Blockade of TNF receptor 1 reduces disease severity but increases parasite transmission during *Plasmodium chabaudi chabaudi* infection

Gráinne H. Long [a,b,*], Brian H.K. Chan [a], Judith E. Allen [a], Andrew F. Read [b], Andrea L. Graham [a]

[a] *Institutes of Evolution, Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JT, Scotland, UK*
[b] *Center for Infectious Disease Dynamics, The Pennsylvania State University, University Park, PA 16802, USA*

Abstract

Reducing host carriage of transmission-stage malaria parasites (gametocytes) is expected to decrease the population-wide burden of malaria. Some malaria disease severity is attributed to the induction of the pro-inflammatory cytokines TNF-α and lymphotoxin-alpha (LT-α), and we are interested in whether anti-malaria interventions which ameliorate the symptoms induced by those cytokines may have the capacity to alter malaria transmission. As many functions of TNF-α and LT-α are exerted through TNF receptor 1 (TNFR1), we investigated the effect TNFR1 blockade exerted on parasite transmission using the rodent malaria *Plasmodium chabaudi chabaudi*. We found that blocking TNFR1 simultaneously increased gametocyte density and infectivity to mosquitoes, whilst reducing disease severity (weight loss). These transmission-enhancing and severity-reducing effects of TNFR1 blockade were independent of asexual parasite load and were observed for several *P. c. chabaudi* genotypes. These results suggest that the effects of candidate malaria interventions on infectivity should be examined alongside effects on disease severity so that the epidemiological consequences of such interventions can be evaluated.
© 2007 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Malaria; Virulence; Gametocytes; Transmission; Immunopathology; Anti-disease; Disease ecology

1. Introduction

Malaria disease severity has both parasite- (e.g., Cross and Langhorne, 1998; Mackinnon and Read, 2004) and immune-mediated (immunopathology) (e.g., Omer and Riley, 1998; Dodoo et al., 2002; Torre et al., 2002; Raberg et al., 2006) components. Clinical protection against severe malaria can therefore be achieved either indirectly, by reducing parasite burden (anti-parasite treatments) (Good, 2005; Good et al., 2005), or directly, by targeting the disease symptoms themselves (anti-disease treatments) (Schofield et al., 2002; Schofield and Mueller, 2006). As the evolutionary success of a parasite is ultimately determined by successful transmission, and disease epidemiology by the infectiousness of individual cases, we are interested in the effects anti-malaria treatments may exert on parasite transmission.

The pro-inflammatory cytokine TNF-α, for example, has dual roles during malaria infection; it has protective anti-parasitic properties that can reduce parasite load (Langhorne et al., 1989; Stevenson et al., 1995; Jacobs et al., 1996; Sam et al., 1999), but unregulated or excessive levels contribute to malaria disease severity (Grau et al., 1987; Clark and Chaudhri, 1988a, 1988b; Kern et al.,

* Corresponding author. Tel.: +44 131 650 5484; fax: +44 131 650 6564.
E-mail address: grainne.h.long@gmail.com (G.H. Long).

1992). Thus, optimal control of malaria infection requires a balanced immune response (Dodoo et al., 2002; Artavanis-Tsakonas et al., 2003). Our laboratory has recently demonstrated that TNFR1 blockade ameliorated the weight loss induced during *Plasmodium chabaudi chabaudi* infection in resistant C57BL/6 mice, but had negligible effects on asexual parasite density (Long et al., 2006). Given that disease severity was decreased despite there being no change in asexual parasite density, it raises the question of what effect TNFR1 blockade may have on parasite transmission. Can anti-malaria interventions which aim to reduce immunopathology, for example, inadvertently affect gametocyte biology and alter the transmission efficiency of malaria?

Compared with the wealth of knowledge regarding the immune response directed against asexual malaria parasites (Good et al., 2005), surprisingly little is known about how host immunity affects gametocytes. The interaction of host serum TNF-α and related cytokines with peripheral blood leucocytes is associated with a substantial loss of gametocyte infectivity to mosquitoes (Naotunne et al., 1991; Karunaweera et al., 1992). This has been attributed to the nitric oxide-dependent inactivation of intracellular gametocytes (Motard et al., 1993; Naotunne et al., 1993). Although a role for TNF-α in targeting gametocytes and reducing their infectiousness to mosquitoes is supported by studies such as these, the exact mode of anti-gametocyte activity, as well as the identity of the downstream effectors, are currently unknown.

In this study, the *P. c. chabaudi* rodent model of malaria infection was used to investigate the effects of in vivo TNF-α and lymphotoxin-alpha (LT-α) – through the administration of a TNF-α receptor fusion protein (TNFR-Ig) – on parasite transmission. Firstly, we investigated the effect of blocking TNFR1 on gametocyte and asexual parasite densities, the infectivity of gametocytes to mosquitoes and disease severity (in terms of weight and red blood cell (RBC) loss). Second, we used a panel of four genetically distinct *P. c. chabaudi* clones known to differ in the disease severity they induce (Mackinnon and Read, 1999a; Long et al., 2006), to determine whether the effects of TNFR1 blockade on gametocyte density are clone-specific. We found evidence that blocking TNFR1 can simultaneously increase the transmission potential and reduce the disease severity, across TNFR-Ig doses and malaria genotypes.

2. Materials and methods

2.1. Hosts and parasites

Both experiments used 6–8-week-old female inbred C57BL/6 mice (bred in-house). We used the rodent malaria, *P. c. chabaudi* (Landau, 1965), because it is one of the best laboratory models for understanding parasite genetic variation in malaria virulence and transmission (Mackinnon and Read, 1999b, 2003; Gandon et al., 2001; Mackinnon et al., 2002) and the molecular basis of innate immunity to malaria infection (Li et al., 2001; Langhorne

et al., 2002; Stevenson and Riley, 2004a). Clones were derived from thicket rat isolates and stored as frozen stabilities (Walliker et al., 1971), with subscript codes denoting their exact clonal history; clone AJ_{4992} was used in experiment one and clones CW_{640}, AS_{11915}, BC_{200} and AJ_{4762} (herein referred to as CW, AS, BC and AJ) in experiment two. Mice were inoculated with 10^6 parasitised RBCs and housed as described previously (Long et al., 2006) and all procedures were carried out according to UK Home Office guidelines.

