{P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right] + N_i\exp\left[-\frac{P_i\beta_N}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}. \quad (5)$$

We fitted eight variations of this model to the data to determine which factors are important for explaining the dynamics of disease. These eight variations are all special cases of the above general model. Briefly, when there is no age structure, all RBCs (i.e., R_1, R_2, R_3, and N) are collapsed into a single class and there is no heterogeneity in burst size or invasion rate. Constant recovery of RBC deficit means that erythropoesis is a linear function of RBC density (θ is constant under normal conditions and during anemia; $\theta_0 = \theta_A$; see app. A), whereas variable recovery assumes that it is a piecewise linear function ($\theta_0 \neq \theta_A$), which accounts for saturation as described earlier. Gametocyte production, governed by the parameter g, is either assumed not to occur ($g = 0$) or to occur at some constant rate throughout infection. The model variations are as follows (see table C1 for the numbers of fitted parameters). (1) No age structure, constant recovery of RBC deficit (RBC recovery), no gametocyte production (GP); $\beta_R = \beta_N$, $\omega_R = \omega_N$, $\theta_0 = \theta_A$, and $g = 0$. (2) No age structure, constant RBC recovery, constant GP; $\beta_R = \beta_N$, $\omega_R = \omega_N$, and $\theta_0 = \theta_A$. (3) No age structure, variable RBC recovery, no GP; $\beta_R = \beta_N$, $\omega_R = \omega_N$, and $g = 0$. (4) No age structure, variable RBC recovery, constant GP; $\beta_R = \beta_N$ and $\omega_R = \omega_n$. (5) Age structure, constant RBC recovery, no GP; $\theta_0 = \theta_A$ and $g = 0$. (6) Age structure, constant RBC recovery, constant GP; $\theta_0 = \theta_A$. (7) Age structure, variable RBC recovery, no GP; $g = 0$. (8) Age structure, variable RBC recovery, constant GP.

Curve Fitting. Initial conditions for individual mice were

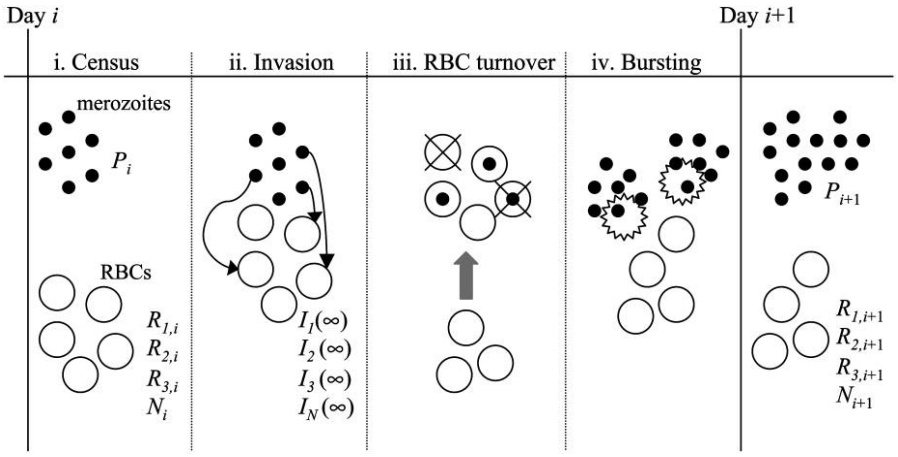

Figure 1: Schematic of asexual blood stage of malaria infections and our model framework. It takes 24 h for *Plasmodium chabaudi* merozoites to invade red blood cells (RBCs), replicate, and burst from infected cells. We model this process in discrete time, tracking the number of merozoites (P_i), reticulocytes of age j ($R_{j,i}$), and normocytes (N_i), on day i. RBC invasion occurs quickly relative to this 1-day cycle. We model this invasion step in continuous time and calculate the number of infected reticulocytes of age j ($I_j(\infty)$) and infected normocytes ($I_N(\infty)$) at the end of this process, when all merozoites are either infecting RBCs or are dead.

determined from experimental measurements of both RBC and parasite densities taken 2 days after inoculation. Parameter estimates were obtained from the literature, and our fitting routine searched a slightly larger parameter space than the biologically reasonable ranges (see table 1). We fitted all parameters except for the merozoite and RBC death rates, which are well known and were fixed for computational practicality (table 1).

The models were fitted to the data from individual mice using the maximum likelihood method. Our approach was to lay a coarse grid over the entire parameter space and, for each parameter set and data point, to calculate the likelihood of the parameters given the data. When the likelihood was maximized over a particular grid, we adjusted the grid using finer-scale steps over a smaller section of parameter space. To find our maximum likelihood parameter estimates, this process was repeated until finer grids failed to achieve a maximum log likelihood greater than that of the previous grid, up to four decimal places. Because we fitted the models to individual mice, we expect the only uncertainties in our data to be caused by measurement error. An earlier experiment estimated the repeatability of RBC densities measured by flow cytometry and parasite densities measured by qPCR. From this we know that the error of the $\log_{10}$-transformed RBC and parasite densities are normally distributed with standard deviations of 0.034 and 0.2, respectively.

Ideally, we would fit the models to both the RBC and the parasite data; however, we have less confidence in our parasite data because of the greater error associated with them. Furthermore, given the discrete-time nature of our model, we can make predictions about the density of merozoites either just after all infected RBCs have burst or just after all merozoites have died or invaded an RBC but before any asexual replication within RBCs has occurred. Our experimental measurements are unlikely to have captured either of these events exclusively, so it is unclear to what we should fit these data. Using only the RBCs to fit the models allows us to avoid this difficulty. Also, because we do not fit our model directly to the parasite data, the parasite density predictions generated from best-fit models provide another qualitative test of the appropriateness of this model. The parasite predictions to which we qualitatively compare our data are those that calculate the density of parasites that have successfully invaded RBCs prior to asexual replication.

With this error structure, the probability of observing 10^D RBCs per μL of blood, given the model that predicts 10^M RBCs per μL of blood, is

$$\Pr(D|M) = \frac{1}{0.034\sqrt{2\pi}}\exp\left[-\frac{(D-M)^2}{2(0.034)^2}\right]. \qquad (6)$$

The likelihood of a particular set of parameters (given the data) is proportional to the product of the probabilities of observing the data (given the model predictions). For numerical accuracy, we take the natural logarithm of the likelihood, so that

$$L = \sum_{q=1}^{t_{max}} \ln\left\{\frac{1}{0.034\sqrt{2\pi}}\exp\left[-\frac{(D_q - M_q)^2}{2(0.034)^2}\right]\right\}, \qquad (7)$$

Table 1: Model parameters, published estimates, and test ranges

Parameter	Definition	Value (range)	Reference
ω_R, ω_N	Mean number of merozoites produced per infected reticulocyte, normocyte	4–10 (2–25)	Garnham 1966; Carter and Walliker 1975; Killick-Kendrick and Peters 1978
β_R, β_N	Invasion rate of reticulocytes, normocytes ($[\text{cells}/\mu\text{L}]^{-1}$ day^{-1})	10^{-6} ($0–2 \times 10^{-5}$)	Hetzel and Anderson 1996[a]; Antia et al. 2008
d	Death rate of RBCs (day^{-1})	.02–.025 (.025)	van Putten 1958[b]; Bannerman 1983
μ	Death rate of merozoites (day^{-1})	40–50 (48)	Garnham 1966; McAlister 1977[a]
g	Proportion of infected RBCs that produce gametocytes	<.1 (0–.5)	Shutler et al. 2005
τ	Lag in RBC production (transit time in bone marrow, days)	2–3 (1–4)	Mary et al. 1980[c]; Chang et al. 2004
θ	Proportion of RBC deficit that is made up each day	.15–.25 (0–1)	Haydon et al. 2003

Note: Unless otherwise noted, estimates are restricted to *Plasmodium chabaudi* and C57 black mice. RBC = red blood cell.

[a] *Plasmodium berghei.*

[b] CBAxC57BL mice.

[c] C3H mice.

where D_q is the $\log_{10}$ of the measured RBC density at time q and M_q is found by simulating the model for a particular data set and taking the $\log_{10}$ of the total density of RBCs (reticulocytes and normocytes) at time q. The best-fit parameters for a given model maximize the log likelihood L.

Statistical Analysis

Model Selection and Goodness of Fit. Given the nested nature of our eight model variations, we use the likelihood ratio test to determine the best-fit model. If the calculated maximum log likelihoods for models A and B are L_A and L_B, respectively, and if model A has fewer fitted parameters, then we define

$$\mathcal{R} = 2(L_B - L_A). \tag{8}$$

If $\mathcal{R}$ is greater than the χ^2 critical value, as determined from a χ^2 distribution with degrees of freedom equal to the difference in fitted parameters between models A and B, then model B is a significantly better fit at the 5% level (e.g., Hilborn and Mangel 1997; Grimshaw et al. 2001; Johnson and Omland 2004). We perform these pairwise model comparisons for each individual mouse to determine the best-fit model. In addition, we reject any models with best-fit parameters that are in strong disagreement with experimentally determined parameter estimates (table 1).

When we choose a best-fit model for each mouse, we test the goodness of fit of the maximum likelihood parameters of this model knowing that, given the data, the likelihood of these parameters is proportional to L_{max}. To perform the goodness-of-fit test, we assume that the model and parameters are true and we simulate 1,000 artificial data sets by generating RBC predictions and incorporating the error structure described in "Curve Fitting." Because we assume that the model is true, any of these artificial data sets could have been the measured one, so we can generate an expected distribution for L_{max} by calculating the likelihoods of the parameters given these artificial data sets. If the observed L_{max} lies within the 95% highest-density region of its expected distribution, then the parameters are considered to be a good fit and are accepted at the $\alpha = 5\%$ level.

Parameter Significance and Parameter Error Estimates. To determine whether fitted parameters are significant, we set each parameter in turn to 0, recalculate the maximum likelihood, and compare this maximum likelihood to the original using the likelihood ratio test, as described in "Model Selection and Goodness of Fit." If the likelihood score of the model including the parameter is significantly better than the model with the parameter set to 0, then the parameter is significant at the 5% level.

To approximate the uncertainty in our estimated parameters, we generate a probability distribution for each. We again assume that the best-fit parameters and model are the true ones, and we generate 100 artificial data sets for each mouse. This gives us 100 data sets, each of which could have been the actual measured data set and would have resulted in a slightly different set of maximum likelihood parameter estimates. With each of these synthetic data sets, we redo the parameter-fitting routine using the best-fit model and we use these maximum likelihood estimates to generate a probability distribution for each parameter.

Coinfection Experiments

The above model fitting and statistical analysis provide a rigorous approach for model selection, but all of these

techniques make use of the original data set for which the models were constructed. Another powerful approach to further examine the validity of the model is to test it with an independent data set. For example, if the model is a valid description of the disease dynamics in $CD4^+$ T cell–depleted mice, then it can be used to predict how the dynamics would differ under different conditions. These predictions can then be tested with new experimental data.

We take this approach by using the best-fit models obtained above to make predictions about the disease dynamics that are expected if both clones simultaneously infect a single host. To generate model predictions, we extend the model to allow for two distinct clones (see app. B) and set appropriate parameter values. For clone-specific burst size and invasion rate parameters, we use the median values from the two 10^6 experiments. For the parameters governing RBC production, we use the mean of the medians from the two clones, except for the time lag, which must be an integer and thus was set at 2.

