Symposium

The Effect of Immunodeficiency on the Evolution of Virulence: An Experimental Test with the Rodent Malaria *Plasmodium chabaudi**

Victoria C. Barclay,[1,†] David A. Kennedy,[1,2] Veronika C. Weaver,[3] Derek Sim,[1] James O. Lloyd-Smith,[4] and Andrew F. Read[1,2,5]

1. Center for Infectious Disease Dynamics, Department of Biology, Pennsylvania State University, University Park, Pennsylvania 16802; 2. Fogarty International Center, National Institutes of Health, Bethesda, Maryland 20892; 3. Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, Pennsylvania 16802; 4. Department of Ecology and Evolutionary Biology, University of California, Los Angeles, California 90095; 5. Department of Entomology, Pennsylvania State University, University Park, Pennsylvania 16802

abstract: Host immunity plays an important role in the evolution of pathogen virulence and disease emergence. There is increasing theoretical and empirical evidence that enhanced immunity through vaccination may have the unfortunate side effect of selecting for more virulent parasites, but the effect of host immune suppression on pathogen evolution is less clear. Here, we use serial passage experiments in mice to test how immune-suppressed hosts may alter pathogen virulence evolution. We passaged *Plasmodium chabaudi* through $CD4^+$ T cell–depleted or control mice every 7 days for 20 weeks and then measured virulence differences during infection of immunologically normal mice. We found that those parasites that had been selected through $CD4^+$ T cell–depleted mice were more virulent than parasites selected through control mice. Virulence increases during serial passage are believed to be caused by pathogen adaptation to the passage host. These data suggest that immune-suppressed hosts could provide a within-host environment that lowers the barrier to parasite adaptation and promotes the evolution of virulence.

Keywords: immune suppression, evolution, virulence.

Introduction

Host populations always contain some individuals that are more vulnerable to infectious diseases than others. This is particularly so in human populations, where genetic deficiencies, malnutrition, immunosuppressive infections, aging, and modern medical practice can all result in immune deficiencies (Morris and Potter 1997). In many parts of the world, immunosuppression in humans is becoming more common. In rich countries, organ transplants and cancer treatments are becoming more widespread, and people are living longer. In poor countries, the human immunodeficiency virus (HIV) epidemic, overlaid on endemic immunosuppressive infections like helminths (Borkow and Bentwich 2004; Brown et al. 2006), continues to generate rising numbers of people with compromised immunity. Both mathematical models and examples from nature have shown that immune-suppressed hosts alter the ecology of infectious disease agents (Lloyd-Smith et al. 2008; Ezenwa et al. 2010; Ezenwa and Jolles 2011), especially since the rise of HIV (Abu-Raddad et al. 2006; Nga et al. 2012). For example, the likelihood that otherwise unsustainable pathogen strains can invade a host population increases with the fraction of immunosuppressed hosts (Lloyd-Smith et al. 2008). Here we empirically ask whether immunosuppressed hosts might also facilitate pathogen adaptation to immunologically normal hosts and whether this adaptation might result in the evolution of more virulent pathogens.

So far as we are aware, this question was first posed by Bruce Wallace in a short note in *The American Naturalist* almost 25 years ago (Wallace 1989, pp. 578–579). Wallace asked whether people with impaired immune systems might provide pathogens "with an opportunity for successfully adapting in a stepwise fashion to increasingly efficient immune systems and, at times, perhaps, for overwhelming them." Noting that there are questions that "cannot be so much as mentioned without violating cherished taboos of our time," he nonetheless asked whether immune-suppressed people might be stepping-stones by which opportunistic or innocuous organisms could adapt to become full-scale human pathogens. Note there is no doubt that infectious agents can be more virulent in immune-suppressed hosts. The question that Wallace asked

* This issue originated as the 2013 Vice Presidential Symposium presented at the annual meetings of the American Society of Naturalists.

† Corresponding author; e-mail: vbarclay@imseinstitute.org.