2.2. Experimental design

TNFR-Ig (kindly provided by the Therapeutic Antibody Centre, University of Oxford, UK) targets and blocks TNFR1 in vivo (Dick et al., 1996). As detailed previously (Long et al., 2006), mice were administered either TNFR-Ig or a human IgG1 isotype control (hu-IgG; Sigma) every morning from days 5 through 8 p.i., before daily disease severity and parasite measurements were collected, as described below. In experiment one, the effect of TNFR-Ig dose on *P. c. chabaudi* AJ infection dynamics was investigated by administrating three different doses of TNFR-Ig or hu-IgG: 19, 38 or 75 µg (four mice per dose, per treatment). In experiment two, the effect of TNFR1 blockade on four genetically distinct *P. c. chabaudi* clones was investigated. Single-clone infections of these four distinct clones were initiated and, based on the results of experiment one, mice received 75 µg of either TNFR-Ig or hu-IgG (five mice per clone, per treatment).

2.3. Monitoring parasitaemia, gametocytaemia and disease severity

Thin blood smears obtained daily from the tail vein were Giemsa stained and microscopically analysed (1,000× magnification) to determine the proportion of RBCs parasitised with gametocytes (gametocytaemia), asexual parasites (parasitaemia) and the proportion of RBCs that were immature (RBC precursors), which was comprised of both erythroblasts and reticulocytes. For 14 days p.i., RBC densities were obtained by flow cytometry (Beckmann Coulter) by standard methods (Long et al., 2006). We calculated asexual parasite, gametocyte and RBC precursor densities (per ml of blood) by multiplying each corresponding proportion by daily RBC densities. Mouse weights were recorded to the nearest 0.1 g on days 0–14 p.i.

2.4. Mosquito transmission

In experiment one, mice receiving the highest antibody dose (75 µg) were used to investigate the relationship between TNFR1 blockade and infectivity to mosquitoes. Mosquito feeds took place 14 days after mouse malaria infection. Groups of 4–5-days-old female *Anopheles stephensi* mosquitoes were arranged in mesh-covered pots and each group fed on an anesthetised mouse (three mice per

treatment group and a mean of 62 mosquitoes per pot), as described previously (Ferguson et al., 2003). Mosquitoes which had not fed were removed from pots and the remainder maintained at 25–30 °C, 70–80% humidity with 12:12 h light–dark cycle and fed 5% glucose, 0.05% *para*-aminobenzoic acid (PABA) solution ad libitum. On days 8 and 9 post blood-feeding, mosquitoes were dissected to assess infection status by counting the number of oocyst in each infected mosquito midgut.

2.5. Trait definition

In both experiments, parasite and gametocyte load were quantified as the cumulative parasite and gametocyte densities produced throughout the infection (days 5–14 p.i. inclusive). In experiment one, the proportion of asexual parasites that produced gametocytes on days 6, 8, 10 and 12 p.i. was estimated using a simple model of exponential growth as described previously (Buckling et al., 1999) and the 'average gametocyte conversion rate' was calculated over this time and used as an estimate of gametocytogenesis. For measures of disease severity, the 'average weight loss' or 'average loss in RBC density' over the course of the primary infection was calculated for each mouse. Previous experiments from our laboratory show weight loss correlates with other measures of malaria-induced morbidity – including anaemia and temperature loss – and is an important surrogate predictor for death (e.g., Mackinnon and Read, 2004). In experiment one, 'average RBC precursor density' was calculated. Prior to statistical analysis, the following transformations were applied to meet the necessary normality and homogeneity-of-variance assumptions: square root (asexual parasite density) and log_{10} (weight, RBC density, gametocyte density and gametocyte conversion rate).

2.6. Statistical analysis

Data were analysed using ANOVAs and ANCOVAs in Minitab (v. 13.30, Minitab Inc.). In experiment one, the explanatory variables used were antibody 'treatment' (TNFR-Ig or hu-IgG) and 'dose' (19, 38 or 75 µg). In experiment two, the explanatory variables used were antibody 'treatment' (TNFR-Ig or hu-IgG) and infecting *P. c. chabaudi* 'clone' (CW, AS, BC or AJ). Mice that died before either the disease severity or parasite load had peaked were removed from the analyses (two TNFR-Ig and two IgG-treated mice in experiment one; and one TNFR-Ig-treated BC-infected mouse and two IgG-treated AJ-infected mice in experiment two). For disease severity analyses, a covariate for either starting weight or RBC density (average days 0 and 1 p.i.) was fitted to control for the mouse's initial values. To determine whether treatment, dose or clone had effects on disease severity and gametocytes beyond those exerted by asexual parasite load, data were analysed with and without asexual parasite load as a covariate. The proportion of mosquitoes infected with parasites was analysed using binomial logistic regression, blocked by mouse to avoid pseudo-replication. The maximal model was fit to the data including, when relevant, covariate, treatment, dose or clone, and an interaction between treatment and dose or clone. The cut-off for significance was $P < 0.05$. For clarity, only significant explanatory variables are reported here.

3. Results

3.1. Malaria-induced weight loss was ameliorated when TNFR1 was blocked, independent of both dose and asexual parasite load

The role TNF-α plays in contributing to malaria disease severity is well established for *P. c. chabaudi* AS infection (Langhorne et al., 2004; Stevenson and Riley, 2004b). In this study we have confirmed malaria disease severity was similarly reduced by blocking TNFR1 during infection with the more virulent *P. c. chabaudi* clone AJ. Following infection with 10^6 *P. c. chabaudi* AJ-infected RBCs, C57BL/6 mice were administered TNFR-Ig on days 5 to 8 p.i. inclusive. Data were statistically analysed to assess whether changes in disease severity – quantified in terms of loss of weight and RBC density, with the initial conditions of each mouse taken into account – could be explained by TNFR-Ig treatment or antibody dose. Inclusion of asexual parasite load into these statistical models allowed us to determine whether changes in asexual density were responsible for driving TNFR-Ig-mediated effects on disease severity.