The predictions from our model are then compared with data from an experiment. Specifically, we obtained data from four mice that received the same anti-$CD4^+$ treatment as in the previous experiments, were contemporaneously inoculated with 10^6 AS and 10^6 DK parasites, and survived to day 19 after inoculation. One mouse (mouse 3) died prematurely in this experiment and was therefore excluded from the analysis. Experimental details are given by Barclay et al. (2008).

Results

Model Development and Data Fitting

Maximum log-likelihood values for each model and mouse are reported in table C1. Using the likelihood ratio test, model 7 was chosen as the best fit for 10 of the 13 mice. The three for which model 7 was not the best fit each had model 8 as a best fit but had maximum likelihood estimates of gametocyte conversion rates that are not easily reconciled with published values (AS 10^5 mouse 1, $g = 0.3$; AS 10^5 mouse 3, $g = 0.19$; AS 10^6 mouse 2, $g = 0.197$). Empirical estimates of gametocyte conversion rates are hard to obtain because only the end product of this process can be counted and the immune system may quickly dispose of maturing gametocytes (Taylor and Read 1997). Instead, some studies have measured the daily proportion of total parasites (gametocytes and merozoites) that are gametocytes (Buckling et al. 1999; Shutler et al. 2005). This serves as a reasonable proxy for conversion rate in the absence of evidence of a strong, gametocyte-specific immune response, considering the relatively long life span in the bloodstream of gametocytes compared with merozoites (gametocyte half-life is estimated to be 8 h for males and 16 h for females; Reece et al. 2003). In experimental infections with the *Plasmodium chabaudi* clone DK under normal conditions, gametocytes make up around 1%–2% of all circulating parasites and, even at their maximum density, do not constitute more than 10% (Shutler et al. 2005). Under maximal stimulation, it is possible that conversion rates reach values above 0.1, but there is no empirical evidence of rates this high being maintained for the duration of the acute phase of infection; therefore, we exclude model 8 for these mice. The next best model for each of these three mice was model 7, so we took this as the best-fit model for these mice for the remainder of the analyses. None of our conclusions about inoculum size or clone effects qualitatively changes by choosing model 8 instead.

The best-fit curves for all mice are in good qualitative agreement with measured RBC densities (see fig. 2). From the best-fit model and parameter estimates, we generated predictions for the parasite dynamics; these are shown, along with experimental data, in figure 3. Considering that we did not use these data for fitting, the model does a good job of qualitatively explaining the parasite dynamics, with a few notable exceptions. For mouse 4 from the AS 10^5 experiment, the model predicts unreasonably high parasite densities for the second peak. There is one potential outlier in the RBC measurements for this mouse occurring on day 10 after inoculation. We omitted this data point, refitted the model, and found much more reasonable predictions for the parasite dynamics (as depicted by the blue line in figs. 2, 3). We denote this modified data set with one outlier omitted as mouse 4′. Also, for mouse 2 from the AS 10^6 experiment, the model fails to capture the timing of parasite peaks. In this data set there were no obvious outliers in the RBC measurements, but the RBC dynamics look very different from those of the other mice in this experiment. In particular, RBC density drops about 2 days later than it does in the other mice, despite similar timing in the parasite peaks, and RBC density fails to show any real increase after the second parasite peak. This mouse was excluded from further statistical analysis.

Statistical Analysis

The goodness-of-fit values of model 7 for all mice are given in the legend of figure 2 and are shown graphically in figure C1. The fits were very good for all mice and all parameters were significant for every mouse. Best-fit parameters for model 7 are shown for individual mice in table C2, and boxplots of estimated parameter distributions are shown in figures C2–C4.

Some trends can be seen when comparing estimated values of certain parameters within individuals. In particular, RBC deficits tend to be made up more quickly under

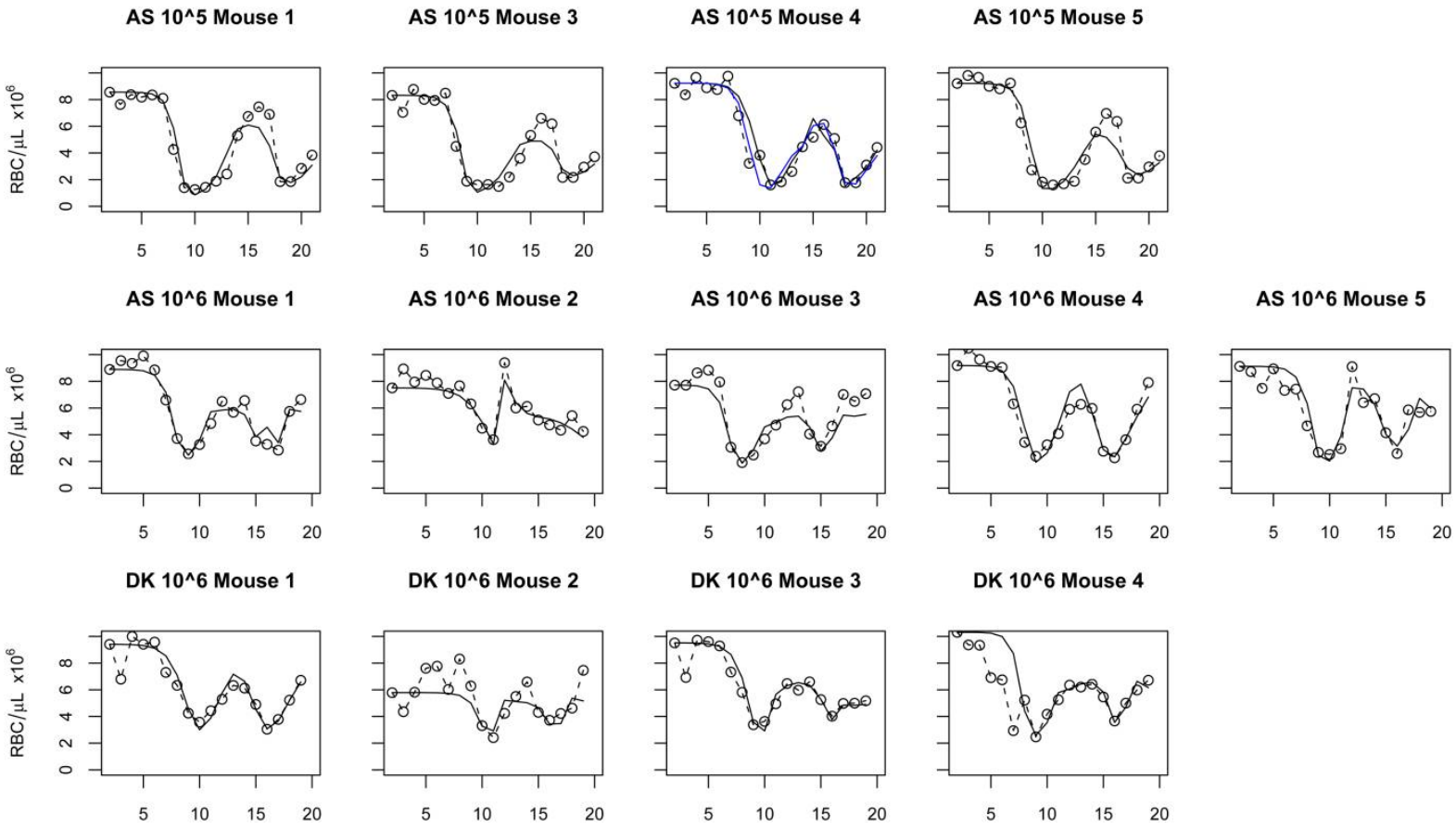

Figure 2: Experimental data and best-fit curves for red blood cell dynamics. *Circles*, observed values; *solid lines*, best fits. Infections are with either 10^5 AS, 10^6 AS, or 10^6 DK parasites. *Blue line*, best-fit curve for mouse 4 in the 10^5 AS experiment with one outlier (day 10) removed (denoted mouse 4′). The fit of the model is very good for all mice. Goodness-of-fit values for the AS 10^5 inoculation: mouse 1 = 388, mouse 3 = 134, mouse 4 = 739, mouse 5 = 134; for the AS 10^6 inoculation: mouse 1 = 824, mouse 3 = 581, mouse 4′ = 590, mouse 5 = 642; for the DK 10^6 inoculation: mouse 1 = 610, mouse 2 = 866, mouse 3 = 727, mouse 4 = 368. Values greater than 50 represent good fits.

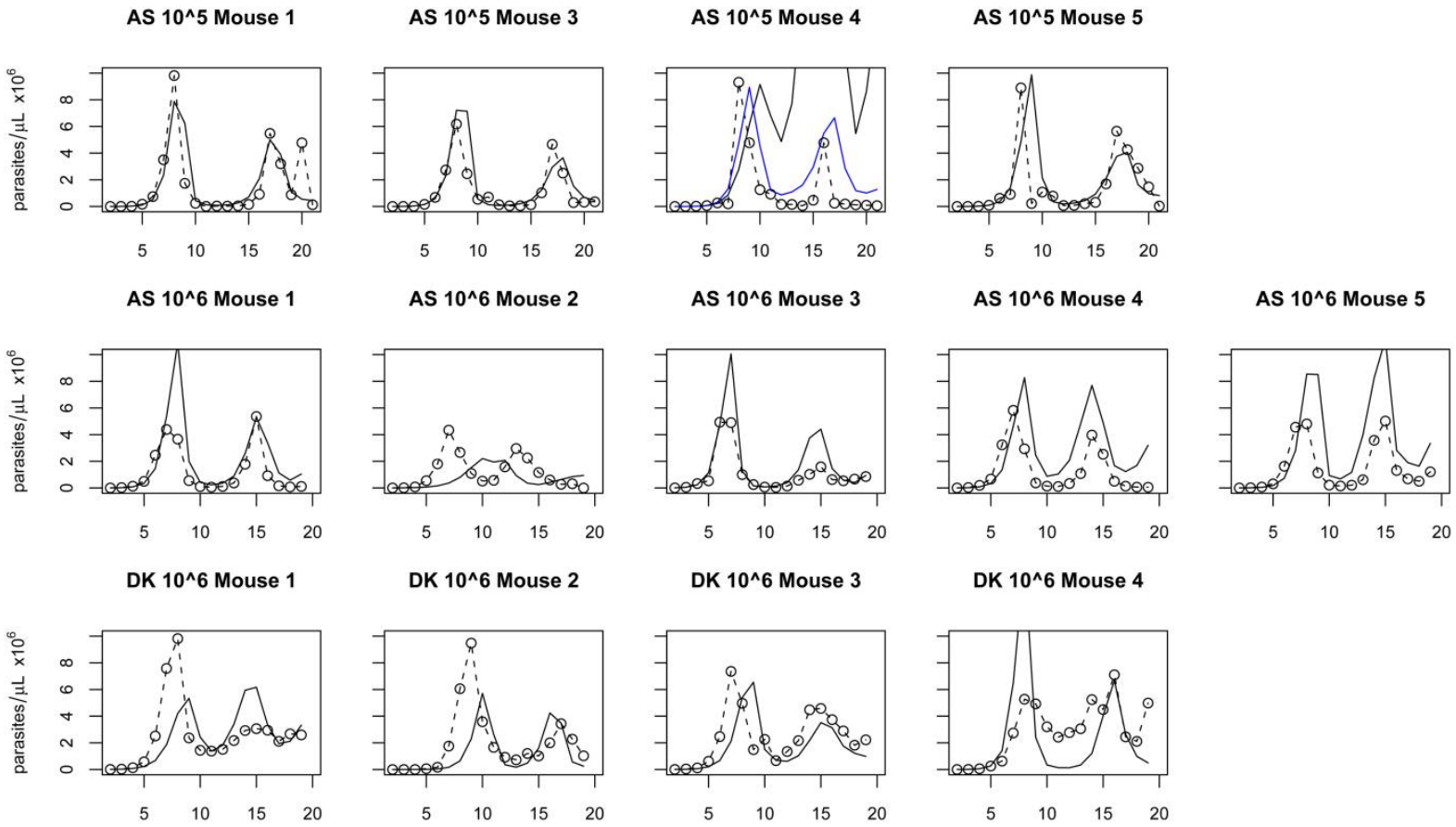

Figure 3: Experimental data and simulations of parasite dynamics. *Circles*, observed values; *solid lines*, model predictions (using parameters fitted to red blood cell data only). Infections are with either 10^5 AS, 10^6 AS, or 10^6 DK parasites. *Blue line*, model predictions for mouse 4 in the 10^5 AS experiment after one outlier (day 10) was removed.

