

## PERSPECTIVE

## Consequences of immunopathology for pathogen virulence evolution and public health: malaria as a case study

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### Abstract

Evolutionary theories explaining virulence—the fitness damage incurred by infected hosts—often focus on parasite strategies for within-host exploitation. However, much virulence can be caused by the host's own immune response: for example, pro-inflammatory cytokines, although essential for killing malaria parasites, also damage host tissue. Here we argue that immune-mediated virulence, or 'immunopathology,' may affect malaria virulence evolution and should be considered in the design of medical interventions. Our argument is based on the ability of immunopathology to disrupt positive virulence-transmission relationships assumed under the trade-off theory of virulence evolution. During rodent malaria infections, experimental reduction of inflammation using reagents approved for field use decreases virulence but increases parasite transmission potential. Importantly, rodent malaria parasites exhibit genetic diversity in the propensity to induce inflammation and invest in transmission-stage parasites in the presence of pro-inflammatory cytokines. If immunopathology positively correlates with malaria parasite density, theory suggests it could select for relatively low malaria virulence. Medical interventions which decrease immunopathology may therefore inadvertently select for increased malaria virulence. The fitness consequences to parasites of variations in immunopathology must be better understood in order to predict trajectories of parasite virulence evolution in heterogeneous host populations and in response to medical interventions.

### Introduction

Parasites are organisms that live on or in another organism—the host—from which they extract some or all of their nourishment and in doing so, cause harm. Figuring out why parasites harm their hosts when they completely rely on hosts for survival has long challenged evolutionary biologists. A favored evolutionary hypothesis to explain parasite virulence (see Glossary for definition of key terms) is the adaptive trade-off hypothesis, which assumes that virulence arises as an unavoidable by-product of host resource exploitation which is needed to maximize the parasite's Darwinian fitness. Under this framework, parasites which are good 'exploiters' are expected to produce more transmission-stage parasites over the duration of infection, with death via over-exploitation the fitness cost

limiting virulence evolution (Anderson and May 1982; Ewald 1983; Read 1994; Ebert and Herre 1996; Frank 1996). The trade-off hypothesis has received empirical support; positive relationships between virulence and transmission have been found for many (for example, Meunier et al. 1985; Mackinnon and Read 1999a; Paul et al. 2004; Masumu et al. 2006; Fraser et al. 2007; Mackinnon et al. 2008; de Roode et al. 2008), but not all host-pathogen systems (Lipsitch and Moxon 1997; Jakel et al. 2001; Medica and Sukhdeo 2001; Ebert and Bull 2003) and direct evidence to support parasite-induced host mortality acting as a brake on virulence evolution is rare (Fenner and Ratcliffe 1965; Mackinnon et al. 2002). For an excellent comprehensive review of the trade-off hypothesis, including current debates in the field, see (Alizon et al. 2009).

**Glossary** Key Terms and Examples of Empirical Measurements

	Definition	What can be measured in a rodent malaria model of infection
Parasite	We use this term to encompass both micro- and macro-parasites.	<i>Plasmodium chabaudi chabaudi</i> .
Virulence	Any harm to host following infection. Theoretical models frequently define virulence as the rate of host mortality. Empirical studies often use morbidity measures as a proxy for host death.	Case fatality rates in some experiments. In others, maximum loss of weight, temperature, or red blood cells during infection, which positively correlate with host death (Timms et al. 2001; Mackinnon et al. 2002; Ferguson et al. 2003).
Transmission Potential	Number of transmission-stage parasites produced during the life-time of infection. Otherwise referred to as the parasites' Darwinian fitness.	Cumulative or peak number of sexual parasites (gametocytes), duration of gametocyte positivity (days gametocyte positive), and/or infectivity to mosquitoes. Cumulative gametocyte density is determined by the rate of gametocyte production and is an excellent indicator of lifetime transmission potential as well as transmission to mosquitoes (Taylor and Read 1997; Mackinnon and Read 1999a; Buckling and Read 2001).
Immunopathology	Harm to host tissue that is caused by the immune response.	Estimated as virulence statistically attributable to inflammatory cytokine levels independent of parasite density.
Recovery rate, estimated as concentration of inflammatory cytokines	Immunological molecules such as cytokines and cells such as macrophages recruited in response to infection. These molecules promote parasite clearance but cause damage.	Pro- and anti-inflammatory cytokine titres in the plasma at the time of peak asexual parasite density.
Exploitation, estimated as parasite density within the host	Extraction of host resources for production of transmission-stage parasites. Empirical studies often use within-host replication or growth rate as a measure of exploitation.	Cumulative or peak density of asexual parasites.
Immunogenicity	Per-parasite immune stimulatory effect.	Intensity of stimulation of Toll Like Receptors on antigen presenting cells.

Here we argue that one reason this apparently conflicting empirical support may arise is because the trade-off hypothesis, until recently (Day et al. 2007; Alizon et al. 2009), did not incorporate parasite-induced immune-mediated virulence, or immunopathology. Here, with a specific focus on malaria, we summarize empirical evidence that immunopathology may shape parasite evolution, with applied consequences. We begin by giving a brief introduction of the malaria life-cycle and immune response to infection. We then describe theoretical work which has extended basic trade-off theory to include certain aspects of the host immune response, including immunopathology. Finally, we outline experimental studies investigating the selective effects of immunopathology on evolution of malaria parasite virulence and discuss the public health implications of results in light of theoretical predictions.