TNFR1 blockade protected against average AJ-induced weight loss (Fig. 1A; treatment: $F_{1,15} = 4.5$, $P = 0.05$. Our choice of average in these analyses is justified in Section 2). Although TNFR-Ig treatment did not significantly affect the average density of RBCs (Fig. 1B), average RBC precursor densities were higher in the TNFR-Ig-treated group (Fig. 1C; treatment: $F_{1,15} = 5.35$, $P = .035$). The dose of treatment did not significantly affect live weight, RBC or reticulocyte density (thus, although dose was always controlled for in our statistical analyses, virulence data are pooled by dose in our figures for pictorial ease; Fig. 1A, B and C). Controlling for asexual parasitaemia did not alter any of these conclusions. Thus, TNFR1 affects malaria-induced weight loss, but not RBC loss, independently of asexual parasite load.

3.2. TNFR1 blockade increased the density of malaria gametocytes, independent of asexual parasite load

Next, we investigated the effect of TNFR1 blockade on malaria transmission potential during infections with virulent *P. c. chabaudi* clone AJ. Data were statistically analysed to assess whether changes in cumulative gametocyte density could be explained by TNFR-Ig treatment or antibody dose. Inclusion of asexual parasite or RBC precursor load into these statistical models allowed us to determine
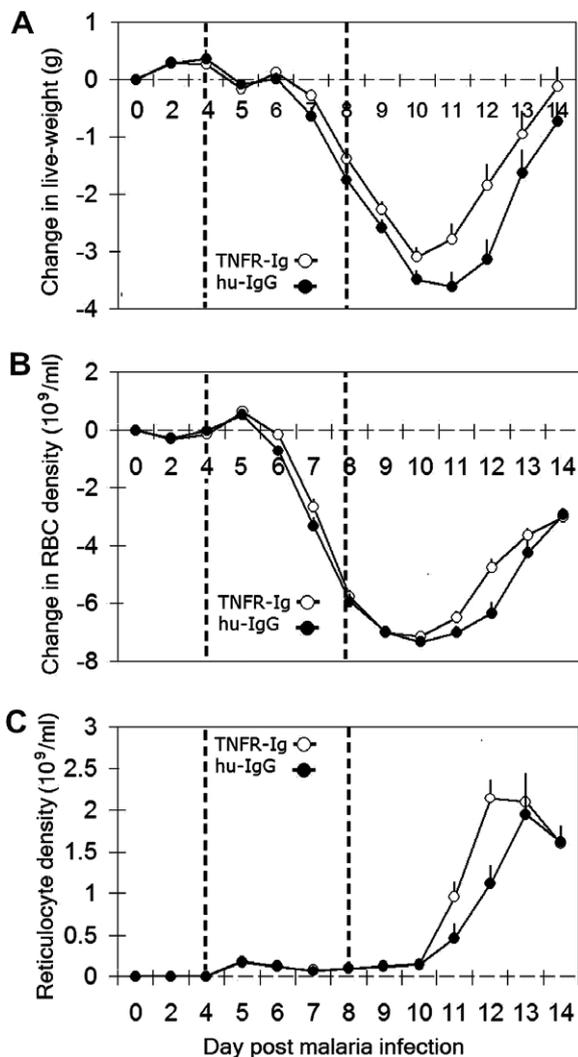
Fig. 1. Effect of tumour necrosis factor receptor (TNFR)-Ig treatment on loss of body mass, anaemia and red blood cell (RBC) precursor density in malaria-infected mice. Plots represent the change in live weight (A), RBC density (B) or RBC precursor density (C) induced by infection with virulent *Plasmodium chabaudi chabaudi* clone AJ in TNFR-Ig treated mice (open symbols) or control IgG-treated mice (solid symbols). Treatment dose, although always controlled for statistically in our analyses, did not significantly affect any virulence parameter measured. Therefore virulence data are pooled by dose in this figure for pictorial ease. Each line represents the mean of 16 mice (±SEM), except where deaths occurred as noted in Section 2. Dotted lines represent periods of antibody administration. hu-IgG, human IgG1 isotype control.

whether changes in these parameters were responsible for driving TNFR-Ig-mediated effects on gametocyte load. Finally, we used a simple model (Buckling et al., 1999) to calculate the rate of gametocytogenesis on days 6 to 12 p.i. inclusive, allowing us to explore whether an increased commitment of asexual parasite to gametocytes was responsible for changes in gametocyte load.

TNFR-Ig treatment significantly increased the cumulative gametocyte density reached during infection (Fig. 2A; treatment: $F_{1, 15} = 10.96$, $P = 0.005$). In addition, gametocyte densities were greatest in mice receiving the highest antibody dose, regardless of antibody type (Fig. 2A; dose: $F_{2, 15} = 14.52$, $P = 0.002$). That the statistically significant effect of TNFR-Ig on the cumulative gametocyte density was observed controlling for antibody dose, confirmed that treatment effects did not depend on dose. However, neither TNFR-Ig treatment nor the dose of TNFR-Ig administered significantly affected asexual parasites (Fig. 2B). In addition, controlling for average asexual parasite or RBC precursor load did not alter the conclusion that TNFR-Ig increased gametocyte load. Finally, the average rate of gametocytogenesis was not affected by TNFR1 blockade (data not shown), suggesting that the increase in gametocytes observed during TNFR-Ig treatment was not due to an increased investment in gametocytes. Thus, the TNFR-Ig-driven increase in cumulative gametocyte density observed here is most consistent with a direct gametocidal role for TNF-α or LT-α in vivo.

3.3. The infectivity to mosquitoes was increased by TNFR1 blockade

Since mice receiving the highest antibody dose (75 μg) had the highest gametocyte densities – which is expected to maximise chances of transmission to mosquitoes (Buckling et al., 1997; Taylor and Read, 1998; Mackinnon and Read, 1999a) – this cohort was chosen to investigate the effect of TNFR1 blockade on malaria infectivity. This provided a stringent test of whether TNFR1 blockade drove changes in transmissibility, rather than merely gametocyte density. Blocking TNFR1 significantly increased the proportion of mosquitoes infected, with TNFR-Ig-treated mice 2.5 times more likely to infect mosquitoes than control mice (Fig. 3; 95% Confidence Interval (C.I.) for odds ratio 2.5: 1.1–5.7, $P = 0.03$). When gametocyte density on the day of the mosquito feed was controlled for statistically, TNFR-Ig-treated mice still supported greater transmission to mosquitoes. These data suggest increased mosquito infectivity of gametocytes in TNFR-Ig treated mice.