anemic conditions than when RBC densities are normal (i.e., $\theta_0 < \theta_A$; see fig. C4). In addition, one aspect of the importance of RBC age structure is borne out in these distributions: for every individual, the invasion rate of fully mature RBCs is higher than for reticulocytes (i.e., $\beta_R < \beta_N$; see fig. C2), often by an order of magnitude. These invasion rates control for differences in availability, so they truly represent a preference for mature RBCs. Despite this preference, burst size is higher for most individuals in reticulocytes than in normocytes (i.e., $\omega_R > \omega_N$; see fig. C3).

Effect of Parasite Clone. By pooling the data from individuals, we are able to compare the distributions of the parameter estimates to see what effect parasite clone has on the infection dynamics and, in particular, to try to identify the basis for differences in virulence between the DK (less virulent) and AS (more virulent) clones. We compared only those data from inoculations with 10^6 parasites to control for possible inoculum size effects. Boxplots of the pooled distributions are shown in figure C5, and median values are listed in table C3. Table 2 contains estimated DK parameter values relative to the AS values, and we assume, somewhat arbitrarily, that differences of greater than 10% of the AS value are evidence of a clone effect. The median invasion rates of reticulocytes are approximately equal for both clones, but the AS clone has a higher invasion rate of normocytes than the DK clone. Median values for burst size are also higher for the AS parasites than for the DK parasites, and this difference is even more pronounced in reticulocytes than in normocyctes. The mice in the AS-infected group have slower rates of RBC production, both at normal RBC densities and during anemia.

Effect of Inoculum Size. We also compared the distributions of the parameter estimates to see what effect inoculum size has on the infection dynamics. We compared only those data from inoculations with AS parasites to control for possible clone effects. Boxplots of the pooled distributions are shown in figure C6, and median values are listed in table C3. Estimated parameter values for the 10^5 experiment relative to those for the 10^6 experiment are given in table 2. Median values of RBC invasion rate and normocyte burst size are approximately equal. However, there is a marked difference in the median reticulocyte burst sizes, with the low inoculum size obtaining almost twice as many merozites per infected reticulocyte than the high inoculum size. Estimates of RBC production rates are higher in the 10^6 inoculum size than in the 10^5 inoculum size, both at normal RBC densities and during anemia.

Table 2: Medians of pooled estimated parameter distributions relative to AS 10^6 estimated values

Parameter	AS 10^5	AS 10^6	DK 10^6	Inoculum size	Strain
θ_0	.759	1	1.312	X	X
θ_A	.381	1	1.18	X	X
β_R	1.02	1	.940		
β_N	.906	1	.791		X
ω_R	1.943	1	.765	X	X
ω_N	1.002	1	.877		X

Note: We assume that a difference of 10% or greater is evidence of an effect, as indicated by X.

Coinfection Experiments

Model predictions and experimental data from coinfection experiments are plotted in figures 4 and 5. Despite not allowing any individual variation except for starting RBC and parasite densities, the model predictions provide a reasonable qualitative fit to the data, particularly for the early phase of the parasite dynamics. The predicted peaks and troughs in the RBC densities have similar amplitude to the data, but the timing is slightly different. This is especially clear in mice 1 and 2, where RBC densities are predicted to rebound faster than they actually do. This suggests that RBC production in mice with mixed infections is slower than in single-clone-infected mice, since we used the single-clone parameter estimates to generate these predictions. Despite these differences, the parasite density predictions are very good, predicting both the correct timing of peaks and the magnitude of the first wave of parasites. Model predictions and data start to diverge after the first peak, with the model overestimating the level of competitive exclusion of the less virulent DK clone by the relatively more virulent AS clone.

Discussion

We have used a comprehensive combination of mathematical modeling and experimental data to explore the relative importance of ecological factors to the within-host dynamics of *Plasmodium chabaudi* infections. From our best-fit model we conclude that RBC availability is an important regulator of parasite growth in $CD4^+$ T cell–depleted mice, and our systematic approach to model fitting uncovered some of the complexities of this resource and its interaction with the parasite.

Consistent with previous work (Hetzel and Anderson 1996), we find that the loss of RBCs due to infection is sufficient to downregulate parasite densities after the initial peak, and the subsequent increase in RBC production alone generates a second wave of parasites. Recrudescences are conventionally explained as outgrowths by antigenic variants able to escape protective immunity, and it is true

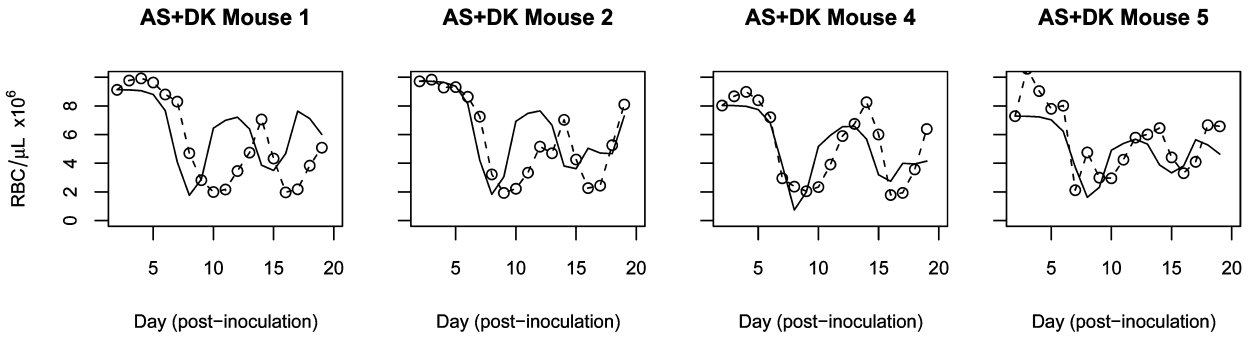

Figure 4: Experimental data and model predictions for red blood cell (RBC) dynamics in competition experiments. *Circles*, observed values; *solid lines*, model predictions. Infections are with 10^6 AS and 10^6 DK parasites; β_R, β_N, ω_R, and ω_N are clone specific and are set to values listed in table C2 for the 10^6 single-clone experiments, and θ_0 and θ_A are set to the mean of the values obtained from these experiments and τ is set to 2, as obtained from the AS 10^6 data.

that recrudescences are antigenically distinct from the primary wave of parasites (Brown and Brown 1965; Phillips et al. 1997). However, because malaria parasites are generating antigenic variation through time, recrudences would be antigenically distinct even if parasite densities were entirely controlled by RBC dynamics. The relative importance of immune evasion and resource limitation remains to be determined in animals with fully intact immune responses.

During periods of anemia, we find that the rate of RBC production is upregulated (similar to findings in Jakeman et al. 1999). Intriguingly, we find that mice infected with the AS clone of *P. chabaudi* have slower rates of RBC production than those inoculated with the DK clone. A similar effect of clone on erythropoesis has been estimated before (Haydon et al. 2003). In our study, it remains unclear whether RBC production is suppressed by the AS clone or enhanced by the DK clone. If future empirical studies can establish a baseline response to anemia, this question could be resolved. Either way, our finding that mice inoculated with the DK clone suffer less anemia may help to explain why the AS clone is relatively more virulent than the DK clone. The details, ubiquity, and adaptive value of this sort of resource manipulation offer many avenues for future investigation.

Recent theory has pointed to a role for RBC age structure in determining relative virulence of *P. chabaudi* clones, with more virulent clones estimated to be able to invade a greater age range of RBCs than less virulent ones (Antia et al. 2008). Our results echo the importance of age structure, but our model takes a different approach and suggests different mechanisms. In particular, Antia et al. (2008) assume that no invasion is possible for RBCs outside a predicted age range and estimate the youngest RBCs each clone is able to infect (8 days for the more virulent clone and 12 days for the less virulent clone). In contrast, we allowed for different invasion rates and burst sizes between types of RBCs and sought to define these types in a biologically meaningful way (i.e., reticulocytes and normocytes). We could then determine whether malaria parasites interact differently with each type of cell and whether these interactions differ between clones.

First, we find that the more virulent clone (AS) has an advantage early on in infections, with a greater invasion rate of and a higher burst size in normocytes than the less virulent clone (DK). Second, although both AS and DK merozoites have lower rates of invasion of reticulocytes, AS gains a fecundity benefit from these invasions, producing more daughter merozoites in younger cells than in fully mature ones. This likely gives AS a big advantage, because RBC production increases when anemia sets in and the system is flushed with reticulocytes. Although no biological mechanism for this higher burst size in reticulocytes is yet known, potential candidates include the fact that the larger size of reticulocytes allows more room for merozoites; that there is a greater structural integrity or deformability of reticulocytes (Taylor-Robinson and Phillips 1994; meaning that older cells may be more rigid and likely to rupture under stress); and, given that reticulocytes are newly introduced into the bloodstream, that their expected circulation time before clearance by the spleen is longer (Loeffler et al. 1989) and the relative risk to the merozoites of undergoing an additional round of asexual multiplication is lower.