The remarkable capacity of *Plasmodium* parasites to adapt, survive and transmit within heterogeneous host populations (Mackinnon and Marsh 2010) goes a long way to explain the success of these parasites, as well as the crippling effect malaria exerts on humanity (Snow et al. 2005). The life-cycle of *Plasmodium* parasites is

complex, consisting of alternating phases in the mosquito vector and the vertebrate host. For a detailed schematic of the *Plasmodium* lifecycle as well as an overview of immune responses to malaria see (Stevenson and Riley 2004). In order to be transmitted to mosquitoes, asexual *Plasmodium* parasites replicating in the red blood cells (RBCs) of a vertebrate host must produce sexual stages (male and female gametocytes). A subset of asexual parasites commit to gametocyte production, so increasing asexual parasite density is expected to correlate with transmission success. Thus in the case of malaria, trade-off theory predicts that the most evolutionarily successful parasite would be one which maximizes gametocyte production and infection length while avoiding host death and immune clearance. Indeed, studies using the *Plasmodium chabaudi* rodent model of malaria infection support several key assumptions of the trade-off hypothesis (Mackinnon and Read 2004a). Specifically, parasite genotypes which inflict the greatest virulence (see Glossary for key traits measured in this malaria model system) enjoy higher transmissibility and, in the absence of host death, longer infectious periods (Mackinnon and Read 1999a,b,

2003; Ferguson et al. 2003). In the presence of infection-induced mortality, the transmission potential is less than if the host lived, suggesting host death is a brake on virulence evolution (Mackinnon et al. 2002).

However, parasite virulence encompasses damage due to infection-induced immunopathology as well as direct parasite-mediated exploitation (Romagnani 1996; Clark et al. 2006; de Jong et al. 2006; Margolis and Levin 2008), among other host and environmental factors (Frank 1996; Little et al. 2010; Magalon et al. 2010). For example, the characteristic anemia associated with malaria infection is driven by a combination of destruction of infected RBCs by parasites, as well as haemolysis (Ritter et al. 1993) and phagocytosis (Waitumbi et al. 2000; Wickramasinghe and Abdalla 2000; Serghides et al. 2003) of both infected and uninfected RBCs by the immune system. In addition, other severe malaria symptoms such as cerebral damage are associated with overzealous infection-induced inflammatory responses (Schofield and Grau 2005). Analogous examples of immunopathological virulence are found in a huge array of infectious diseases (Graham et al. 2005a).

The immune response against malaria (and indeed a wide array of pathogens) thus requires a delicate balance to control parasites on the one hand and avoid immunopathological damage on the other, in both rodent (Li et al. 2001; Stevenson and Riley 2004) and human malaria infection (Dodoo et al. 2002; Clark et al. 2006). The importance of pro-inflammatory responses, including tumour necrosis factor [TNF]- $\alpha$ , in cell- and antibody-mediated immunity to malaria is well established (see Li et al. 2001 and Stevenson and Riley 2004 for good reviews in this area). However, excessive levels of pro-inflammatory cytokines cause much of the characteristic malaria inflammation and anti-inflammatory cytokines such as interleukin [IL]-10 play important roles in limiting infection-induced immunopathology (Li et al. 2001; Dodoo et al. 2002). The tight immune regulation necessary for optimal defense against infectious agents has been likened to the porridge sampled by Goldilocks in the home of the three bears (Germain 2001): whereas a slow or weak immune response risks being overwhelmed by rising parasitaemia—‘too cold’—an overzealous one may lead to severe disease because of the destructive effects it exerts on host tissue—‘too hot’. The optimal response must be robust, rapid and properly regulated—‘just right’.

### Effects of protective versus pathological immune responses on virulence evolution

Theoreticians increasingly recognize the pivotal influence host immunity exerts on parasite dynamics at both the within-host and epidemiological scales and figuring out the best ways to incorporate host immunity into the

trade-off model has been the focus of much recent work (Alizon and van Baalen 2008; Alizon et al. 2009). In some scenarios, immune responses are assumed to be purely protective, acting solely to decrease parasite density and thus to reduce disease severity and risk of host death (Antia et al. 1994; Antia and Lipsitch 1997; Gandon et al. 2001). This type of immune system activity alleviates the reduced transmission potential associated with early host death. Thus, theory predicts that highly immune host populations can maintain more virulent parasites relative to naïve host populations (Gandon et al. 2001). Empirical evidence from rodent malaria infections supports this prediction, with immune selection accelerating the rate of virulence evolution (Mackinnon and Read 2003, 2004b).

What selective pressures do pathological immune responses exert on the trajectory of parasite virulence evolution, beyond those exerted by protective immunity? The key issue is that immunopathology can alter the relationship between virulence and exploitation (including within-host replication and/or between-host transmission). For example, excessive inflammation might alter virulence-exploitation relationships by: (i) increasing the rate of host death even when the rate of parasite killing also increases (Graham et al. 2005a; Day et al. 2007); (ii) disproportionately killing transmission-stage parasites; or (iii) altering investment in transmission-stage parasites. The applicability of these scenarios to the malaria case study is discussed below.

Theoretical work has investigated how parasite exploitation strategies should evolve in the presence of (i) immune-mediated host death (Day et al. 2007). According to this theory, virulence  $\alpha(\epsilon, c)$  comprises direct effects ( $\gamma$ ) of exploitation ( $\epsilon$ ) plus effects of immunopathology  $f(\epsilon, c)$ , which are in turn broken into four biologically plausible components that scale with exploitation  $\epsilon(\varphi_1)$ , with recovery rate  $c$  ( $\varphi_2$ ), with their statistical interaction  $\epsilon c$  ( $\varphi_3$ ), or are independent of both ( $\varphi_0$ ) (Day et al. 2007). Immunopathology is plausibly a function of recovery rate because both depend on the extent of immune activation (e.g., high plasma concentrations of pro-inflammatory cytokines such as TNF- $\alpha$  are associated with rapid malaria clearance but elevated risk of collateral inflammatory damage; Li et al. 2001; Stevenson and Riley 2004). Thus, in this framework (Day et al. 2007), overall virulence can be represented as follows:

$$\begin{aligned}\alpha(\epsilon, c) &= \gamma\epsilon + f(\epsilon, c) = \gamma\epsilon + \varphi_0 + \varphi_1\epsilon + \varphi_2c + \varphi_3\epsilon c \\ &= \varphi_0 + (\varphi_1 + \gamma)\epsilon + \varphi_2c + \varphi_3\epsilon c\end{aligned}$$