3.4. The TNFR-Ig driven increase in transmission potential was observed across several malaria genotypes

We were interested in whether the transmission potential of distinct parasite genotypes is differentially affected by TNFR1 blockade. After infection with 10^6 parasites from one of four genetically distinct *P. c. chabaudi* clones, C57BL/6 mice were treated with TNFR-Ig on days 5–8 p.i. As in experiment one, and now across genetically distinct *P. c. chabaudi* infections, TNFR-Ig-treated mice had higher gametocyte densities compared with control mice (Fig. 4; treatment: $F_{1, 29} = 4.5$, $P = 0.042$, clone: $F_{3, 29} = 3.02$, $P = 0.046$). Although the clones appeared to vary in their response to TNFR-Ig treatment – for example, a TNFR-Ig driven increase in gametocytes is evident for clone CW, but not for clone AS (Fig. 4) – there is no evidence to suggest that this clone to clone variation is
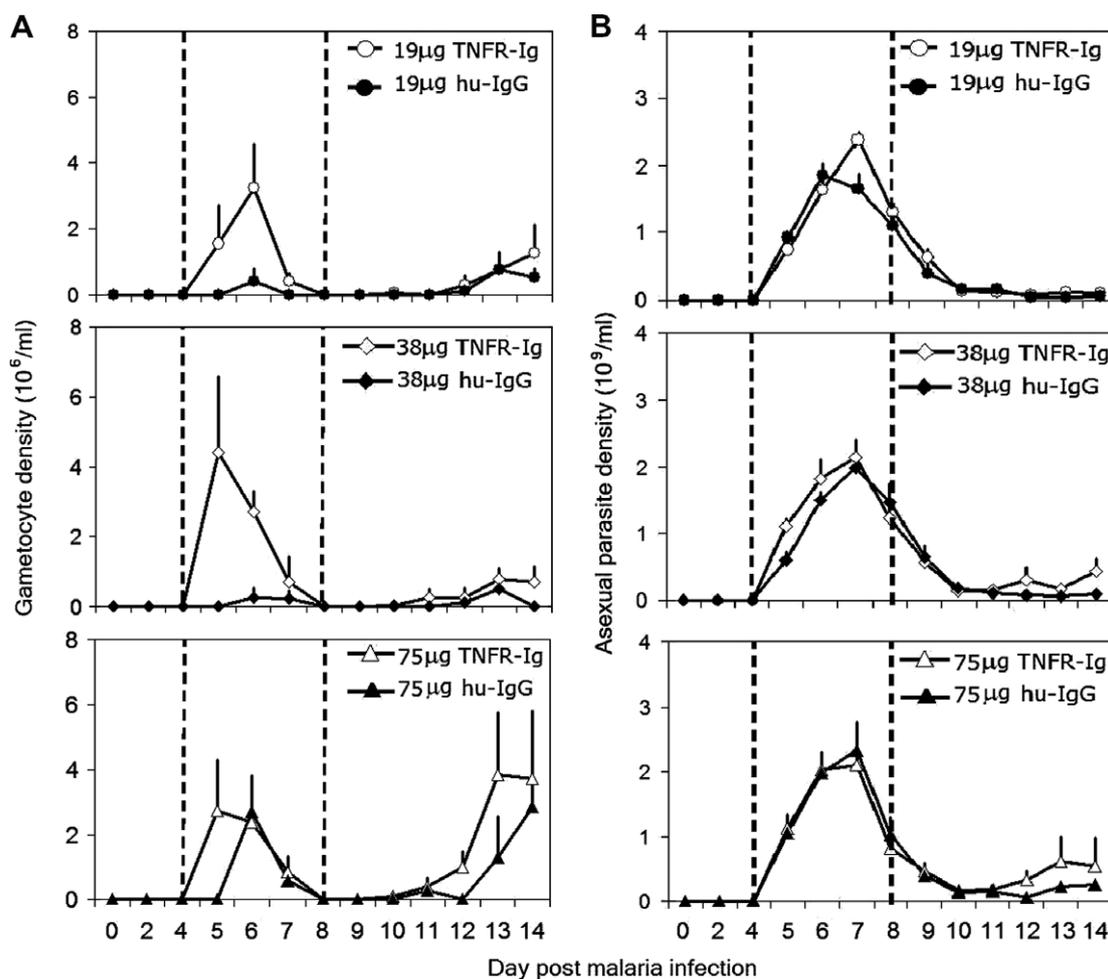
Fig. 2. Effect of tumour necrosis factor receptor (TNFR)-Ig treatment and dose on the kinetics of gametocyte and asexual parasite density. Plots represent the kinetics of gametocyte density (A) and asexual parasite density (B) during infection with virulent *Plasmodium chabaudi chabaudu* clone AJ in TNFR-Ig-treated mice (open symbols) or control IgG-treated mice (solid symbols). Three different doses of antibody were used (19, 38 and 75 μg) and dotted lines represent periods of antibody administration. Each line represents the mean of 5 mice (±SEM), except where deaths occurred as noted in Section 2. hu-IgG, human IgG1 isotype control.
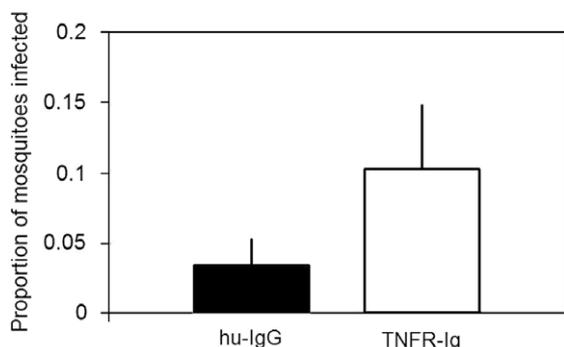

Fig. 3. Effect of tumour necrosis factor receptor (TNFR)-Ig treatment on transmission of the *Plasmodium chabaudi chabaudi* clone AJ to mosquitoes. Bars represent the proportion of mosquitoes infected after feeding on AJ-infected mice, treated with either 75 μg of control IgG (solid bar) or TNFR-Ig (open bar). Means are based on six mice from which totals of 217 (hu-IgG) and 185 (TNFR-Ig) mosquitoes took a blood meal (±95% Confidence Interval). Infection with oocysts of clone AJ was assessed by microscopic dissection of the mosquito midgut. hu-IgG, human IgG1 isotype control.

more than we would expect by chance alone (clone × treatment: $F_{3, 29} = 1.01$, $P = 0.40$). Regardless of parasite clone, TNFR-Ig-treated mice produced on average 44% more gametocytes than control mice. Again, TNFR-Ig treatment did not significantly affect asexual parasite density (Long et al., 2006), and controlling for asexual parasite load did not alter either clone or treatment effects on gametocyte density. TNFR1 blockade ameliorated the malaria-induced weight loss, but not RBC loss, regardless of asexual parasite load or parasite genotype (Long et al., 2006). Thus, across several *P. c. chabaudi* clones, TNFR1 blockade simultaneously increased the density of gametocytes and protected against malaria-induced weight loss, independently of asexual parasite load.