In mice with intact immune systems, *P. chabaudi* merozoites infect the most abundant cell type, showing a preference for normocytes early in infections and then switching to reticulocytes when the mouse becomes anemic and the bloodstream flushes with new RBCs (Jarra and Brown 1989; Taylor-Robinson and Phillips 1994). In $CD4^+$ T cell–

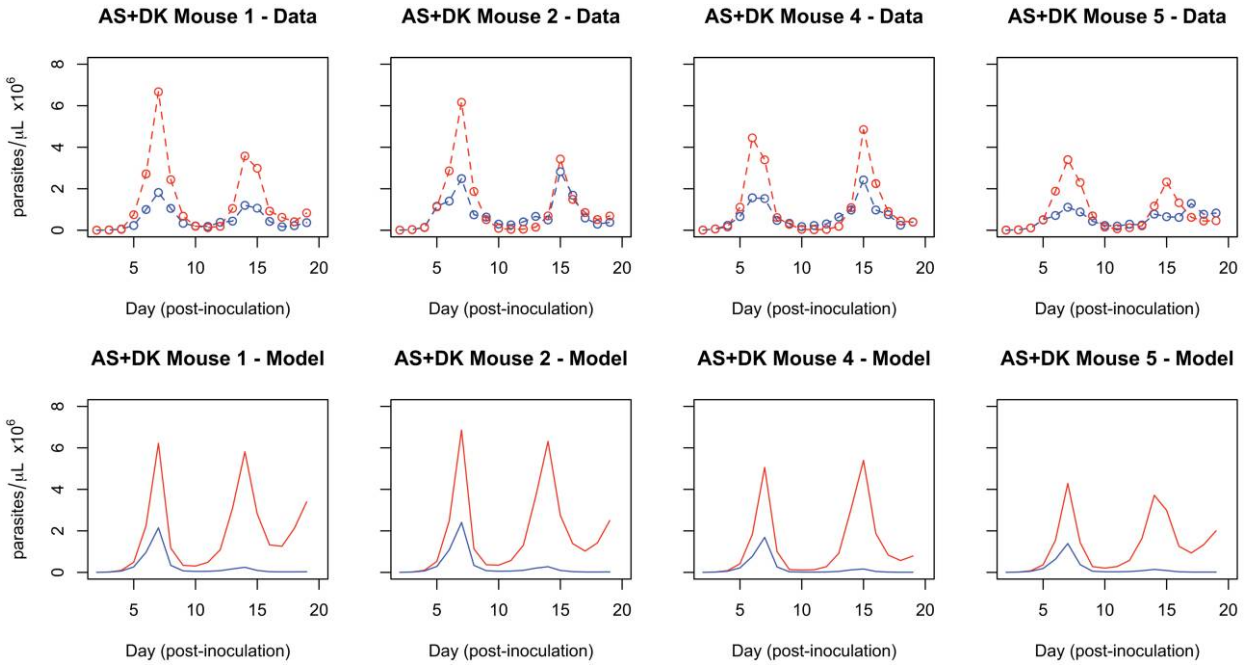

Figure 5: Experimental data and model predictions for parasite dynamics in competition experiments. *Red*, AS parasites; *blue*, DK parasites; *circles*, observed values; *solid lines*, model predictions. Parameters are the same as in figure 4.

depleted mice, infections become chronic, RBC densities remain depressed, and the proportion of circulating reticulocytes remains higher than under normal conditions, although they remain less abundant than mature RBCs (Taylor-Robinson and Phillips 1994). In broad agreement with our "fecundity benefit" hypothesis of reticulocyte invasion, other experimental studies have shown that, in immune-depleted mice, AS parasites continue to show a preference for reticulocytes (Taylor-Robinson and Phillips 1994); that is, they invade reticulocytes more often than would be expected given their relative density. In these mice, the cost of overcoming a lower invasion rate in reticulocytes may be less than the burst size benefit, whereas in the presence of an immune response, the cost of not infecting the most easily accessible cell may be prohibitively high. Predicting a facultative adjustment of a parasite life-history trait in response to the host environment may be novel in the case of RBC preference and host immunity, but it is not unprecedented. Rodent malaria parasites have been shown to alter either their investment in gametocytes or the gametocyte sex ratio in response to host stress (e.g., Buckling et al. 1999; Paul et al. 2003; Reece et al. 2005).

Given this empirical evidence of variable investment in gametocytes throughout infections, and depending on the host environment, modeling conversion rate as a constant throughout the course of infection is inadequate. However, our results suggest that, on average, conversion to gametocytes has no significant effect on the infection dynamics. An alternative approach would be to allow this parameter to vary over time. Although this would perhaps be more biologically realistic, it would also make the parameter fitting process computationally impractical. In its simplest form, our model need not explicitly track gametocytogenesis, as this loss of asexual individuals could be subsumed by the estimates for burst sizes or invasion rates.

Our results present some unexpected differences due to parasite inoculum size. First, inoculum size matters for RBC production, with higher inoculum sizes inducing higher production rates. These parameter estimates are likely due to the fact that, in the experimental data, mice inoculated with 10^5 AS parasites reached lower minimum RBC densities than those who received 10^6 parasites. Greater anemia with a lower inoculum size is not consistent with previous results on inoculum size effects (Timms et al. 2001), although that earlier study was performed in mice with intact immune systems and with different *P. chabaudi* clones. Second, reticulocyte burst sizes are about twofold higher in the 10^5 inoculum size experiment than in the 10^6 inoculum size experiment. One potential explanation of this is the rigidity of the model framework. It could be that, with the larger inoculum size as well as an increased RBC production, the RBCs are maturing

faster so that what we are modeling as reticulocytes are functionally fully mature RBCs; we would then expect the best-fit β_R value to be closer to the β_N value for the 10^6 inoculum size experiment. A more intriguing possibility is that burst size is density dependent, so that the merozoites regulate their usage of RBCs.

We found no consistent estimate of the lag in RBC production from our individual mice. Although published estimates of the transit time of blood cell precursors to the bloodstream tend to be around 3 days (Mary et al. 1980; Chang et al. 2004), this process has been estimated to take as little as 33 h under maximal erythropoetic stimulation (Loeffler et al. 1989). Again, our model did not allow any flexibility in this parameter over time because the discretized description of RBC production meant it had to be an integer. The medians from the pooled distributions do match up with the predictions about RBC production rates: the DK clone induced the highest production rate and had the shortest lag, whereas inoculation with a smaller number of merozoites induced the lowest production rate and had the longest lag. Although the medians from both high-inoculum-size experiments are different, the means were more similar (AS, 2.0 days; DK, 1.7 days) and the true transit time need not be an integer.

Both empirical and theoretical studies have attempted to understand the mechanisms of competition in genetically diverse malaria infections. Experiments with *P. chabaudi* in athymic mice revealed some evidence for immune-mediated apparent competition when the initial wave of parasites receded (Råberg et al. 2006), but when the $CD4^+$ T cell–depleted mice used in the analyses reported here were compared with immune-system-intact control mice, evidence for $CD4^+$ T cell–mediated competition could not be found (Barclay et al. 2008). In the presence of a weak immune response, theory predicts that competition for RBCs is a limiting factor for coinfecting parasites (Hellriegel 1992). Our results confirm this: RBC availability alone can explain the first peak of parasites in $CD4^+$ T cell–depleted mice. The differences in burst sizes and invasion rates of the clones that were derived from our single-strain model generate the appropriate relative heights of the first AS and DK merozoite peaks in coinfections. Beyond the initial peak, our model predictions and the data start to deviate. The model predicts much stronger competitive suppression of the less virulent DK clone by the AS clone than what is seen in the data. Our data are consistent with previous studies in immune-intact mice, which showed that, after the initial peaks, parasite densities were no different or higher in competition than they were in single infections (Bell et al. 2006). The authors of that article point to the immune response becoming more clone specific as an explanation of why the competitively superior clone cannot completely suppress the other. Here, we propose that a similar but $CD4^+$ T cell–independent specific immune response is responsible for the competitive release of the DK clone for which our model could not account. Further insights into the dynamics of coinfections could be found by fitting our competition model to the available data to see whether differences in infection dynamics are solely due to differences in parameters or, alternatively, whether other mechanisms (like our proposed immune response) are in operation.

Although we believe that the ability to predict the outcome of the acute phase of competition experiments provides strong validation of our model, it could be further tested in a number of ways. One option is to empirically test our parameter estimates, although our results show that there will be substantial variation between individual mice. Another option is to experiment with other clones to see whether their relative virulence can be explained by the mechanisms we have proposed. It would also be interesting to see whether there was more evidence of density-dependent regulation of RBC usage and burst sizes, perhaps through experimental manipulation of RBC availability.

The results presented here take a significant first step toward a comprehensive model of the within-host dynamics of malaria infections. We have shown that ecological factors are crucial for explaining infection dynamics and, with this foundation, we can build up the complexity toward a better understanding of the dynamics in the more biologically interesting setting of immune-intact hosts.

Acknowledgments

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APPENDIX A

Model Derivation

The basic structure of the discrete-time model in tracking merozoite density and RBC density is derived by considering the series of events occurring during a single day. Suppose the number of merozoites and susceptible RBCs of each age class per microliter of blood at the census point on day i are given by P_i, $R_{1,i}$, $R_{2,i}$, $R_{3,i}$, and N_i. The next event in the replication cycle is the invasion of susceptible RBCs. We model this as a continuous time process that occurs quickly, relative to the 24 h discrete-time burst cycle. Free-living merozoites invade reticulocytes and normocytes at rates β_R and β_N, respectively, and experience a natural mortality rate μ while in the bloodstream. We assume that merozoites can infect both susceptible and already infected RBCs, but for simplicity (and similar to Hetzel and Anderson 1996) we assume that secondary invasions are lost and do not change the behavior of the primary invasion. Given these assumptions, we can write differential equations to describe the invasion phase. For $j = 1, 2$, and 3 (i.e., the day after the release of the reticulocyte in the bloodstream),

$$\frac{dP}{dt} = -P\left[\beta_R \sum_{j=1}^{3} (R_j + I_j) + \beta_N(N + I_N)\right] - \mu P, \tag{A1}$$

$$\frac{dR_j}{dt} = -\beta_R P R_j, \tag{A2}$$

$$\frac{dN}{dt} = -\beta_N P N, \tag{A3}$$

$$\frac{dI_j}{dt} = \beta_R P R_j, \tag{A4}$$

$$\frac{dI_N}{dt} = \beta_N P N. \tag{A5}$$

Pay special attention to the notation in equations (A1)–(A5). The variables in these equations are missing the subscript i referring to the day in question because these are, in fact, different variables than the P_i, $R_{j,i}$, N_i, $I_{j,i}$, and $I_{N,i}$ introduced above. The variables in equations (A1)–(A5) track the dynamics of the different kinds of cells during the RBC invasion phase only, which occurs as one of the events during each day. The variables subscripted with an i, however, track these values from one day to the next. Thus, the initial conditions for the above system of differential equations on day i are $P(0) = P_i$, $R_j(0) = R_{j,i}$, $N(0) = N_i$, $I_j(0) = 0$, and $I_N(0) = 0$. The solution to this system is readily found, allowing us to calculate the number of each type of cell after this invasion phase is complete; that is, $R_j(\infty)$, $N(\infty)$, $I_j(\infty)$, and $I_N(\infty)$ (note that $P(\infty) = 0$).

The next event is RBC turnover through death and erythropoesis. We suppose that a fraction d of all RBCs die, then RBC aging occurs, and then finally newly produced RBCs enter the age 1 class at a rate that depends on the RBC density τ days earlier. Specifically, we model RBC production in a density-dependent fashion as in Haydon et al. (2003) but with a time lag of τ days between the onset of anemia and the body's response. This represents the time it takes for new blood cells to develop in the bone marrow before being released into the bloodstream. Thus, the number of susceptible age 1 RBCs produced on day i is $\theta[K - (R_{1,i-\tau} + R_{2,i-\tau} + R_{3,i-\tau} + N_{i-\tau})]$, where K represents the equilibrium density of RBCs in the absence of both merozoites and natural death and θ is the proportion of the RBC deficit that is made up in one day. Other forms of RBC production, such as a saturating Hill function, have been suggested (Mackey 1997), but we use this linear form of density dependence for simplicity and we account for a type of saturation of production by having two separate values of θ: one for when RBC level is above 50% of its normal density (θ_0) and another for when it is below 50% (θ_A). We have performed all of the analyses below with a Hill function as well, and this tended to result in qualitatively reasonable but statistically poorer fits to the data (N. Mideo, unpublished results). Last, with the above assumptions, the number of susceptible RBCs of age 2 and age 3 on day i becomes $(R_{1,i} - I_1(\infty))(1 - d)$ and $(R_{2,i} - I_2(\infty))(1 - d)$, respectively, and the number of normocytes becomes $(R_{3,i} - I_3(\infty) + N_i - I_N(\infty))(1 - d)$.