Am. Nat. 2014. Vol. 184, pp. S47–S57.
DOI: 10.1086/676887

and we are asking here is whether immunosuppressed hosts are an environment in which natural selection will favor strains of pathogens that cause more severe disease in hosts that are immunologically "normal."

It is hard to know a priori what the evolutionary impact of immunosuppression might be on rates of adaptation to novel hosts or to virulence evolution. Immune-suppressed hosts might indeed be the stepping-stones envisaged by Wallace, allowing pathogens to adapt to other aspects of host physiology and biochemistry without having to survive the onslaught of a full immune attack. Alternatively, immune-suppressed hosts might instead be very specialized environments in which pathogen adaptation results in a trade-off, with pathogens less able to deal with intact host immunity (Kubinak et al. 2012). In that case, adaptation to immune-deficient hosts might retard adaptation to immune-intact hosts.

Many mathematical models have demonstrated that host immunity can be a strong evolutionary force shaping patterns of pathogen virulence (Anderson and May 1982; Antia et al 1994; Nowak and May 1994; Casadevall and Pirofski 2001; Gandon et al. 2001, 2003; Mackinnon and Read 2004*a*, 2004*b*; Day et al. 2007). One group of models predicts that greater host immunity or resistance will favor the evolution of increased pathogen virulence. These models assume that pathogens can transmit only while their hosts are alive, and because survival time increases with host immunity, vaccine-induced immunoenhancement may allow more virulent strains to circulate (Gandon and Michalakis 2000; Gandon et al. 2001, 2003; Mackinnon and Read 2004*a*, 2004*b*; Barclay et al. 2012).

This evolutionary response to host immunity most likely explains why the myxoma virus became more virulent in the latter part of the twentieth century in Australia. Following decades of selection by this highly fatal virus, rabbit populations evolved more effective innate immunity, which in turn selected for increased virulence in the virus (when measured in naive laboratory rabbits; Fenner and Fantini 1999; Best and Kerr 2000; Kerr and McFadden 2002; Kerr 2012). An extension of these models, therefore, is that reductions in immune competence would then favor less virulent parasites. Other models, however, have suggested the opposite. For instance, Antia et al. (1994; see also King et al. 2009) suggested that the adaptive immune response may selectively target faster-growing virulent pathogen strains, which would provide a selective advantage to slower-growing, less virulent strains. In this case, reductions in immune competence would favor more virulent parasites.

Even in well-studied systems where there are good data on the relative fitness of more or less virulent parasites under immune pressure, it is difficult to predict the impact of immunodeficiency on host adaptation and virulence evolution. For instance, in the system we work on, the rodent malaria *Plasmodium chabaudi* in laboratory mice, serial passage through immunized hosts enhanced virulence as assayed in immunologically naive animals (Mackinnon and Read 2004*a*; Barclay et al. 2012). This is likely because more virulent malaria parasites are less rapidly cleared by host immunity (Gandon et al. 2003; Barclay et al. 2012). If better immune escape is indeed associated with virulence, as those experimental evolution studies suggest, it is tempting to conclude that immunosuppression will weaken selection for high virulence. However, within infections there is intense within-host competition among parasites. This competition is for resources (Barclay et al. 2008) or enemy-free space (Read and Taylor 2001; Råberg et al. 2006; Barclay et al. 2008). Competitive ability in this experimental system is correlated with virulence (de Roode et al. 2005*a*, 2005*b*; Bell et al. 2006). In $CD4^+$ T cell–depleted mice, competition between rodent malaria parasites is enhanced (Barclay et al. 2008), in part due to resource limitation (Hellriegel 1992; Hetzel and Anderson 1996; Haydon et al. 2003; Mideo et al. 2008). This suggests high virulence will be promoted by immunosuppression. In contrast, immune-mediated apparent competition has been shown to contribute to competitive suppression of avirulent malaria strains (Råberg et al. 2006). Under this scenario, immunosuppression may favor a reduction in virulence by alleviating competitive suppression of avirulent strains.