In summary, although there were quantitative differences between the experiments in terms of the average gametocyte density and loss in body weight, in both experiments TNFR1 blockade was associated with increased gametocyte density and protection against weight loss (Table 1). Thus, in vivo TNFR1 blockade simultaneously
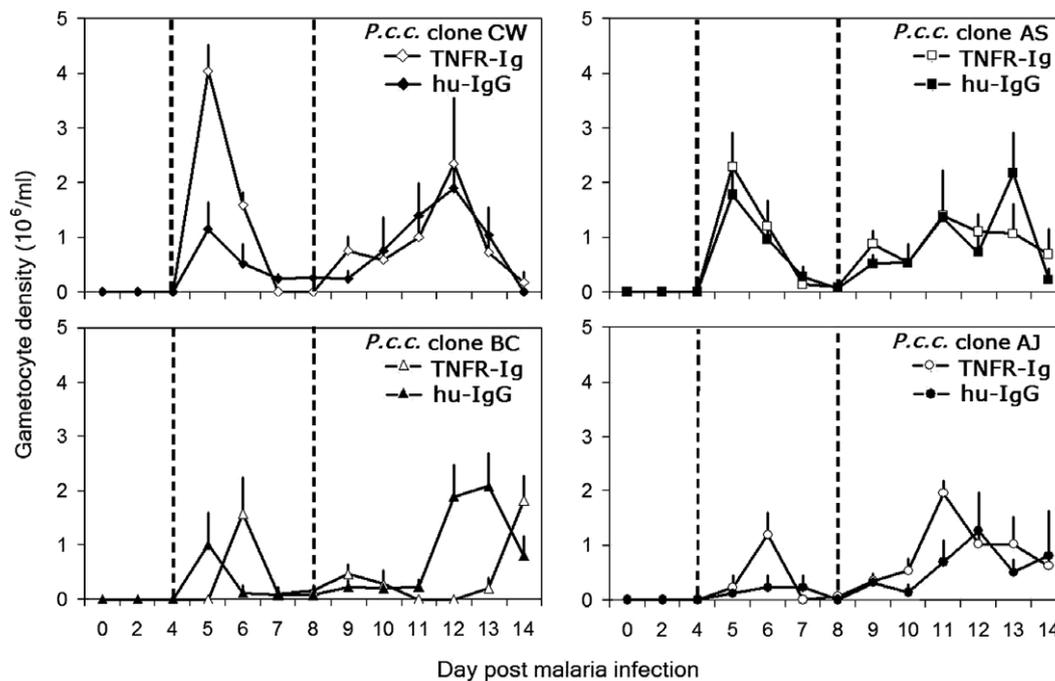
Fig. 4. Effect of tumour necrosis factor receptor (TNFR)-Ig treatment and parasite clone on the kinetics of gametocyte density. Plots represent the kinetics of gametocyte density during infection with one of four *Plasmodium chabaudi chabaudi* clones (CW, AS, BC or AJ) in TNFR-Ig-treated mice (open symbols) or control IgG-treated mice (solid symbols) and dotted lines represent periods of antibody administration. Each line represents the mean of five mice (±SEM), except where deaths occurred as noted in Section 2. hu-IgG, human IgG1 isotype control.

Table 1
The effect of TNF receptor 1 (TNFR1) blockade on the average gametocyte density ($\times 10^6$/ml) and average loss in live weight (g) during *Plasmodium chabaudi chabaudi* infection

Experiment and trait	TNFR-Ig means ± SE	Control means ± SE	Significance of treatment effects
Experiment 1			
Gametocyte density	1.13 ± 0.13	0.30 ± 0.14	$F_{1,15} = 10.96$, $p = 0.005$
Body weight lost	0.98 ± 0.19	1.60 ± 0.21	$F_{1,15} = 4.5$, $p = 0.05$
Experiment 2			
Gametocyte density	0.82 ± 0.08	0.67 ± 0.08	$F_{1,29} = 4.5$, $p = 0.042$
Body weight lost	0.24 ± 0.18	0.94 ± 0.19	$F_{1,28} = 7$, $p = 0.01$

Data are least-squares means ± standard errors (SE).

increased gametocyte density and reduced weight loss across doses of TNFR-Ig (Fig. 2A) and clones of *P. c. chabaudi* (Fig. 4).

4. Discussion

The disease severity caused by *P. c. chabaudi* infection has both parasite- (Cross and Langhorne, 1998) and immune-mediated components (Li et al., 1999, 2003; Clark et al., 2004). Here, we evaluated the effect an anti-TNFR1-based immunotherapy (TNFR-Ig) exerts on the transmission potential and disease severity of *P. c. chabaudi* infection. We showed that administering TNFR-Ig early during primary infection protected against weight loss (Fig. 1A). Despite the lack of protection against malaria-induced anaemia (Fig. 1B), TNFR1 blockade significantly increased RBC precursor burdens from day 10 p.i. onwards (Fig. 1C), consistent with the proposed inhibitory role for TNF-α on erythropoeisis (Johnson et al., 1989). Importantly, we found that TNFR1 blockade significantly increased the cumulative gametocyte density (Figs. 2A and 4) and infectivity to mosquitoes (Fig. 3), independently of asexual parasite load. Indeed, asexual parasite load was not significantly affected by TNFR-Ig (Fig. 2B), consistent with a major role for TNFR1-independent factors in controlling asexual parasite burdens (Jacobs et al., 1996; Li and Langhorne, 2000; Long et al., 2006). These data suggest that during *P. c. chabaudi* infection, therapies which block TNFR1 may have antagonistic effects on ameliorating malaria disease burden, reducing some infection-induced pathologies while increasing parasite transmission.