The final event is the bursting of RBCs and the resulting production of merozoites, at which point a new census

occurs. Using ω_R and ω_N as the burst size of infected retoculocytes and normocytes, respectively, the total number of merozoites produced on day i is then $[\omega_R \sum_{j=1}^{3} I_j(\infty) + \omega_N I_N(\infty)](1 - d)(1 - g)$, where g is the proportion of infected RBCs that produce gametocytes rather than merozoites (referred to as the conversion rate). Thus, the discrete time system of equations from one day to the next is

$$P_{i+1} = \left(\omega_R \sum_{j=1}^{3} I_j(\infty) + \omega_N I_N(\infty)\right)(1 - d)(1 - g), \tag{A6}$$

$$R_{1,i+1} = \theta[K - (R_{1,i-\tau} + R_{2,i-\tau} + R_{3,i-\tau} + N_{i-\tau})], \tag{A7}$$

$$R_{2,i+1} = (R_{1,i} - I_1(\infty))(1 - d), \tag{A8}$$

$$R_{3,i+1} = (R_{2,i} - I_2(\infty))(1 - d), \tag{A9}$$

$$N_{i+1} = (R_{3,i} - I_3(\infty) + N_i - I_N(\infty))(1 - d). \tag{A10}$$

Finally, we derive explicit expressions for the number of each type of RBC invaded in a particular cycle by finding general solutions to the differential equations (A1)–(A5). Because the number of total RBCs (uninfected plus infected) remains constant during a bout of invasion (because RBC production and natural death occur in the next step of the discrete cycle), the parasite dynamics do not depend on the dynamics of either. Using this fact and the initial conditions $R_j(0) = R_{j,i}$, $N(0) = N_i$, $P(0) = P_i$, $I_j(0) = 0$, and $I_N(0) = 0$, we can first find an expression for $P(t)$ by solving equation (A1). This gives us

$$P(t) = P_i \exp\{-t[(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu]\}. \tag{A11}$$

Substituting this into equations (A4) and (A5) we find

$$I_j(t) = R_{j,i}\left\{1 - \exp\left[-\frac{(1 - \exp\{-t[(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu]\})P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\} \tag{A12}$$

for $j = 1, 2$, and 3, and

$$I_N(t) = N_i\left\{1 - \exp\left[-\frac{(1 - \exp\{-t[(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu]\})P_i\beta_N}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}. \tag{A13}$$

Further simplification of these expressions is possible because, given our parameters, the number of parasites declines to 0 within a single day. This makes biological sense, because free-living parasites have only a matter of minutes to infect a RBC before they will die naturally. Thus, it is a reasonable approximation to assume that the numbers of infected cells just before bursting are given by the limits of equations (A12) and (A13) as t goes to infinity. Thus,

$$I_j(\infty) = R_{j,i}\left\{1 - \exp\left[-\frac{P_i\beta_R}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}, \tag{A14}$$

$$I_N(\infty) = N_i\left\{1 - \exp\left[-\frac{P_i\beta_N}{(R_{1,i} + R_{2,i} + R_{3,i})\beta_R + N_i\beta_N + \mu}\right]\right\}. \tag{A15}$$

Substituting the solutions for $I_j(\infty)$ and $I_N(\infty)$ into the above discrete time system, equations (A6)–(A10), gives the complete model of the main text.

APPENDIX B

Competition Model with Two Clones

We can extend our original discrete time framework (eqq. [A6]–[A10]) to allow for two different clones of a parasite, P_A and P_B. Densities of reticulocytes (on their jth day in the bloodstream) and normocytes infected with parasite A are given by I_{jA} and I_{NA}. Reticulocytes and normocytes infected with parasite B are given by I_{jB} and I_{NB}. As before, we assume that only the first parasite to infect an RBC matters. Subsequent invasions are essentially lost, so we have to consider only singly infected RBCs. The basic model tracking parasite and RBC densities is given by:

$$P_{A,i+1} = \left[\omega_{RA}\sum_{j=1}^{3} I_{jA}(P_{A,i}, R_{j,i}) + \omega_N I_{NA}(P_{A,i}, N_i)\right](1-d)(1-g), \tag{B1}$$

$$P_{B,i+1} = \left[\omega_{RB}\sum_{j=1}^{3} I_{jB}(P_{B,i}, R_{j,i}) + \omega_N I_{NB}(P_{B,i}, N_i)\right](1-d)(1-g), \tag{B2}$$

$$R_{1,i+1} = \theta[K - (R_{1,i-\tau} + R_{2,i-\tau} + R_{3,i-\tau} + N_{i-\tau})], \tag{B3}$$

$$R_{2,i+1} = [R_{1,i} - I_{1A}(P_{A,i}, R_{1,i}) - I_{1B}(P_{B,i}, R_{1,i})](1-d), \tag{B4}$$

$$R_{3,i+1} = [R_{2,i} - I_{2A}(P_{A,i}, R_{2,i}) - I_{2B}(P_{B,i}, R_{2,i})](1-d), \tag{B5}$$

$$\begin{aligned} N_{i+1} = {} & [R_{3,i} - I_{3A}(P_{A,i}, R_{3,i}) - I_{3B}(P_{B,i}, R_{3,i})](1-d) \\ & + [N_i - I_{NA}(P_{A,i}, N_i) - I_{NB}(P_{B,i}, N_i)](1-d). \end{aligned} \tag{B6}$$

The dynamics of the invasion phase are now described by the following set of differential equations:

$$\frac{dP_A}{dt} = -P_A\left[\beta_{RA}\sum_{j=1}^{3}(R_j + I_{jA} + I_{jB}) + \beta_{NA}(N + I_{NA} + I_{NB})\right] - \mu P_A, \tag{B7}$$

$$\frac{dP_B}{dt} = -P_B\left[\beta_{RB}\sum_{j=1}^{3}(R_j + I_{jA} + I_{jB}) + \beta_{NB}(N + I_{NA} + I_{NB})\right] - \mu P_B, \tag{B8}$$

$$\frac{dR_j}{dt} = -\beta_{RA}P_A R_j - \beta_{RB}P_B R_j, \tag{B9}$$

$$\frac{dN}{dt} = -\beta_{NA}P_A N - \beta_{NB}P_B N, \tag{B10}$$

$$\frac{dI_{jA}}{dt} = \beta_{RA}P_A R_j, \tag{B11}$$

$$\frac{dI_{NA}}{dt} = \beta_{NA}P_A N, \tag{B12}$$

$$\frac{dI_{jB}}{dt} = \beta_{RB}P_B R_j, \tag{B13}$$

$$\frac{dI_{NB}}{dt} = \beta_{NB}P_B N. \tag{B14}$$

As before, we can find expressions for $P_A(t)$ and $P_B(t)$. These expressions are equivalent to equation (A11), except with strain-specific invasion rates. Given the initial conditions $R_k(0) = R_{k,i}$, $N(0) = N_i$, $P_A(0) = P_{A,i}$, $P_B(0) = P_{B,i}$, $I_{jA}(0) = 0$, $I_{jB}(0) = 0$, $I_{NA}(0) = 0$, and $I_{NB}(0) = 0$,

$$P_A(t) = P_{A,i}\exp\{-t[(R_{1,i} + R_{2,i} + R_{3,i})\beta_{RA} + N_i\beta_{NA} + \mu]\}, \tag{B15}$$

$$P_B(t) = P_{B,i}\exp\{-t[(R_{1,i} + R_{2,i} + R_{3,i})\beta_{RB} + N_i\beta_{NB} + \mu]\}. \tag{B16}$$

Substituting these results into equations (B9) and (B10), we can find expressions for the $R_j(t)$ values and for $N(t)$. Letting $Y_i = R_{1,i} + R_{2,i} + R_{3,i}$,

$$R_j(t) = R_{j,i}\exp\left(-\frac{\{1 - \exp[-t(N_i\beta_{NA} + Y_i\beta_{RA} + \mu)]\}P_{A,i}\beta_{RA}}{N_i\beta_{NA} + Y_i\beta_{RA} + \mu}\right.$$

$$\left.-\frac{\{1 - \exp[-t(N_i\beta_{NB} + Y_i\beta_{RB} + \mu)]\}P_{B,i}\beta_{RB}}{N_i\beta_{NB} + Y_i\beta_{RB} + \mu}\right), \tag{B17}$$

$$N(t) = N_i\exp\left(-\frac{\{1 - \exp[-t(N_i\beta_{NA} + Y_i\beta_{RA} + \mu)]\}P_{A,i}\beta_{NA}}{N_i\beta_{NA} + Y_i\beta_{RA} + \mu}\right.$$

$$\left.-\frac{\{1 - \exp[-t(N_i\beta_{NB} + Y_i\beta_{RB} + \mu)]\}P_{B,i}\beta_{NB}}{N_i\beta_{NB} + Y_i\beta_{RB} + \mu}\right). \tag{B18}$$

To a good approximation,

$$\exp[-t(N_i\beta_{NA} + Y_i\beta_{RA} + \mu)] \approx \exp[-t(N_i\beta_{NB} + Y_i\beta_{RB} + \mu)], \tag{B19}$$

which allows us to solve for the numbers of different kinds of infected cells just before bursting. As earlier, we take the limits as t goes to infinity. Thus,

$$I_{jA}(\infty) = \frac{P_{A,i}R_{j,i}\beta_{RA}(N_i\beta_{NB} + Y_i\beta_{RB} + \mu)\left\{1 - \exp\left[-\left(\frac{P_{A,i}\beta_{RA}}{Y_i\beta_{RA} + N_i\beta_{NA} + \mu} + \frac{P_{B,i}\beta_{RB}}{Y_i\beta_{RB} + N_i\beta_{NB} + \mu}\right)\right]\right\}}{N_i(P_{A,i}\beta_{RA}\beta_{NB} + P_{B,i}\beta_{RB}\beta_{NA}) + P_{B,i}\beta_{RB}(Y_i\beta_{RA} + \mu) + P_{A,i}\beta_{RA}(Y_i\beta_{RB} + \mu)}, \tag{B20}$$

$$I_{NA}(\infty) = \frac{P_{A,i}N_i\beta_{NA}(N_i\beta_{NB} + Y_i\beta_{RB} + \mu)\left\{1 - \exp\left[-\left(\frac{P_{A,i}\beta_{NA}}{Y_i\beta_{RA} + N_i\beta_{NA} + \mu} + \frac{P_{B,i}\beta_{NB}}{Y_i\beta_{RB} + N_i\beta_{NB} + \mu}\right)\right]\right\}}{N_i(P_{A,i} + P_{B,i})\beta_{NA}\beta_{NB} + P_{B,i}(Y_i\beta_{RA} + \mu) + P_{A,i}\beta_{NA}(Y_i\beta_{RB} + \mu)}, \tag{B21}$$

$$I_{jB}(\infty) = \frac{P_{B,i}R_{j,i}\beta_{RB}(N_i\beta_{NA} + Y_i\beta_{RA} + \mu)\left\{1 - \exp\left[-\left(\frac{P_{A,i}\beta_{RA}}{Y_i\beta_{RA} + N_i\beta_{NA} + \mu} + \frac{P_{B,i}\beta_{RB}}{Y_i\beta_{RB} + N_i\beta_{NB} + \mu}\right)\right]\right\}}{N_i(P_{A,i}\beta_{RA}\beta_{NB} + P_{B,i}\beta_{RB}\beta_{NA}) + P_{B,i}\beta_{RB}(Y_i\beta_{RA} + \mu) + P_{A,i}\beta_{RA}(Y_i\beta_{RB} + \mu)}, \tag{B22}$$

$$I_{NB}(\infty) = \frac{P_{B,i}N_i\beta_{NB}(N_i\beta_{NA} + Y_i\beta_{RA} + \mu)\left\{1 - \exp\left[-\left(\frac{P_{A,i}\beta_{NA}}{Y_i\beta_{RA} + N_i\beta_{NA} + \mu} + \frac{P_{B,i}\beta_{NB}}{Y_i\beta_{RB} + N_i\beta_{NB} + \mu}\right)\right]\right\}}{N_i(P_{A,i} + P_{B,i})\beta_{NA}\beta_{NB} + P_{B,i}(Y_i\beta_{RA} + \mu) + P_{A,i}\beta_{NA}(Y_i\beta_{RB} + \mu)}. \tag{B23}$$