(below we use empirical data to estimate parameters of the rightmost version of the equation). The theory arising from this dissection of the causes of immunopathology predicts that when the risk of immune-mediated host

death increases with within-host exploitation (for example, when  $\varphi_1$  or  $\varphi_3 > 0$ ), natural selection favors parasites with prudent within-host exploitation strategies because of the increased survival cost of exploitation (Day et al. 2007). On the other hand, if immunopathological host death is independent of exploitation (e.g., when  $\varphi_1 = \varphi_3 = 0$ ), then selection is expected to lead to increased virulence. In this scenario, immune-mediated host death has the effect of increased background mortality—i.e., the host will die regardless of what the parasite does (Long et al. 2008a)—which weakens the survival benefit of prudent exploitation and may select for increased virulence (Day et al. 2007). The trade-off hypothesis thus can readily accommodate immunopathology, and the predicted evolutionary trajectories depend upon the mechanism(s) by which immunopathology is generated.

The association of host-damaging immune responses with mechanisms that (ii) disproportionately kill- or (iii) alter investment in transmission-stage parasites has not to our knowledge been explored explicitly by theoreticians, but could also exert powerful effects on parasite virulence evolution by altering virulence-transmission relationships. At a minimum, (ii) should decrease transmission and (iii) should increase transmission, for a given level of virulence. For example, certain pro-inflammatory cytokines implicated in driving malaria virulence are also associated with gametocyte killing (Naotunne et al. 1991, 1993). If immunopathological virulence is associated with gametocyte death, then the relationship between virulence and transmission could become negative, thus defying a major assumption of basic theory (Anderson and May 1982). On the other hand, immunopathology could increase transmission potential if it increases the proportion of asexual parasites which become committed to the sexual stage. It has long been observed that commitment of malaria asexuals to gametocytes is influenced by their environment (Carter and Miller 1979). Indeed, 'stress cues' in the form of host immune responses against asexual parasites have been suggested to correlate with altered gametocyte loads and below we discuss one study where we find such evidence (Long et al. 2008b), in contrast to a previous study which did not (Buckling and Read 2001). If inflammation triggers gametocytogenesis, the positive relationship between virulence and transmission assumed by basic theory would be maintained along with the predictions arising thereof.

We have experimentally examined each of these possible selective effects of pathological immune responses in rodent malaria. The majority of previous work on protective and pathological responses to malaria (for example, as reviewed by Li et al. 2001) focused on a single parasite clone and did not investigate transmission potential or

other proxies for parasite fitness. We therefore undertook a series of experiments in which we explored whether immune factors central to the protection-pathology balancing act help to determine the virulence induced during infection with genetically distinct malaria parasites, and if so, what effect this may have on parasite fitness. We used the same rodent malaria system for which the adaptive trade-off hypothesis has empirical support (Mackinnon and Read 2004a); *P. chabaudi* in a resistant strain of laboratory mice C57BL/6 (Stevenson et al. 1982). To seek evidence of parasite genetic differences in immunopathological virulence and transmission determinants on which selection might operate, we used a toolbox of between four and eight genetically distinct *P. chabaudi* clones—which have been shown to differ in the *in vivo* virulence they induce (Mackinnon and Read 1999a; Ferguson et al. 2003)—and tracked asexual and sexual parasite densities, virulence, and systemic inflammation during infection (see Glossary for traits measured). Furthermore, we experimentally manipulated the host immune response itself, thanks to the availability of suitable reagents for laboratory mice (Li et al. 2001; Stevenson and Riley 2004). Indeed, via the systemic administration of immunological reagents we were able to push hosts in both pro-inflammatory (Long et al. 2008a) and anti-inflammatory (Long et al. 2006, 2008b) directions.

Before outlining our published results in light of possibilities (i)–(iii), we describe the statistical dissection of immunopathological mechanisms—for example, the overproduction of pro-inflammatory cytokines—from direct effects of parasite replication in determining variation in virulence. Such methods also enable identification of exploitation-dependent versus exploitation-independent immunopathology, which is critical to predicting evolutionary trajectories (Day et al. 2007).

### Statistical separation of immune- from direct parasite-mediated virulence

An evolutionary immunologist interested in how immunopathology affects virulence evolution must distinguish immune- from directly parasite-mediated host damage. Once data on parasite densities, inflammation, and virulence are obtained (see Glossary), statistical separation of the causes of virulence is achievable using analysis of covariance (Graham et al. 2005b). Predictor variables may include immune manipulation, parasite genotype, and their interaction, as well as linear and quadratic terms for the density of both parasites and pro-inflammatory cytokines.

For example, we found that the characteristic virulence of parasite genotypes positively correlated with the inflammatory response induced during the acute phase of infection (Long et al. 2006). To determine whether

this simply reflected known differences in asexual replication rates among clones (Mackinnon and Read 1999a), data were analyzed with maximum parasitaemia as a covariate (though we thereafter used parasite density; see Glossary). More virulent malaria clones induced a greater plasma TNF- $\alpha$  response relative to their less virulent counterparts, even when any linear and (in this case non-significant) quadratic effects of asexual parasite load were statistically controlled for (Long et al. 2006). Thus, we found evidence that the clones differed significantly in the per-parasite inflammation they induced. Whether *P. falciparum* malaria exhibits similar genetic diversity is not yet clear—for example see (Corrigan and Rowe 2010)—but parasite genetic differences in immunopathological virulence have been demonstrated for various other infections, including protozoa (Kebaier et al. 2001; Mordue et al. 2001) and viruses (de Jong et al. 2006). To directly test whether the propensity to induce a large inflammatory response was driving some *P. chabaudi* clones to higher virulence, we applied anti-inflammatory treatments—i.e., we blocked TNF- $\alpha$  signaling through the administration of soluble TNF- $\alpha$  receptor fusion protein, TNFR-Ig, early during infection—which reduced the virulence of all clones, independent of parasite load (Long et al. 2006, 2008b). Thus although there was variation among clones in the induction of inflammation, TNF- $\alpha$  signaling contributed to the virulence of all clones.