A public health goal of malaria treatments is to reduce the infectious reservoir. Although the biological details could be quite different in human malaria, we have shown here that in at least one malaria system, anti-cytokine receptor therapy can enhance malaria transmission potential while alleviating symptoms. We suggest there is a strong case that, in clinical trials, the effect of candidate interventions on infectivity be examined alongside effects on disease severity so that treatment efficacy can be more fully evaluated – i.e., at the population as well as the individual patient levels. In a recent field study, careful monitoring of the efficacy of intermittent anti-parasitic

preventive treatment revealed that intervention significantly decreased both asexual and gametocyte prevalence, as well as the incidence of anaemia, compared with placebo control groups (Schellenberg et al., 2005). That particular anti-parasite treatment thus benefited both the individual and the population. Indeed, in principle, anti-parasite treatments such as the cinchona alkaloids quinine and quinidine or conventional vaccines which are targeted at killing the parasite or reducing its growth rate, pose relatively little risk of leading to increased transmission (Gandon et al., 2001; Mackinnon et al., in press). This is because, by reducing the pool of asexual parasites from which gametocytes are derived, anti-parasite treatments are expected to greatly reduce both disease severity and transmission potential. We would encourage studies to test this prediction by routinely monitoring gametocytes following anti-parasite clinical interventions, particularly those expected to alter immune response induction. In contrast, because anti-disease therapies are thought to protect the host without necessarily killing the parasite, their effects on malaria transmission are far from clear. Thus, the case for monitoring gametocyte biology is particularly strong for interventions which target symptoms rather than parasites (Gandon et al., 2001; Mackinnon et al., in press) with anti-toxin vaccines (Schofield, 2002) potentially falling into this category. Indeed, anti-glycosylphosphatidylinositol (GPI) vaccination ameliorated the virulence and mortality induced by *Plasmodium berghei* infection (Schofield et al., 2002), but its effects on transmission stage parasites have yet to be determined. Just as individuals with reduced immune competence may act as reservoirs for gametocytes (Abu-Raddad et al., 2006) so too might individuals treated with anti-cytokine, anti-cytokine receptor, or other anti-disease therapies.

The increased gametocyte density and mosquito infectivity we observed during TNFR-Ig treatment may be caused by several mechanisms. An increase in the proportion of asexual parasites becoming committed to the sexual stage might drive increases in lifetime transmission potential. However, as the commitment of asexual parasites to gametocytes and development to full maturity takes 24–48 h during rodent malaria infections (Carter and Walliker, 1975), the rapidity with which TNFR1 blockade caused gametocyte densities in both experiments to increase – levels were elevated within 4 h of TNFR-Ig treatment (Figs. 2A and 4) – rules out gametocyte conversion being the main causal factor driving increased gametocyte load. In support of this, neither asexual parasite nor RBC precursor density (Trager et al., 1999) helped to explain gametocyte load in our statistical models and no difference in gametocyte conversion rate was found between treatments. Instead, we propose that protection from anti-gametocyte activity (Naotunne et al., 1991, 1993) explains the TNFR-Ig-driven increase in gametocyte density and infectivity. Gametocyte-specific immune responses during *Plasmodium falciparum* infection have been implicated in regulating gametocyte density, independently of asexual

stage immunity (Baird et al., 1991) and our data are consistent with TNF-α or LT-α being directly gametocidal in vivo; however, this was not directly tested.

As with all models, there are caveats with our experimental system. For example, our treatment represented the addition of an extrinsic molecule – albeit one currently in use for autoimmune conditions (Doan et al., 2006; Woolacott et al., 2006) – which may avoid normal homoeostatic regulation. Therefore, exactly how our treatment relates to, for example, vaccine-induced changes in immunity have yet to be elucidated. In addition, TNFR-Ig does not distinguish between TNF-α and the closely related cytokine LT-α, and as both cytokines can signal through TNFR1, we have been careful to attribute treatment effects in these experiments to TNFR1 blockade. Recent findings support distinct roles for these cytokines during malaria infection; LT-α, and not TNF-α, is thought to play a more important role in driving severe disease during *P. berghei* infection (Engwerda et al., 2002) and in regulating early immune responses against *P. chabaudi* parasites (Clark et al., 2007). These data suggest that TNF-α may not be on the critical path for toxin-mediated pathogenesis or immune regulation in these particular model systems. It would be very interesting to determine whether these cytokines possess different anti-gametocytic properties in vivo during infection with *P. c. chabaudi* as well as other malarias. LT-α appears to be particularly associated with T-cell-mediated immunity (McCarthy et al., 2006); therefore immune responses induced by anti-parasite vaccines – particularly if T-cells are skewed away from LT-α production – may have similar effects on gametocytes to those documented here. Finally, we did not distinguish the form or the cellular source of TNF-α/LT-α affected by our experimental manipulation; macrophages (Stevenson et al., 1992), dendritic cells (Seixas et al., 2001) and CD4^{+} T-cells (Hirunpetcharat et al., 1999) have been shown to produce TNF-α during malaria infection and we would encourage future experiments aimed at deducing the cellular source of these cytokines. Regardless of the exact mechanism, the TNFR-Ig-driven increase in gametocyte load and infectivity to mosquitoes strongly suggests a TNFR1-dependent gametocidal pathway exists in vivo and highlights the need for a more complete understanding of gametocyte biology in the context of the mammalian immune system.

We suggest that any benefit afforded by anti-disease treatments that reduce malaria-induced immunopathology or influence T-cell priming must be evaluated with respect to their effect on transmission-stage parasites. Indeed, measuring gametocyte carriage rates should probably be routine for malaria interventions planned for use in the wider community (for example, during trials of vaccines (Mackinnon and Read, 2004) or drugs). This would enable careful consideration of whether the benefits associated with wide-spread treatments (reduced clinical symptoms), outweigh any detrimental side-effects associated with their application (increased gametocyte carriage). Routine monitoring of how such therapies affect gametocyte density as

well as disease severity would allow more informed decisions to be made regarding the wide-spread application of putative anti-malaria drugs. Another benefit of routine gametocyte monitoring may be sensitive early warning of emerging drug resistance (Hallett et al., 2006). The transmission consequences of interventions aimed at blocking immunopathology in other infectious (Aslam et al., 2005; Holmgren et al., 2005) and non-infectious (Crum et al., 2005) contexts should be similarly evaluated.