APPENDIX C

Supplementary Results

We use the maximum likelihood method of fitting our model variations to the individual mouse data. Details of the methodology are presented in the main text. The best-fit parameters for a given model maximize the log likelihood L; the maximum likelihood values are presented in table C1. We use the likelihood ratio test to compare the fit of our nested model variations.

As described in "Model Development and Data Fitting," we reject model 8 as the best fit for the three mice data sets that chose this model on the basis of the estimated gametocyte conversion rate g. Thus, the best-fit model for each mouse is model 7 and the best-fit parameters for each individual are given in table C2.

We test the goodness-of-fit of the maximum likelihood parameters of model 7 to the data sets of each individual mouse. The likelihood of these parameters, given the data, is proportional to L_{max}. For this test, we assume that the model and parameters are true and simulate 1,000 artificial data sets by generating RBC predictions and incorporating the error structure described in "Curve Fitting." Each of these data sets represents one that could have been the measured one; we can generate an expected distribution for L_{max} by calculating the likelihoods of the parameters given these artificial data sets. If the observed L_{max} value lies within the 95% highest-density region (HDR) of its expected distribution, then the parameters are considered to be a good fit and are accepted at the $\alpha = 5\%$ level. In figure C1, we plot these distributions and highlight the location of the observed L_{max} within the 95% HDR. The fit is good for each mouse.

We can use the approach described above for generating artificial data sets to approximate the uncertainty in our estimated parameters. We generate 100 artificial data sets for each mouse, each of which again could have been the measured one and would have resulted in a slightly different set of maximum likelihood parameter estimates. With each of these synthetic data sets, we perform the parameter-fitting routine again using the best-fit model, and we use these maximum likelihood estimates to generate a probability distribution for each parameter. These distributions are shown in figures C2–C4.

By pooling the individual probability distributions for each parameter according to experiment (i.e., AS 10^5, AS 10^6, or DK 10^6), we attempt to identify any effects of parasite clone or inoculum size. Median parameter values, according to treatment, are presented in table C3 and are shown graphically, along with distributions, in figures C5 and C6.

Table C1: Maximum log likelihoods (L_{max}) for each model and the best-fit model, as chosen by the likelihood ratio test, for each mouse.

Treatment, mouse	Model (no. parameters)								Best-fit model
	1 (4)	2 (5)	3 (5)	4 (6)	5 (6)	6 (7)	7 (7)	8 (8)	
AS 10^5:									
1	−252.0354	−254.2259	−198.3124	−188.9497	−71.9943	−60.4726	−29.2523	−16.5551	8
3	−145.7672	−148.5523	−102.6568	−99.7949	−74.8265	−58.1948	−33.6190	−31.6321	8
4	−41.778	−41.3139	−34.0503	−33.1802	−32.7567	−31.4321	−15.2876	−17.1742	7
5	−91.2966	−97.2707	−68.2014	−73.0514	−65.1489	−65.4983	−30.5617	−30.1121	7
AS 10^6:									
1	−95.3124	−95.3124	2.5347	3.3676	8.1647	8.1713	21.1703	20.1022	7
2	−28.5043	−28.1106	−17.2592	−20.8733	−15.0168	−13.7768	28.2615	25.6421	8
3	−205.2719	−205.2719	−82.4215	−81.7086	−21.2089	−19.6881	2.6447	3.8579	7
4	−101.6202	−101.6202	−61.5321	−56.9027	2.4884	3.0744	14.3611	14.3611	7
5	−87.6311	−88.0733	−33.816	−33.816	−6.2623	−6.2629	.7489	.276	7
DK 10^6:									
1	5.1129	5.376	20.2243	19.9075	22.3498	23.4571	24.9077	25.5997	7
2	−113.2633	−113.261	−96.7325	−96.7376	−93.2548	−93.25448	−22.9551	−22.9441	7
3	−32.8633	−32.9051	−7.8646	−7.7993	5.5394	8.3251	24.2722	24.3229	7
4	−260.4084	−259.8688	−130.6609	−128.4231	−133.3927	−120.12560	−86.8485	−87.2826	7

Note: Models that share the same number of fitted parameters can be compared directly; that is, a model with a larger L_{max} value is significantly better. For models that differ by one fitted parameter, twice the difference in L_{max} values must be greater than 3.84 for a more complex model to provide a significantly better fit. See "Statistical Analysis" for details.

Table C2: Estimated parameter values for the best-fit model

Treatment, mouse	θ_0	θ_A	$\beta_R \times 10^6$	$\beta_N \times 10^6$	ω_R	ω_N	τ
AS 10^5:							
1	.06901	.18889	1.0889	7.08999	13.8193	9.1826	3
3	.05124	.128	.3918	15.06	21	6.072	3
4	.83897	.5099	9.997	.9889	11.995	22	2
5	.05181	.1309	.7839	12.0610	19.890	6.030	2
AS 10^6:							
1	.04942	.41099	1.0009	16.28	4.649	6.001	3
2	.01877	.5	.589	1.489	2	13.889	1
3	.07664	.5	.2263	12.206	10.999	7.732	3
4	.5	.3715	.8309	3.5002	19	11.021	1
5	.0875	.4491	.843	11.749	15.89	6	2
DK 10^6:							
1	.5	.4389	.8508	4.104	18	7.111	1
2	.04139	.5	.2759	19.825	11.799	5.991	1
3	.1739	.5	.725	7.499	9.46	6.014	1
4	.04983	.3889	.889	10.3891	2.98	8.078	3

Table C3: Medians of pooled estimated parameter distributions

Parameter	AS 10^5	AS 10^6	DK 10^6
θ_0	.0759	.10	.1312
θ_A	.1999	.52509	.6196
β_R	1.086×10^{-6}	1.065×10^{-6}	1.001×10^{-6}
β_N	10.14×10^{-6}	11.189×10^{-6}	8.861×10^{-6}
ω_R	15.778	8.12	6.21
ω_N	7.998	7.979	7.0
τ	3	2	1

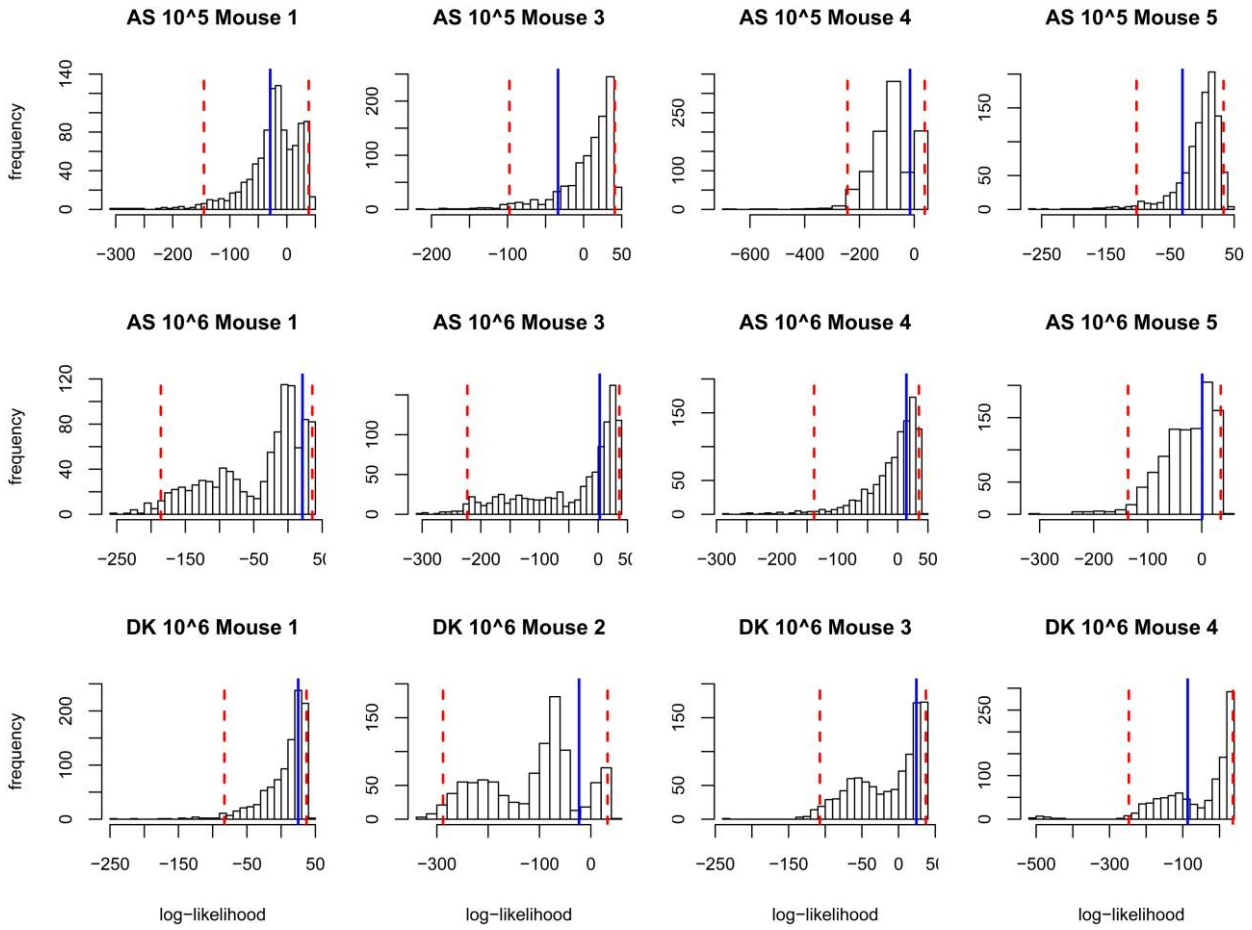

Figure C1: Expected distributions of L_{max}, the maximum log likelihood of the best-fit model given the data, for each individual mouse. These distributions are generated by calculating the log likelihood of the best-fit model and parameters given each of 1,000 artificial data sets. *Blue lines*, the observed L_{max} (log likelihood of best-fit model and parameters given the measured data). In each case, the blue line is within the 95% highest-density region of its expected distribution (*dashed red lines*); therefore, the model is a good fit for each data set.