Here, to illustrate how such data may be aligned with theory to estimate the contribution of immunopathology to virulence evolution (Day et al. 2007), we have combined and re-analysed our published data on the virulence of four malaria clones in un-manipulated versus anti-inflammatory treated hosts (Long et al. 2006, 2008b). However, several caveats are essential from the outset. We agree in principle that it should be achievable to study immunopathology-induced mortality *per se* (Day et al. 2007), but our data were not collected for that purpose. Instead, our data comprise all-cause mortality and morbidity. For example, we measured RBC density daily and observed substantial declines during acute malaria, but we did not partition the anaemia into portions attributable to lysis of infected RBCs versus bystander killing (Waitumbi et al. 2000; Wickramasinghe and Abdalla 2000; Serghides et al. 2003) or removal from circulation of uninfected RBCs (Safeukui et al. 2008) by the immune system. Such partitioning is possible via analysis of parasite population dynamics (Haydon et al. 2003) or detailed mechanistic analysis (Chang and Stevenson 2004). It would also be possible, in future experiments, to measure virulence traits that more purely represent immunopathology—for example through quantifiable inflammatory damage to the brain of malaria-infected mice (Amante

et al. 2010). But to make use of the existing all-cause virulence data, we redistribute terms from Day et al.'s theoretical development (Day et al. 2007) as outlined above, to generate the following expression for all-cause virulence:  $\alpha(\varepsilon, c) = \varphi_0 + (\varphi_1 + \gamma)\varepsilon + \varphi_2c + \varphi_3\varepsilon c$ . The dual coefficients for exploitation  $\varepsilon$  represent dual contributions of immunopathology  $\varphi_1$  and direct damage  $\gamma$  to the per-parasite virulence (see also Little et al. 2010), though the two are often indistinguishable in practice. Finally, we used peak parasite density as our proxy for parasite exploitation and peak plasma pro-inflammatory cytokine concentration as our proxy for recovery rate (see Glossary). The latter is justified by the common observation that elevated pro-inflammatory cytokines are associated with rapid malaria clearance (Li et al. 2001; Stevenson and Riley 2004). Both proxies accord with the empirical expectations of (Day et al. 2007) and are rendered essential by the available data, even if the proxies are imperfect: the precise variables that are theoretically interesting are often empirically inaccessible.

With these caveats, we proceeded with statistical analysis as described in Box 1. Our analyses of anaemia and cachexia (Table 1A and B, respectively within Box 1) partitioned virulence increasing with parasite density from that increasing with inflammation. Even when virulence did increase with parasite density (and, in some cases, quadratic parasite density), some virulence independently increased with inflammation. Box 1 further illustrates diversity among clones in the relative importance of the two mechanisms. For example, whereas virulent clone AJ causes RBC loss via both exploitation-dependent and -independent mechanisms, virulent clone BC appears to cause virulence independent of exploitation, but strongly dependent upon the inflammatory response induced (for example, 3.3 billion RBCs were lost for every log increase in picograms of pro-inflammatory cytokines per milliliter of plasma). Overall, clones exhibited significant variation in the slopes of the relationships described (see Box 1). If further empirical data showed that immunopathology (and not just total virulence) scaled with exploitation for the majority of malaria genotypes in the host type most important for transmission (see below), then natural selection would be predicted to reduce malaria virulence (Day et al. 2007).

### How much parasite transmission is gained or lost in hosts with immunopathology?

The transmission potential of rodent malaria parasites can also be empirically estimated (Mackinnon and Read 1999a). Indeed, the theoretician's definition of exploitation comprises production of transmission stage parasites (Glossary). Therefore, to elucidate potential selective

**Box 1.** An illustration of how the contributions to virulence of exploitation (proxy: parasite density) and recovery rate (proxy: inflammation) can be statistically distinguished.

Data are drawn from experiments on rodent malaria (Long et al. 2006, 2008b), in which (Table 1 A) anemia (maximum RBC loss compared to initial RBC density) and (1B) cachexia (maximum loss of body mass compared to initial mass) were measured. Coefficients of the linear model for virulence (i.e., slopes) were calculated within treatment type (unmanipulated versus anti-inflammatory) based upon the framework of Day et al. (2007). Given the limited statistical power of this analysis, priority was given to estimating the following terms critical to trajectories of virulence evolution:  $\varphi_1 + \gamma$  describes how virulence relates to exploitation;  $\varphi_2$  describes how virulence relates to recovery rate (see text for details plus justification of proxies). Linear and quadratic parasite density, inflammation, and their interactions with genotype were fitted simultaneously as fixed explanatory variables, with parasite genotype as a random effect. Minimal models were obtained, and included no quadratics. In unmanipulated hosts, estimates for  $\varphi_1 + \gamma$  ( $F_{3,116} = 3.26$ ;  $P = 0.024$ ) and  $\varphi_2$  ( $F_{3,116} = 4.69$ ;  $P = 0.0039$ ) varied significantly among genotypes for anemia. Estimates for  $\varphi_2$  exhibited a trend for variation among genotypes for cachexia ( $F_{3,116} = 2.05$ ;  $P \sim 0.11$ ).