Acknowledgements

References

Abu-Raddad, L.J., Patnaik, P., Kublin, J.G., 2006. Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa. Science 314, 1603–1606.

Artavanis-Tsakonas, K., Tongren, J.E., Riley, E.M., 2003. The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. Clin. Exp. Immunol. 133, 145–152.

Aslam, S., Hamill, R.J., Musher, D.M., 2005. Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies. Lancet Infect. Dis. 5, 549–557.

Baird, J.K., Jones, T.R., Masbar, P.S., Ratiwayanto, S., Leksana, B., 1991. Evidence for specific suppression of gametocytemia by *Plasmodium falciparum* in residents of hyperendemic Irian Jaya. Am. J. Trop. Med. Hyg. 44, 183–190.

Buckling, A., Crooks, L., Read, A., 1999. *Plasmodium chabaudi*: effect of antimalarial drugs on gametocytogenesis. Exp. Parasitol. 93, 45–54.

Buckling, A.G.J., Taylor, L.H., Carlton, J.M.R., Read, A.F., 1997. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. Philos. Trans. Roy. Soc. Lond. B Biol. Sci. 264, 553–559.

Carter, R., Walliker, D., 1975. New observations on the malaria parasites of rodents of the Central African Republic – *Plasmodium vinckei petteri* subsp. nov. and *Plasmodium chabaudi* Landau, 1965. Ann. Trop. Med. Parasitol. 69, 187–196.

Clark, I.A., Chaudhri, G., 1988a. The balance of useful and harmful effects of TNF, with special reference to malaria. Ann. Inst. Pasteur Immunol. 139, 305–306.

Clark, I.A., Chaudhri, G., 1988b. Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. Br. J. Haematol. 70, 99–103.

Clark, I.A., Alleva, L.M., Mills, A.C., Cowden, W.B., 2004. Pathogenesis of malaria and clinically similar conditions. Clin. Microbiol. Rev. 17, 509–539.

Clark, I.A., Kulk, N., Amante, A., Haque, A., Engwerda, C.R., 2007. Lymphotoxin alpha and tumour necrosis factor are not required for control of parasite growth, but differentially regulate cytokine production during *Plamodium chabaudi chabaudi* AS infection. Parasite Immunol. 29, 153–158.

Cross, C.E., Langhorne, J., 1998. *Plasmodium chabaudi chabaudi* (AS): inflammatory cytokines and pathology in an erythrocytic-stage infection in mice. Exp. Parasitol. 90, 220–229.

Crum, N.F., Lederman, E.R., Wallace, M.R., 2005. Infections associated with tumor necrosis factor-alpha antagonists. Medicine (Baltimore) 84, 291–302.

Dick, A.D., McMenamin, P.G., Korner, H., Scallon, B.J., Ghrayeb, J., Forrester, J.V., Sedgwick, J.D., 1996. Inhibition of tumor necrosis factor activity minimizes target organ damage in experimental autoimmune uveoretinitis despite quantitatively normal activated T cell traffic to the retina. Eur. J. Immunol. 26, 1018–1025.

Doan, Q.V., Chiou, C.F., Dubois, R.W., 2006. Review of eight pharmacoeconomic studies of the value of biologic DMARDs (adalimumab, etanercept, and infliximab) in the management of rheumatoid arthritis. J. Manag. Care Pharm. 12, 555–569.

Dodoo, D., Omer, F.M., Todd, J., Akanmori, B.D., Koram, K.A., Riley, E.M., 2002. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. J. Infect. Dis. 185, 971–979.

Engwerda, C.R., Mynott, T.L., Sawhney, S., De Souza, J.B., Bickle, Q.D., Kaye, P.M., 2002. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J. Exp. Med. 195, 1371–1377.

Ferguson, H.M., Mackinnon, M.J., Chan, B.H., Read, A.F., 2003. Mosquito mortality and the evolution of malaria virulence. Evolution Int. J. Org. Evolution 57, 2792–2804.

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

Good, M.F., 2005. Vaccine-induced immunity to malaria parasites and the need for novel strategies. Trends Parasitol. 21, 29–34.

Good, M.F., Xu, H., Wykes, M., Engwerda, C.R., 2005. Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. Annu. Rev. Immunol. 23, 69–99.

Grau, G.E., Fajardo, L.F., Piguet, P.F., Allet, B., Lambert, P.H., Vassalli, P., 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science 237, 1210–1212.

Hallett, R.L., Dunyo, S., Ord, R., Jawara, M., Pinder, M., Randall, A., Alloueche, A., Walraven, G., Targett, G.A., Alexander, N., Sutherland, C.J., 2006. Chloroquine/sulphadoxine–pyrimethamine for gambian children with malaria: transmission to mosquitoes of multidrug-resistant *Plasmodium falciparum*. PLoS Clin. Trials 1, e15.

Hirunpetcharat, C., Finkelman, F., Clark, I.A., Good, M.F., 1999. Malaria parasite-specific Th1-like T cells simultaneously reduce parasitemia and promote disease. Parasite Immunol. 21, 319–329.

Holmgren, J., Adamsson, J., Anjuere, F., Clemens, J., Czerkinsky, C., Eriksson, K., Flach, C.F., George-Chandy, A., Harandi, A.M., Lebens, M., Lehner, T., Lindblad, M., Nygren, E., Raghavan, S., Sanchez, J., Stanford, M., Sun, J.B., Svennerholm, A.M., Tengvall, S., 2005. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. Immunol. Lett. 97, 181–188.

Jacobs, P., Radzioch, D., Stevenson, M.M., 1996. A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. Infect. Immun. 64, 535–541.

Johnson, R.A., Waddelow, T.A., Caro, J., Oliff, A., Roodman, G.D., 1989. Chronic exposure to tumor necrosis factor in vivo preferentially inhibits erythropoiesis in nude mice. Blood 74, 130–138.