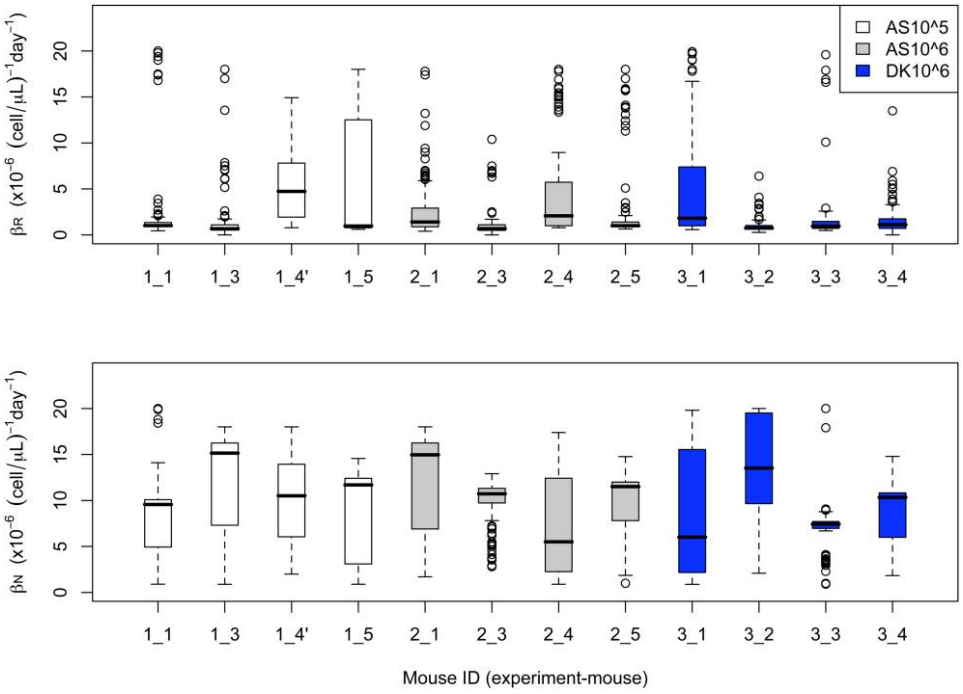

Figure C2: Boxplots of the estimated distributions of invasion rate for reticulocytes (*top*) and normocytes (*bottom*). Each boxplot contains 100 parameter estimates obtained from refitting simulated data sets (100 data sets per mouse). *Horizontal line*, median; *circles*, outliers; each box contains 50% of the values.

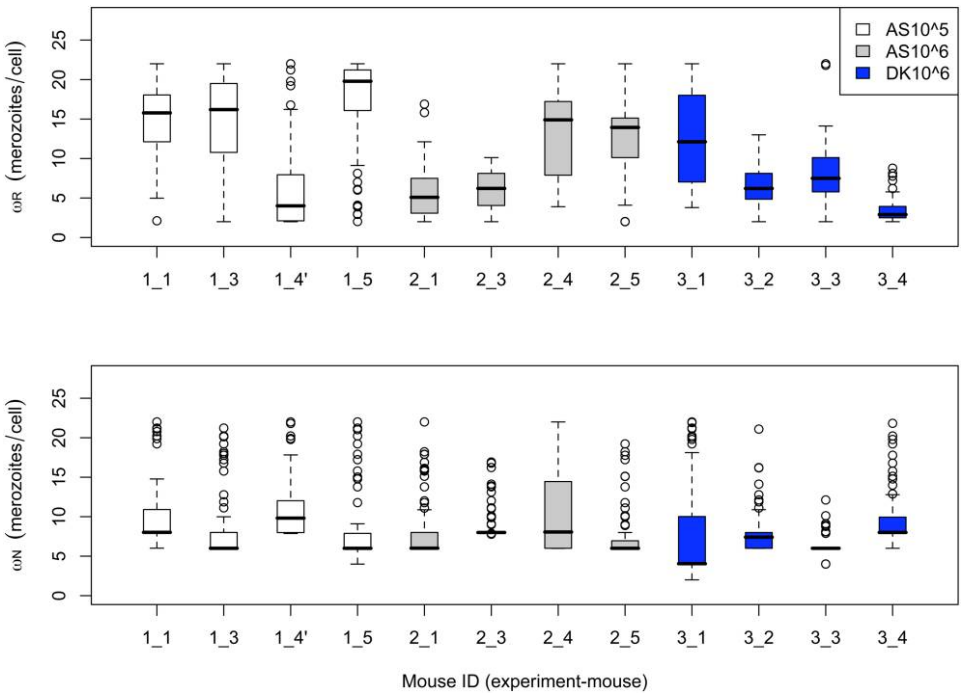

Figure C3: Boxplots of the estimated distributions of burst size for reticulocytes (*top*) and normocytes (*bottom*). Each boxplot contains 100 parameter

estimates obtained from refitting simulated data sets (100 data sets per mouse). *Horizontal line*, median; *circles*, outliers; each box contains 50% of the values.

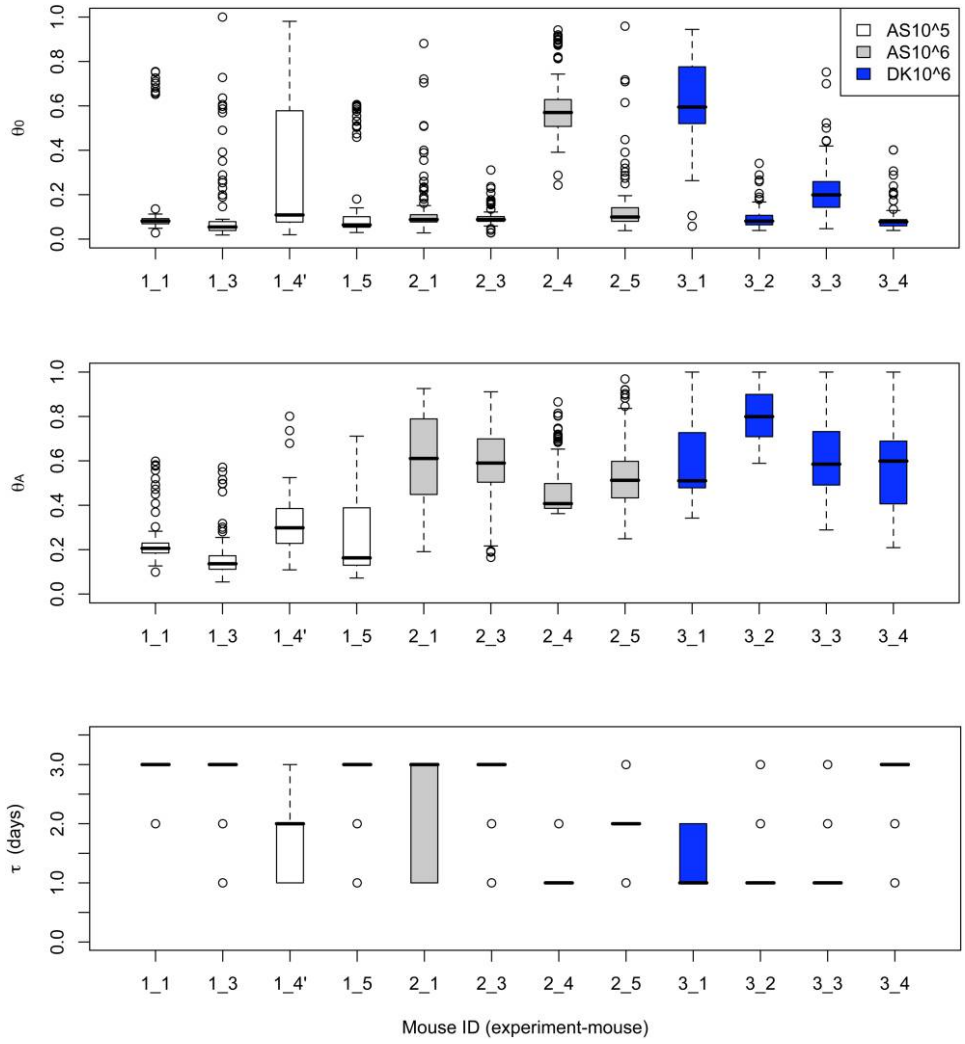

Figure C4: Boxplots of the estimated distributions of RBC production parameters: proportion of RBC deficit made up in one day under normal conditions (*top*), proportion of RBC deficit made up in one day under anemic conditions (*center*), and time lag due to maturation of RBC (*bottom*). Each boxplot contains 100 parameter estimates obtained from refitting simulated data sets (100 data sets per mouse). *Horizontal line*, median; *circles*, outliers; each box contains 50% of the values.

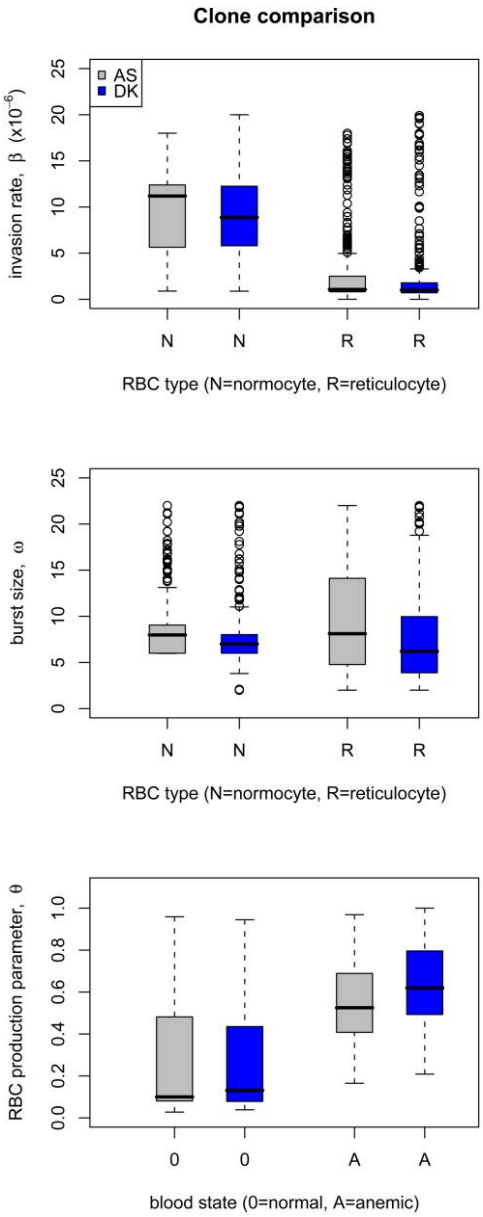

Figure C5: Boxplots of the combined estimated parameter distributions for the AS 106 (*gray*) and DK 106 (*blue*) experiments. Each boxplot contains 400 parameter estimates (100 data sets per mouse × 4 mice per strain). *Horizontal line*, median; *circles*, outliers; each box contains 50% of the values.