Data were then broken down by clone within treatment type. Linear and quadratic terms for parasite density and inflammation were simultaneously fitted as fixed explanatory variables for the virulence response variables. Initially, intercepts were not fitted, to conserve statistical power for this illustrative analysis. Estimated slopes in boldface type in Tables 1 A (for anaemia) and 1B (for cachexia) differed significantly from zero in minimal models;  $P$  values appear in parentheses. Models were then re-run with intercepts fitted. This led to similar results, except that quadratic parasite density was never a significant contributor to virulence, and all  $P$  values increased. However, of the 16 slopes that differed significantly from zero in the original analysis, only 5 lost significance once the intercepts were fitted. These are reflected in the  $P$ -values of the original analysis, presented below. Analysis was conducted in PROC MIXED for SAS Systems 9.1.

**Table 1.** (A) Peak anaemia (loss of red blood cell (RBC) density; in  $10^9/\text{mL}$ ) (B) Peak cachexia (loss of body mass; in grams).

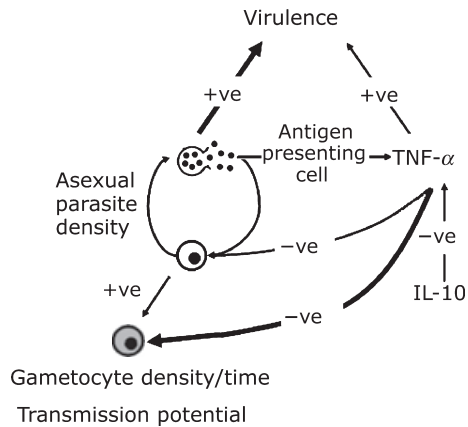
Parasite genotype	Unmanipulated hosts		Anti-inflammatory hosts	
	$\varphi_1 + \gamma$ (RBCs lost per log increase in parasite density)	$\varphi_2$ (RBCs lost per log increase in pro-inflammatory cytokines)	$\varphi_1 + \gamma$ (RBCs lost per log increase in parasite density)	$\varphi_2$ (RBCs lost per log increase in pro-inflammatory cytokines)
<b>A</b>				
AJ (N = 31; 21)	<b>4.7</b> (0.0028) <sup>Q</sup>	<b>2.3</b> (<0.0001) <sup>Q</sup>	<b>8.5</b> (<0.0001)	<b>3.0</b> (<0.0001)
AS (N = 21; 9)	5.5 (0.074)	<b>2.3</b> (<0.0001)	<b>7.5</b> (0.039)	<b>1.5</b> (0.030)
BC (N = 16; 5)	0 (~0.5)	<b>2.9</b> (0.0001)		NE (N too small)
CW (N = 18; 5)	<b>6.2</b> (0.026) <sup>Q</sup>	<b>1.5</b> (0.0049) <sup>Q</sup>		NE (N too small)
<b>B</b>				
	$\varphi_1 + \gamma$ (grams lost per log increase in parasite density)	$\varphi_2$ (grams lost per log increase in pro-inflammatory cytokines)	$\varphi_1 + \gamma$ (grams lost per log increase in parasite density)	$\varphi_2$ (grams lost per log increase in pro-inflammatory cytokines)
AJ (N = 31; 21)	<b>1.9</b> (0.036) <sup>Q</sup>	<b>1.1</b> (<0.0001) <sup>Q</sup>	2.7 (0.063)	<b>1.5</b> (<0.0001)
AS (N = 21; 9)	0 (~0.5)	<b>1.1</b> (0.0048)	0 (~0.9)	0 (~0.3)
BC (N = 16; 5)	0 (~0.6)	<b>1.7</b> (0.0001)		NE (N too small)
CW (N = 18; 5)	2.0 (0.13)	<b>0.5</b> (0.012)		NE (N too small)

N = number of mice per treatment group infected with each parasite genotype; NE = not estimated; <sup>Q</sup> = quadratic parasite density (PD) was a significant contributor to virulence; the slope and  $P$  value shown in such cases are for linear PD and inflammation with quadratic PD retained in the model; slopes in bold differ significantly from zero.

pressures posed by immunopathology, we now compare malaria transmission potential for hosts receiving pro-inflammatory, null, and anti-inflammatory treatments.

As outlined above, one way immunopathological virulence can affect parasite fitness and hence the trajectory of virulence evolution is by (i) increasing the rate of host mortality. To explore this scenario, we experimentally reduced activity of the cytokine interleukin [IL]-10—which helps prevent immune hyperactivity during malaria infection (see Fig. 1 and Li et al. 2001; Stevenson

and Riley 2004)—and in doing so made immunopathology the primary cause of virulence (Long et al. 2008a). Specifically, neutralizing IL-10 signaling expedited time-to-death for eight *P. chabaudi* genotypes, and death curtailed transmission potential (Long et al. 2008a). Using similar analyses to those presented in Box 1, we statistically investigated whether linear or quadratic effects of asexual parasite load, the pro-inflammatory cytokine TNF- $\alpha$ , or key morbidity measurements explained variation in mortality rates. We found that parasite



**Figure 1** Tumour necrosis factor (TNF)- $\alpha$  and other pro-inflammatory cytokines help govern both virulence and transmission of malaria parasites. Here we want to illustrate how an increased understanding of the relationship between virulence mechanisms (in this case immunopathological virulence) and how they relate to parasite transmission could have major implications for virulence evolution. ‘-ve’ denotes an inhibitory effect, while ‘+ve’ denotes synergy. Arrows in bold are considered especially potent effects. The gametocidal effects of TNF- $\alpha$  can reduce transmission potential (Long et al. 2008b), but unregulated or excessive levels can lead to immunopathological virulence (Long et al. 2006, 2008b). These data show that virulence and transmission are not always positively correlated. Such new information can be incorporated into the trade-off model to provide quantitative answers to how virulence might evolve (Day et al. 2007).