Thus, 25 years after Wallace asked whether immunosuppressed hosts might speed the rate of adaptation to new hosts and hence generate more virulent pathogens, we still have no clear idea of whether his concern is warranted. Here we report what we believe are the first experimental data addressing the issue. Increases in virulence during serial passage demonstrate that parasites can quickly adapt to novel hosts. We therefore ask whether passage through immune-suppressed hosts is accompanied by increases in parasite densities in immunologically normal hosts, our measure of host adaptation. *Plasmodium chabaudi* is naturally found in thicket rats (*Thamnomys rutilans*), so serial passage in laboratory mice gives them the opportunity for adaptation to mice, a novel host. Further, virulence and parasite densities in this system are correlated (reviewed by Mackinnon and Read 2004*b*, Mackinnon et al. 2008). We depleted mice of $CD4^+$ T cells as a measure of immune suppression, because these cells are pivotal in cellular immunity (Good et al. 2013). We then repeatedly passaged *P. chabaudi* through $CD4^+$ T cell–depleted or immune intact control mice and tested the effect of immune suppression on evolved pathogen virulence, expressed in immunologically normal mice. We found that parasites evolved in mice with suppressed im-

mune systems became more virulent to hosts with normal immunity.

Material and Methods

Parasites and Hosts

We used the DK clone of *Plasmodium chabaudi adami*, which was originally collected from thicket rats (*Thamnomys rutilans*) in the Congo Brazzaville (Landau 1965; Carter and Walliker 1976) and subsequently cloned by limiting dilution. Laboratory genotypes are stored as stable isolates in liquid nitrogen with subscript codes used to identify their position in clonal history (Mackinnon and Read 1999). Mice in our experiments were female C57Bl/6, at least 6–8 weeks old. Parasite densities were estimated from samples of tail blood using Giemsa-stained thin smears and red blood cell density estimated by flow cytometry (Beckman Coulter) or by genotype-specific real-time quantitative polymerase chain reaction (qPCR) assays as described previously (Bell et al. 2006). For amplification of the DK genotype, we used the forward primer previously used to amplify AS/AJ genotypes (Bell et al. 2006) and the DK genotpe-specific reverse primer 5′ GATTGTAGAGAAGTAGAAAATACAGATACAACTAA 3′.

$CD4^+$ T Cell Depletion

We use $CD4^+$ T cell depletion in mice as our model of immunosuppression. In this mouse–*P. chabaudi* model, $CD4^+$ T cells are important in the immune response to malaria, both for their cytokines and their help for antibody production (Stephens et al. 2005).

The antimouse, rat monoclonal antibody GK1.5 (ATCC TIB 207) was grown in Iscove's Modified Dulbecco's Medium (ATCC-formulated) plus fetal bovine serum (Thermo Scientific) in CELLine Bioreactor flasks (Argos Technologies). Cells were harvested after 7 days, and the antimouse $CD4^+$ T cell antibody was purified on a HiTrap Protein G affinity column (GE Healthcare). Mice were injected intraperitoneally with 200 μg of the purified antibody in phosphate-buffered saline 5, 4, and 1 days before parasite challenge and then once weekly after parasite challenge. A fluorescent-activated cell sorter was used to confirm $CD4^+$ T cell depletion as previously described (Barclay et al. 2008), and FlowJo analyses demonstrated depletion of $CD4^+$ T cells similar to that described by Barclay et al. (2008).

Serial Passages

Serial passage protocols were similar to those described previously (Barclay et al. 2012). The data we report here comes from an experiment in which parasites were passaged through three contemporaneous selection treatments: mice vaccinated with a recombinant malaria antigen (V-lines), through normal mice (C-lines), and through immunodeficient mice (D-lines; fig. 1). At the start (generation 1), five mice in each selection treatment were infected with 5×10^5 *P. c. adami* genotype DK_{247} (generation 0). Parasites from each one of the five mice at generation 1 were then used to infect at least two mice at generation 2 (forming a total of 10 sublines per treatment). Duplicate infections helped reduce the possibility of losing lines during the selection phase. Elsewhere, we reported the effect of vaccination (Barclay et al. 2012); here we report the impact of immunodeficiency. Thus, the data we report here on the C-lines are those reported earlier (Barclay et al. 2012); the new data here come from the D-lines and associated analysis.