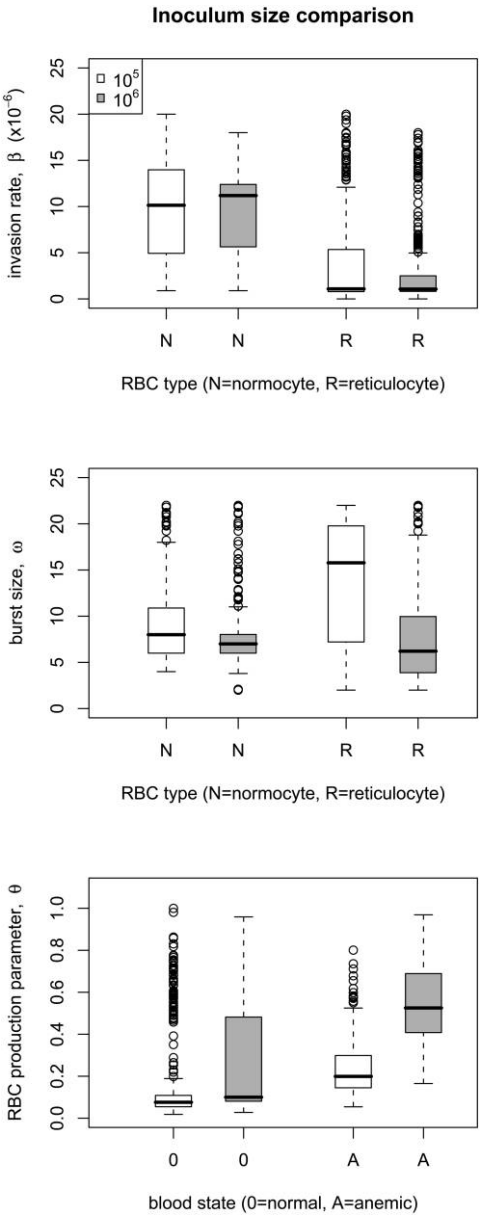

Figure C6: Boxplots of the combined estimated parameter distributions for the AS 105 (*white*) and AS106 (*gray*) experiments. Each boxplot contains 400 parameter estimates (100 data sets per mouse × 4 mice per dose). *Horizontal line*, median; *circles*, outliers; each box contains 50% of the values.

Literature Cited

Antia, R., A. Yates, and J. C. de Roode. 2008. The dynamics of acute malaria infections. I. Effect of the parasite's red blood cell preference. Proceedings of the Royal Society B: Biological Sciences 275: 1449–1458.

Bannerman, R. M. 1983. Hematology. Pages 293–312 *in* J. D. Small, H. L. Foster, and J. G. Fox, eds. The mouse in biomedical research. Academic Press, New York.

Barclay, V. C., L. Råberg, B. H. K. Chan, S. Brown, D. Gray, and A. F. Read. 2008. $CD4^+$ T cells do not mediate within-host competition between genetically diverse malaria parasites. Proceedings of the Royal Society B: Biological Sciences 275:1171–1179.

Beale, G. H., R. Carter, and D. Walliker. 1978. Genetics. Pages 213–245 *in* R. Killick-Kendrick and W. Peters, eds. Rodent malaria. Academic Press, London.

Bell, A. S., J. C. D. Roode, D. Sim, and A. F. Read. 2006. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution 60:1358–1371.

Brown, K. N., and I. N. Brown. 1965. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. Nature 208:1286–1288.

Buckling, A., L. C. Ranford-Cartwright, A. Miles, and A. F. Read. 1999. Chloroquine increases *Plasmodium falciparum* gametocytogenesis in vitro. Parasitology 118:339–346.

Carter, R., and D. Walliker. 1975. New observations on malaria parasites of rodents of Central African Republic: *Plasmodium vinckei petteri* subsp. nov. and *Plasmodium chabaudi* Landau, 1965. Annals of Tropical Medicine and Parasitology 69:187–196.

Chang, K. H., M. Tam, and M. M. Stevenson. 2004. Modulation of the course and outcome of blood-stage malaria by erythropoietin-induced reticulocytosis. Journal of Infectious Diseases 189:735–743.

Cromer, D., K. J. Evans, L. Schofield, and M. P. Davenport. 2006. Preferential invasion of reticulocytes during late-stage *Plasmodium berghei* infection accounts for reduced circulating reticulocyte levels. International Journal for Parasitology 36:1389–1397.

de Roode, J. C., A. F. Read, B. H. K. Chan, and M. J. Mackinnon. 2003. Rodent malaria parasites suffer from the presence of conspecific clones in three-clone *Plasmodium chabaudi* infections. Parasitology 127:411–418.

de Roode, J. C., R. Culleton, S. J. Cheesman, R. Carter, and A. F. Read. 2004. Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. Proceedings of the Royal Society B: Biological Sciences 217:1073–1080.

de Roode, J. C., M. E. H. Helinski, M. A. Anwar, and A. F. Read. 2005*a*. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. American Naturalist 166:531–542.

de Roode, J. C., R. Pansini, S. J. Cheesman, M. E. H. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, et al. 2005*b*. Virulence and competitive ability in genetically diverse malaria infections. Proceedings of the National Academy of Sciences of the USA 102:7624–7628.

Eichner, M., H. H. Diebner, L. Molineaux, W. E. Collins, G. M. Jeffery, and K. Dietz. 2001. Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malaria therapy data. Transactions of the Royal Society of Tropical Medicine and Hygiene 95:497–501.

Ganzoni, A., R. S. Hillman, and C. A. Finch. 1969. Maturation of the macroreticulocyte. British Journal of Haematology 16:119–134.

Garnham, P. C. C. 1966. Malaria parasites and other hæmosporidia. Blackwell Scientific, Oxford.

Good, M. F., and D. L. Doolan. 1999. Immune effector mechanisms in malaria. Current Opinion in Immunology 11:412–419.

Grimshaw, S. D., D. G. Whiting, and T. H. Morris. 2001. Likelihood ratio tests for a mixture of two von Mises distributions. Biometrics 57:260–265.

Haydon, D. T., L. Matthews, R. Timms, and N. Colegrave. 2003. Top-down or bottom-up regulation of intra-host blood-stage malaria: do malaria parasites most resemble the dynamics of prey or predator? Proceedings of the Royal Society B: Biological Sciences 270:289–298.

Hellriegel, B. 1992. Modeling the immune response to malaria with ecological concepts: short-term behavior against long-term equilibrium. Proceedings of the Royal Society B: Biological Sciences 250:249–256.

Hetzel, C., and R. M. Anderson. 1996. The within-host cellular dynamics of bloodstage malaria: theoretical and experimental studies. Parasitology 113:25–38.

Hilborn, R., and M. Mangel. 1997. The ecological detective: confronting models with data. Princeton University Press, Princeton, NJ.

Jakeman, G. N., A. Saul, W. L. Hogarth, and W. E. Collins. 1999. Anæmia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology 119:127–133.

Jarra, W., and K. N. Brown. 1989. Protective immunity to malaria: studies with cloned lines of rodent malaria in Cba. Parasite Immunology 11:1–13.

Johnson, J. B., and K. S. Omland. 2004. Model selection in ecology and evolution. Trends in Ecology & Evolution 19:101–108.

Killick-Kendrick, R., and W. Peters. 1978. Rodent malaria. Academic Press, London.

Langhorne, J., B. Simon-Haarhaus, and S. J. Meding. 1990. The role of $CD4^+$ T cells in the protective immune response to *Plasmodium chabaudi* in vivo. Immunology Letters 25:101–108.

Loeffler, M., K. Pantel, H. Wulff, and H. E. Wichmann. 1989. A mathematical model of erythropoiesis in mice and rats. I. Structure of the model. Cell and Tissue Kinetics 22:13–30.

Mackey, M. C. 1997. Mathematical models of hematopoietic cell replication and control. Pages 149–178 *in* H. G. Othmer, F. Adler, M. A. Lewis, and J. Dallon, eds. Case studies in mathematical modeling: ecology, physiology, and cell biology. Prentice Hall, Englewood Cliffs, NJ.

Mary, J. Y., A. J. Valleron, H. Croizat, and E. Frindel. 1980. Mathematical analysis of bone-marrow erythropoiesis: application to C3H mouse data. Blood Cells 6:241–254.

McAlister, R. O. 1977. Time-dependent loss of invasive ability of *Plasmodium berghei* merozoites in vitro. Journal of Parasitology 63:455–463.

McQueen, P. G., and F. E. McKenzie. 2004. Age-structured red blood cell susceptibility and the dynamics of malaria infections. Proceedings of the National Academy of Sciences of the USA 101: 9161–9166.

Molineaux, L., and K. Dietz. 1999. Review of intra-host models of malaria. Parasitologia 41:221–231.

Paul, R. E. L., F. Ariey, and V. Robert. 2003. The evolutionary ecology of *Plasmodium*. Ecology Letters 6:866–880.

Phillips, R. S., L. R. Brannan, P. Balmer, and P. Neuville. 1997. Antigenic variation during malaria infection: the contribution from the murine parasite *Plasmodium chabaudi*. Parasite Immunology 19:427–434.

Pombo, D. J., G. Lawrence, C. Hirunpetcharat, C. Rzepczyk, M. Bryden, N. Cloonan, K. Anderson, et al. 2002. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. Lancet 360:610–617.

Råberg, L., J. C. de Roode, A. S. Bell, P. Stamou, D. Gray, and A. F. Read. 2006. The role of immune-mediated apparent competition in genetically diverse malaria infections. American Naturalist 168: 41–53.

Reece, S. E., A. B. Duncan, S. A. West, and A. F. Read. 2003. Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*. Parasitology 127:419–425.

———. 2005. Host cell preferences and variable transmission strategies in malaria parasites. Proceedings of the Royal Society B: Biological Sciences 272:511–517.

Shutler, D., S. E. Reece, A. Mullie, P. F. Billingsley, and A. F. Read. 2005. Rodent malaria parasites *Plasmodium chabaudi* and *P. vinckei* do not increase their rates of gametocytogenesis in response to

mosquito probing. Proceedings of the Royal Society B: Biological Sciences 272:2397–2402.

Stephens, R., and J. Langhorne. 2006. Priming of CD4(+) T cells and development of CD4(+) T cell memory: lessons for malaria. Parasite Immunology 28:25–30.

Stevenson, M. M., and E. M. Riley. 2004. Innate immunity to malaria. Nature Reviews Immunology 4:169–180.

Taylor, L. H., and A. F. Read. 1997. Why so few transmission stages? reproductive restraint by malaria parasites. Parasitology Today 13: 135–140.

Taylor-Robinson, A. W., and R. S. Phillips. 1994. Predominance of infected reticulocytes in the peripheral-blood of CD4(+) T-cell-depleted mice chronically infected with *Plasmodium chabaudi chabaudi*. Parasitology Research 80:614–619.

Timms, R., N. Colegrave, B. H. K. Chan, and A. F. Read. 2001. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitology 123:1–11.

Urban, B. C., R. Ing, and A. M. Stevenson. 2005. Early interactions between blood-stage *Plasmodium* parasites and the immune system. Current Topics in Microbiology and Immunology 297:25–70.

van Putten, L. M. 1958. The life span of red cells in the rat and the mouse as determined by labeling with Dfp32 in vivo. Blood 13: 789–794.

WHO and UNICEF. 2005. World malaria report 2005. http://rbm.who.int/wmr2005/index.html. Accessed November 2007.

Wiczling, P., and W. Krzyzanski. 2007. Method of determination of the reticulocyte age distribution from flow cytometry count by a structured-population model. Cytometry 71A:460–467.

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