density could not explain time-to-death ( $F_{1,52} = 1.08$ ,  $P = 0.3$ ). Instead, the plasma concentration of TNF- $\alpha$  during acute infection and minimum body-temperature significantly predicted survival time, across treatment groups and independent of parasite load: higher TNF- $\alpha$  ( $F_{1,52} = 9.42$ ,  $P = 0.003$ ) and greater temperature losses ( $F_{1,52} = 30.7$ ,  $P < 0.001$ ) were associated with a quicker time-to-death. This is not surprising as mortality actually increased with decreasing parasite burden in IL-10 neutralized hosts (Long et al. 2008a), and the over-production of pro-inflammatory cytokines can mediate a toxic shock-like syndrome (Florquin et al. 1994; Holscher et al. 2000). Similarly, the enhanced disease severity of mice deficient in IL-10 and infected with the parasites *Trypanosoma cruzi* (Hunter et al. 1997), *Toxoplasma gondii* (Gazzinelli et al. 1996), or *Helicobacter hepaticus* (Kullberg et al. 1998) did not correlate with increased parasite load, but rather with an overzealous inflammatory response. If hosts with strong pro-inflammatory responses are common and if they die without transmitting infections in nature, this should affect virulence evolution. The direction of selection depends upon whether immunopathology positively correlates with exploitation in such hosts (Day et al. 2007).

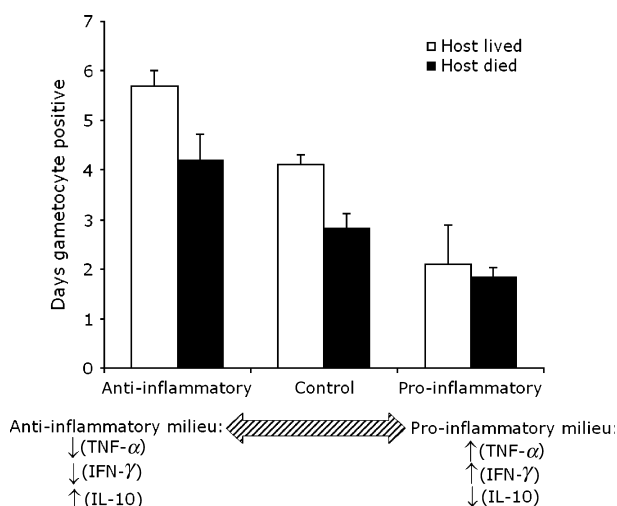
Beyond causing host death, immunopathology might also affect the direction of malaria virulence evolution by (ii) killing or (iii) altering investment in gametocytes. In other words, inflammatory immune responses can disproportionately affect gametocytes, independent of asexual parasite density (Fig. 1). We found that the anti-inflammatory treatment by which we neutralised TNF- $\alpha$  increased transmission potential over the duration of infection (Long et al. 2008b), independent of asexual parasite load and regardless of *P. chabaudi* genotype (see Glossary for details of traits measured). Indeed, over the course of infection TNFR-Ig treated mice had on average 44% more gametocytes than control mice and, importantly, were more infectious to mosquitoes (Long et al. 2008b). Commitment and development of asexuals to gametocytes takes 24–48 h in *P. chabaudi* (Carter and Walliker 1975). The speed with which TNF- $\alpha$  neutralization increased gametocyte densities—within 4 h of treatment—thus effectively excluded gametocyte conversion as the main factor driving increased gametocyte load. Rather, the increase in gametocyte density and infectivity in hosts receiving anti-inflammatory treatment may be explained by protection of gametocytes from immune attack (Naotunne et al. 1991, 1993). In agreement with our findings, excessive induction of TNF- $\alpha$  in human malaria has been proposed to contribute to severe disease on the one hand (Clark et al. 2004, 2006), and reduce infectiousness to mosquitoes on the other (Karunaweera et al. 1992). These gametocidal effects (ii) of TNF- $\alpha$  suggest that inflammation may contribute to virulence while reducing transmission potential.

At the same time, gametocyte production is highly susceptible to environmental changes (Carter and Miller 1979). For example, it has been hypothesized that host immune responses may trigger gametocytogenesis (Dyer and Day 2000). Although a previous direct test of this hypothesis failed to find supporting evidence (Buckling and Read 2001), we have additional results which suggest a pro-inflammatory environment is associated with gametocytogenesis cues for some malaria genotypes. The experimental enhancement of immunopathology during infection via IL-10 neutralisation incurred a uniform fitness cost to all *P. chabaudi* clones by reducing the lifetime transmission potential via host death (Long et al. 2008a), as described above. However, *early* in infection, parasite genotype was important in determining how increases in inflammation affected the density of transmission-stage parasites; whether inflammation cued gametocytogenesis depended on *P. chabaudi* parasite genotype (Long et al. 2008a). More generally, it is possible that parasites experience trade-offs for inducing inflammation, if the costs of provoking pro-inflammatory responses at one point in

infection are outweighed by the benefits at other time points.

These empirical results for gametocyte density are corroborated by a new, combined analysis of the duration of gametocyte positivity across the three treatment groups (see Glossary for details of traits measured). Figure 2 illustrates the effects of host death and each of our immune manipulations on the duration of gametocytemia, and argues that both death and host heterogeneity in the inflammatory state may affect malaria transmission potential. For example, anti-inflammatory treatment increased the duration of gametocytemia by  $1.53 \pm 0.33$  days on average, an effect comparable in magnitude (though opposite in sign) to the effects of host death on transmission

( $-1.22 \pm 0.28$  days on average; Fig. 2). Overall, hosts presenting an anti-inflammatory milieu were infectious for longest, while 'pro-inflammatory hosts' had the briefest infectious periods. These effects were independent of host death (Fig. 2) and asexual parasite density (Long et al. 2008a,b), although we acknowledge that the duration of asexual parasitaemia would probably affect duration of gametocytemia during chronic infections (Bell et al. 2006). Collectively, our empirical results thus far suggest that immunopathological virulence has the potential to strongly affect malaria fitness, via increased host death rates (Long et al. 2008a) and/or via associations with altered production (Long et al. 2008a) or survival (Long et al. 2008b) of transmission-stage parasites themselves.