Virulence Phenotyping

Virulence (minimum red blood cell densities) and clone performance (total parasite densities) were assessed in two separate "evaluation" experiments conducted after the serial passages. In all cases, frozen lines of *P. c. adami*–infected erythrocytes (IRBC) were first introduced into immune-intact donor mice, and then exact inoculations of 1×10^6 IRBC were introduced into immune-intact experimental (test) mice. Immune-intact donors are used because exact doses to initiate experimental infections cannot be obtained from frozen stock.

The first evaluation experiment compared the performance of parasites derived from 10 rounds of serial passage. The second evaluation experiment compared the performance of parasites derived from 21 rounds of serial passage with their unpassaged ancestral progenitors. In each of these experiments we compared the five C-lines with the five D-lines, with each line used to infect three mice. In the second experiment, nine mice were also infected with the ancestral parasites. During evaluation experiment 2, one mouse infected with C-line parasites died on day 5, and one mouse infected with C-line parasites received a lower parasite dose (peak parasite density was 1 order of magnitude lower than other C-line infected mice). Both mice were excluded from all analyses.

Statistical Analysis

All analyses were conducted in R 2.10.1 (R Development Core Team 2009). All parasite density data were log transformed to meet normality assumptions of the models.

Parasite densities were recorded on the day of serial passage. Log linear models were used to calculate differences in densities between $CD4^+$ T cell–depleted and con-