Karunaweera, N.D., Carter, R., Grau, G.E., Kwiatkowski, D., Del Giudice, G., Mendis, K.N., 1992. Tumour necrosis factor-dependent parasite-killing effects during paroxysms in non-immune *Plasmodium vivax* malaria patients. Clin. Exp. Immunol. 88, 499–505.

Kern, P., Hemmer, C.J., Gallati, H., Neifer, S., Kremsner, P., Dietrich, M., Porzsolt, F., 1992. Soluble tumor necrosis factor receptors correlate with parasitemia and disease severity in human malaria. J. Infect. Dis. 166, 930–934.

Landau, I., 1965. Description of *Plasmodium Chabaudi* N. sp., parasite of African rodents. C R Hebd. Seances Acad. Sci. 260, 3758–3761.

Langhorne, J., Meding, S.J., Eichmann, K., Gillard, S.S., 1989. The response of CD4^{+} T cells to *Plasmodium chabaudi chabaudi*. Immunol. Rev. 112, 71–94.

Langhorne, J., Quin, S.J., Sanni, L.A., 2002. Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. Chem. Immunol. 80, 204–228.

Langhorne, J., Albano, F.R., Hensmann, M., Sanni, L., Cadman, E., Voisine, C., Sponaas, A.M., 2004. Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. Immunol. Rev. 201, 35–47.

Li, C., Corraliza, I., Langhorne, J., 1999. A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice. Infect. Immun. 67, 4435–4442.

Li, C., Langhorne, J., 2000. Tumor necrosis factor alpha p55 receptor is important for development of memory responses to blood-stage malaria infection. Infect. Immun. 68, 5724–5730.

Li, C., Seixas, E., Langhorne, J., 2001. Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. Med. Microbiol. Immunol. (Berl.) 189, 115–126.

Li, C., Sanni, L.A., Omer, F., Riley, E., Langhorne, J., 2003. Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies. Infect. Immun. 71, 4850–4856.

Long, G.H., Chan, B.H., Allen, J.E., Read, A.F., Graham, A.L., 2006. Parasite genetic diversity does not influence TNF-mediated effects on the virulence of primary rodent malaria infections. Parasitology 133, 673–684.

Mackinnon, M.J., Read, A.F., 1999a. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Evolution Int. J. Org. Evolution 53, 689–703.

Mackinnon, M.J., Read, A.F., 1999b. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Evolution Int. J. Org. 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.J., Read, A.F., 2003. The effects of host immunity on virulence–transmissibility relationships in the rodent malaria parasite *Plasmodium chabaudi*. Parasitology 126, 103–112.

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

Mackinnon, M.J., Gandon, S., Read, A.F., in press. Virulence evolution in response to vaccination: the case of malaria. Vaccine.

McCarthy, D.D., Summers-Deluca, L., Vu, F., Chiu, S., Gao, Y., Gommerman, J.L., 2006. The lymphotoxin pathway: beyond lymph node development. Immunol. Res. 35 (1–2), 41–54.

Motard, A., Landau, I., Nussler, A., Grau, G., Baccam, D., Mazier, D., Targett, G.A., 1993. The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites. Parasite Immunol. 15, 21–26.

Naotunne, T.S., Karunaweera, N.D., Del Giudice, G., Kularatne, M.U., Grau, G.E., Carter, R., Mendis, K.N., 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J. Exp. Med. 173, 523–529.

Naotunne, T.S., Karunaweera, N.D., Mendis, K.N., Carter, R., 1993. Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates. Immunology 78, 555–562.

Omer, F.M., Riley, E.M., 1998. Transforming growth factor beta production is inversely correlated with severity of murine malaria infection. J. Exp. Med. 188, 39–48.

Raberg, 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.

Sam, H., Su, Z., Stevenson, M.M., 1999. Deficiency in tumor necrosis factor alpha activity does not impair early protective Th1 responses against blood-stage malaria. Infect. Immun. 67, 2660–2664.

Schellenberg, D., Menendez, C., Aponte, J.J., Kahigwa, E., Tanner, M., Mshinda, H., Alonso, P., 2005. Intermittent preventive antimalarial treatment for Tanzanian infants: follow-up to age 2 years of a randomised, placebo-controlled trial. Lancet 365, 1481–1483.

Schofield, L., 2002. Antidisease vaccines. Chem. Immunol. 80, 322–342.

Schofield, L., Hewitt, M.C., Evans, K., Siomos, M.A., Seeberger, P.H., 2002. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. Nature 418, 785–789.

Schofield, L., Mueller, I., 2006. Clinical immunity to malaria. Curr. Mol. Med. 6, 205–221.

Seixas, E., Cross, C., Quin, S., Langhorne, J., 2001. Direct activation of dendritic cells by the malaria parasite, *Plasmodium chabaudi chabaudi*. Eur. J. Immunol. 31, 2970–2978.

Stevenson, M.M., Huang, D.Y., Podoba, J.E., Nowotarski, M.E., 1992. Macrophage activation during *Plasmodium chabaudi* AS infection in resistant C57BL/6 and susceptible A/J mice. Infect. Immun. 60, 1193–1201.

Stevenson, M.M., Tam, M.F., Wolf, S.F., Sher, A., 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J. Immunol. 155, 2545–2556.

Stevenson, M.M., Riley, E.M., 2004a. Innate immunity to malaria. Nat. Rev. Immunol. 4, 169–180.

Stevenson, M.M., Riley, E.M., 2004b. Innate immunity to malaria. Nat. Rev. Immunol. 4, 169–180.

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.

Torre, D., Speranza, F., Martegani, R., 2002. Role of proinflammatory and anti-inflammatory cytokines in the immune response to *Plasmodium falciparum* malaria. Lancet Infect. Dis. 2, 719–720.

Trager, W., Gill, G.S., Lawrence, C., Nagel, R.L., 1999. *Plasmodium falciparum*: enhanced gametocyte formation in vitro in reticulocyte-rich blood. Exp. Parasitol. 91, 115–118.

Walliker, D., Carter, R., Morgan, S., 1971. Genetic recombination in malaria parasites. Nature 232, 561–562.

Woolacott, N.F., Khadjesari, Z.C., Bruce, I.N., Riemsma, R.P., 2006. Etanercept and infliximab for the treatment of psoriatic arthritis: a systematic review. Clin. Exp. Rheumatol. 24, 587–593.