**Figure 2** Inflammatory status of the host shapes rodent malaria transmission potential. *In vivo* treatments extended the range of inflammatory phenotypes exhibited during *Plasmodium chabaudi* infection. We applied two types of treatments: 'anti-inflammatory' in the form of TNFR-Ig which decreased immunopathology (Long et al. 2008b), or 'pro-inflammatory' treatments in the form of IL-10R (Long et al. 2008a) which enhanced immunopathology. We always included a control (unmanipulated) group. In a combined data analysis, we found that the number of days gametocyte positive (an estimate of the duration of infectiousness) was strongly affected by host inflammatory status ( $F_{2,207} = 25.1$ ;  $P < 0.0001$ ). Hosts receiving anti-inflammatory treatment were gametocyte positive for longest, followed by un-manipulated hosts and then hosts receiving pro-inflammatory treatment (Tukey-corrected pair-wise differences between anti-inflammatory hosts and both other types  $P < 0.0001$ ; un-manipulated and pro-inflammatory mice also differed significantly,  $P = 0.0011$ ). Death of the host further reduced gametocyte positivity by an average of  $1.2 \pm 0.3$  days ( $F_{1,207} = 19.0$ ;  $P < 0.0001$ ), but did not interact significantly with treatment type ( $P \sim 0.4$ ). Thus, the duration of gametocyte patency associated with inflammatory status was independent of the significant infection-shortening effects of host death. Test statistics and  $P$ -values for main effects come from the minimal model, including a random effect for clone, run in PROC MIXED for SAS Systems 9.1.

### Immunopathology and virulence evolution; insights from malaria

Reasons why immunopathology might shape the evolution of malaria virulence are summarized in Table 2. In essence, the extra mortality imposed by immunopathology may inflict an extra cost of virulence, which cannot be explained by host exploitation alone. Such extra death may not be positively related to host exploitation or transmission and may alter the cost/benefit ratio of virulence. Indeed, for *P. chabaudi*, the capacity of inflammation to kill both hosts and gametocytes may generate negative virulence-transmission relationships (or, at a minimum, alter transmission potential for a given level of virulence). Furthermore, the suite of genotypes studied here exhibits relevant genetic variation and thus raw material for natural selection: parasite genotype shapes the amount of inflammation induced, the extent to which virulence positively correlates with parasite density, and gametocytogenesis cues. These traits could in turn affect the outcome of competition within hosts. For example, genotypes that strongly induce inflammation and transmit efficiently in the face of high concentrations of cytokines such as TNF- $\alpha$  may outcompete genotypes that would otherwise (i.e., in the context of weak inflammatory responses) achieve high within-host densities. Virulent immune-provoking behaviours and enhanced immune resistance have been proposed as tactics adopted by invading pathogens to eliminate competitors (Brown et al. 2008). Indeed, within host competition among genotypes can either increase or decrease immediate virulence and is also a major driver of virulence evolution (Mideo 2009).

Before these findings (Table 2) are used to predict evolutionary trajectories with confidence, however, much work remains to be done. For example, the simultaneous contributions of host and parasite genotypes (Little et al. 2010) to immunopathology would be important to



**Table 2.** Summary of why immunopathology may be relevant to Malaria Virulence Evolution, based on evidence from experiments on *Plasmodium chabaudi* in laboratory mice.

Possible outcomes	Negative correlation between transmission and virulence?	Inflammation can kill/damage host, independent of PD	Inflammation can kill gametocytes, independent of PD	Variation in induction of inflammation	Variation in how virulence correlates with PD (per-parasite virulence)	Variation in whether inflammation cues gametocytogenesis
Empirical observations	Applies to all tested genotypes (Long et al. 2006, 2008a,b)	Applies to all tested genotypes (Long et al. 2006, 2008a,b)	Applies to all tested genotypes (Long et al. 2008b); Fig. 2	Dependent on parasite genotype (Long et al. 2006)	Dependent on parasite genotype (Long et al. 2006); Table 1	Dependent on parasite genotype (Long et al. 2008a)
Trait distribution						
References						

PD = parasite density.

explore, and tolerance traits whereby hosts alleviate the fitness effects of a given parasite burden may be critical (Raberg et al. 2009). It would also be of great interest to estimate  $\phi_3$ , the coefficient of the statistical interaction between exploitation and recovery rate (Day et al. 2007), and to obtain firmer estimates of the other coefficients in a better-powered analysis in future. Such analysis of a human malaria species would be ideal, and the literature suggests that suitable data exist (Akanmori et al. 2000). Finally, it would be of interest to explore the effects of immunopathology upon trajectories of virulence evolution that are predicted by theories other than the trade-off model (Levin and Bull 1994; Alizon et al. 2009).

For now, the outcome of immunopathology for malaria virulence evolution remains difficult to predict. The extent to which malaria in the laboratory represents malaria in the field, plus the relative importance of inflammation- versus exploitation-dependent immunopathological virulence, will determine whether we expect increased or decreased ESS virulence due to immunopathology. For example exploitation-dependent immunopathology, could select for decreased virulence (Day et al. 2007), by decreasing parasite immunogenicity and/or replication rate. However, protective immune responses against malaria impose the opposite selection pressure (Mackinnon and Read 2004b), as does immunopathological virulence that is independent of exploitation (Day et al. 2007). The relative strengths of these potentially conflicting effects of immunity remain to be elucidated.