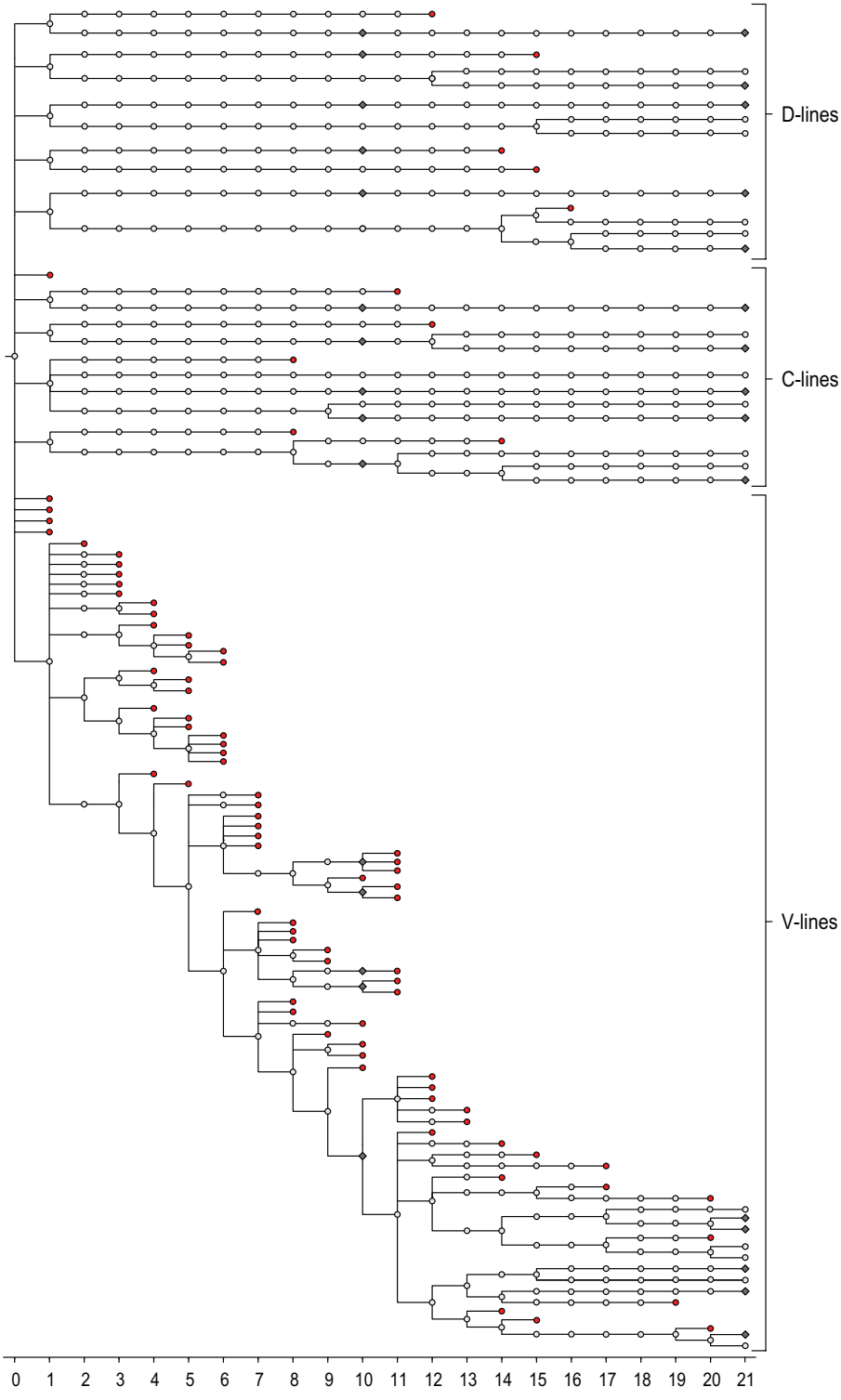

Figure 1: Experimental evolution in control, $CD4^+$ T cell–depleted, and AMA-1-vaccinated animals. Figure adapted from Barclay et al. 2012, figure S1. Control mice, mice previously depleted of $CD4^+$ T cells, or mice immunized with the AMA-1 vaccine were infected with *Plasmodium chabaudi adami* genotype DK_{247} (passage 1) to initiate the C-lines, D-lines, and V-lines, respectively. We previously reported the comparison of the C- and V-lines (Barclay et al. 2012); here we report on the comparison of the C- and D-lines. Red circles indicate where lines were lost due to insufficient parasites; in such instances, blood from a mouse in another line within that treatment group was used to infect at least two other mice in the next generation. Filled diamonds represent parasite lines used in the two different evaluation experiments.

trol mice across the selection phase (0 to 21). Maximal models (parasite densities = treatment ($CD4^+$ T cell depleted or control) + serial passage number + all higher-order interactions) were tested in the first instance, and minimal models were obtained by dropping nonsignificant terms successively, beginning with highest-order interactions, to obtain the significant minimal model.

For the evaluation phases, differences in minimum red blood cell densities and log peak parasite densities among the five independent replicate sublines (C-lines and D-lines) were first analyzed using mixed effect linear models, with subline as a random effect (Pinheiro and Bates 2000). In both evaluation experiments, there were no subline variances, therefore ANOVA models were used to calculate between treatment effects.