Furthermore, it will be essential to consider the role of host heterogeneity in the evolution of malaria virulence (Mackinnon and Marsh 2010). Immunopathology is likely to be highest in non-immune individuals (for example, children in areas of high malaria transmission) whose immune response is not yet primed against malaria infection and who might suffer more immunopathology as a result of non-specific anti-parasitic clearance mechanism(s), such as the systemic induction of inflammatory host cytokines (Grau et al. 1989). Theoretical studies have revealed that the cost of host death is greatest in children as they are both more likely to die- and transmit- malaria parasites than their adult counterparts (Mackinnon and Marsh 2010). An immunopathological arm to virulence in non-immune hosts has been shown (Schofield and Grau 2005) and in such a host population, mutant parasites that are less immunogenic would be expected to be favored by natural selection as highly immunogenic strains would be removed by host death. But host populations are a mixture of host ‘types’ and because parasites do not chose their hosts and evolve to suit the average host (Mackinnon and Marsh 2010), the optimal level of virulence which evolves in a population will ultimately

depend on the relative frequencies of non-immune versus semi-immune hosts in the population. Thus, the results depicted in Fig. 2, although suggestive of a key role of host heterogeneity in malaria transmission potential, must be placed firmly in an epidemiological perspective before the full implications become clear.

As far as we are aware, ours is the first empirical evidence supporting malaria genetic variation in both the induction of inflammation that contributes to virulence (Long et al. 2006) and in the response to inflammation that affects gametocytogenesis and hence transmission potential (Long et al. 2008a). Indeed, outside of malaria, no studies we could find explicitly examine the transmission consequences of genetic differences in immunopathological virulence. More data are required in both human malaria and other parasitic diseases to determine whether the immunopathology, virulence, transmission relationships are encoded by parasite genes, and to determine how much virulence variation exists in the field. It is our contention that expanding the trade-off theory to incorporate immunopathology, coupled with empirical studies which test that theory will help improve our understanding of the evolution of parasite virulence. For example, investigating whether immunopathological virulence schedules (for example, see Table 1 within Box 1) differ between distinct parasite strains and if so, what the fitness consequences are for transmission potential. Such studies would allow more informed decisions to be made regarding medical interventions which would be optimal for long term disease control.

### Evolutionary responses to malaria interventions that alter immunopathology

A public health goal for malaria treatments is to reduce the infectious reservoir, while the reduction of disease severity and prevention of death is a major clinical goal. Our results suggest that interventions aimed at reducing symptoms of disease but not parasites, might not be beneficial at the population level. Reducing immunopathological disease severity in individual hosts could in fact increase transmission potential in the short term (Long et al. 2008b). When immunopathology arises independently of exploitation, this might increase evolved virulence in the longer term (Day et al. 2007), in keeping with predictions for malaria evolution in response to anti-disease vaccines that alleviate the cost of virulence for parasites (Gandon et al. 2001; Williams 2009). In the past, the association of elevated serum TNF- $\alpha$  levels with malaria disease severity (Grau et al. 1989; Kwiatkowski 1990) led to the use of anti-TNF- $\alpha$  therapies for acute human malaria. These therapies significantly reduced body temperature (Kwiatkowski et al. 1993; van

Hensbroek et al. 1996; Looreesuwan et al. 1999), but increased the severity of neurological sequelae (van Hensbroek et al. 1996) and are not currently recommended because of lack of consistent clinical benefit. However, current work developing anti-toxin malaria vaccines may raise similar concerns about transmission, due to downstream effects on TNF- $\alpha$ . For example, anti-GPI vaccines aim to neutralise the toxin which triggers the release of TNF- $\alpha$  and related cytokines from host immune cells (Schofield et al. 2002).

Just as vaccine evaluation has been proposed to include evolutionary assessments—such as effects of vaccination on parasite transmission (Read and Mackinnon 2008)—we feel therapies which target immune-mediated virulence should likewise evaluate evolutionary risk. Routine monitoring of how any new anti-malaria therapy affects 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. Where medical interventions fail to prevent parasite transmission, archiving of parasites to provide a detailed account of parasite diversity pre-, during- and post-treatments might help identify potential areas of concern and point towards the optimal strategy for control and ultimately parasite eradication (Read and Mackinnon 2008). Finally, empirical studies examining the within-host dynamics and transmission potential of pathogens in response to drug or vaccine pressure provide a fruitful avenue with which to investigate evolution of vaccine escape/facilitation (Grech et al. 2008; Long et al. 2010) or drug-resistance (Wargo et al. 2007; Huijben et al. 2010) and may also help make more informed decisions on optimal intervention for disease control in the presence of immunopathology.

### Future directions and outlook

The current lack of cross talk between molecular and evolutionary biologists regarding the causes of virulence and how they affect transmission makes predicting the trajectory of virulence evolution difficult. A more in-depth knowledge of the biological system at hand might very well lead to better quantitative predictions (Gandon et al. 2001), and here the trade-off is open to addition of much biological detail (and there is an abundance of biomedical knowledge available). Indeed, a deeper understanding of the mechanisms driving parasite virulence will help define relationships between parasite traits of key importance to the trade-off theory (Frank and Schmid-Hempel 2008). Work focused on *P. falciparum* show how layers of biological detail can be added to the trade-off model in order to provide a framework that can predict more quantitative outcomes (Alizon and van Baalen 2005) and

test hypotheses about virulence evolution in the field (Mackinnon and Marsh 2010). Tests of the nature and strength of relationships between parasite-encoded virulence (of any mechanism) and transmission are well within grasp of many empiricists. Such tests would go a long way in elucidating which selection pressures are the most powerful drivers of evolutionary change and what interventions are optimal for long-term parasite control.

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