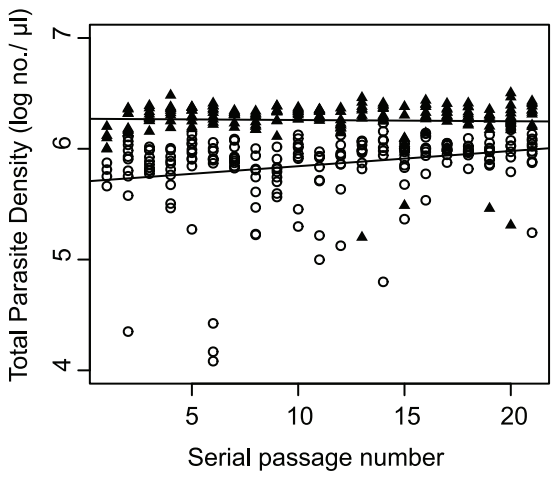

Figure 2: Parasite densities of each mouse during serial passage in control and $CD4^+$ T cell–depleted animals. Each data point represents the log parasite density of each mouse in the C-lines (open circles) or D-lines (filled triangles) from passage 1 to 21. Solid lines represent the log linear regression change in parasite density per selection treatment over time. Across serial passage, D-line parasites were always higher than C-line parasites ($F_{1,393} = 177.7$, $P < .001$), although C-line parasites did increase in density across time ($F_{3,391} = 66.5$, $P < .001$).

Results

We contemporaneously passaged *Plasmodium chabaudi adami* DK parasites every 7 days for 20 weeks through either control mice or through $CD4^+$ T cell–depleted mice. We refer to the parasite lines evolved under these contrasting conditions as C-lines and D (depleted)-lines respectively. We set out to measure the evolutionary response in five independent replicate lines of each type. We recorded the parasite densities in each individual mouse on the day of passage. Across the 20 weeks, parasite densities were higher in mice depleted of $CD4^+$ T cells than control mice (fig. 2; $F_{3,391} = 66.5$, $P < .001$). C-line parasites increased in density across the selection phase ($F_{1,196} = 13.8$, $P < .001$), but D-lines did not ($F_{1,195} = 0.1$, $P < .73$).

To assess the influence of passage on parasite virulence, we evaluated the virulence of the C- and D-lines in immune-intact mice at two time points during the evolution of the lines, once after 10 rounds of serial passage (evaluation experiment 1), and again after 21 rounds (evaluation experiment 2). In that latter experiment, we also assayed the virulence of the ancestral parasites (passage 0). In each of these evaluation experiments, virulence was measured in two ways: as the mean total parasite density and as the mean minimum red blood cell density (anemia).

Parasites passaged through both $CD4^+$ T cell–depleted mice (D-lines) and immunologically normal mice (C-lines) had higher total parasite densities than the ancestral parasites from which they were derived (fig. 4*D*). D-line parasites were also more virulent to immune-intact animals than C-line parasites (figs. 3*B*, 3*D*, 4*B*, 4*D*). This difference had already arisen by the tenth passage and was still apparent after 21 passages. Thus, in immune-intact mice, D-line parasites from both the tenth and twenty-first passage "generations" caused greater anemia than did C-line parasites (figs. 3*B*, 4*B*; $F_{1,28} = 31.6$, $P < .001$, and $F_{1,26} = 30.2$, $P < .001$, respectively). Thus, over the course of the experiment, parasites that evolved in $CD4^+$ T cell–depleted mice became more virulent to immune-intact animals than parasites that evolved in control mice.

The virulence differences apparent at the tenth round of selection were associated with differences in parasite densities (fig. 3). D-line parasites produced more parasites in total than did C-line parasites (fig. 3*D*; $F_{1,28} = 15.42$, $P < .001$). However, D-line parasites from 21 passages, while still inducing more anemia, did not achieve significantly higher densities than C-line parasites (fig. 4; D-lines vs. C-lines: $F_{1,26} = 3.4$, $P = .075$), even though they did achieve higher densities than ancestral parasites (fig. 4*D*; D-lines vs. ancestral: $F_{1,22} = 23.6$, $P < .001$).

Discussion

Twenty-five years ago, Wallace (1989) first raised the question of whether people with impaired immune systems could act as evolutionary gateways for opportunistic pathogens to become better able to replicate in people with normal immune systems. His concern seems all the more relevant today, where changing medical practices, aging populations, the HIV epidemic, and other immunosuppressive conditions reduce the efficacy of host immunity. Our findings go some way to reinforcing Wallace's concerns: we found that serial passage through immunosuppressed mice resulted in parasites that induced greater vir-

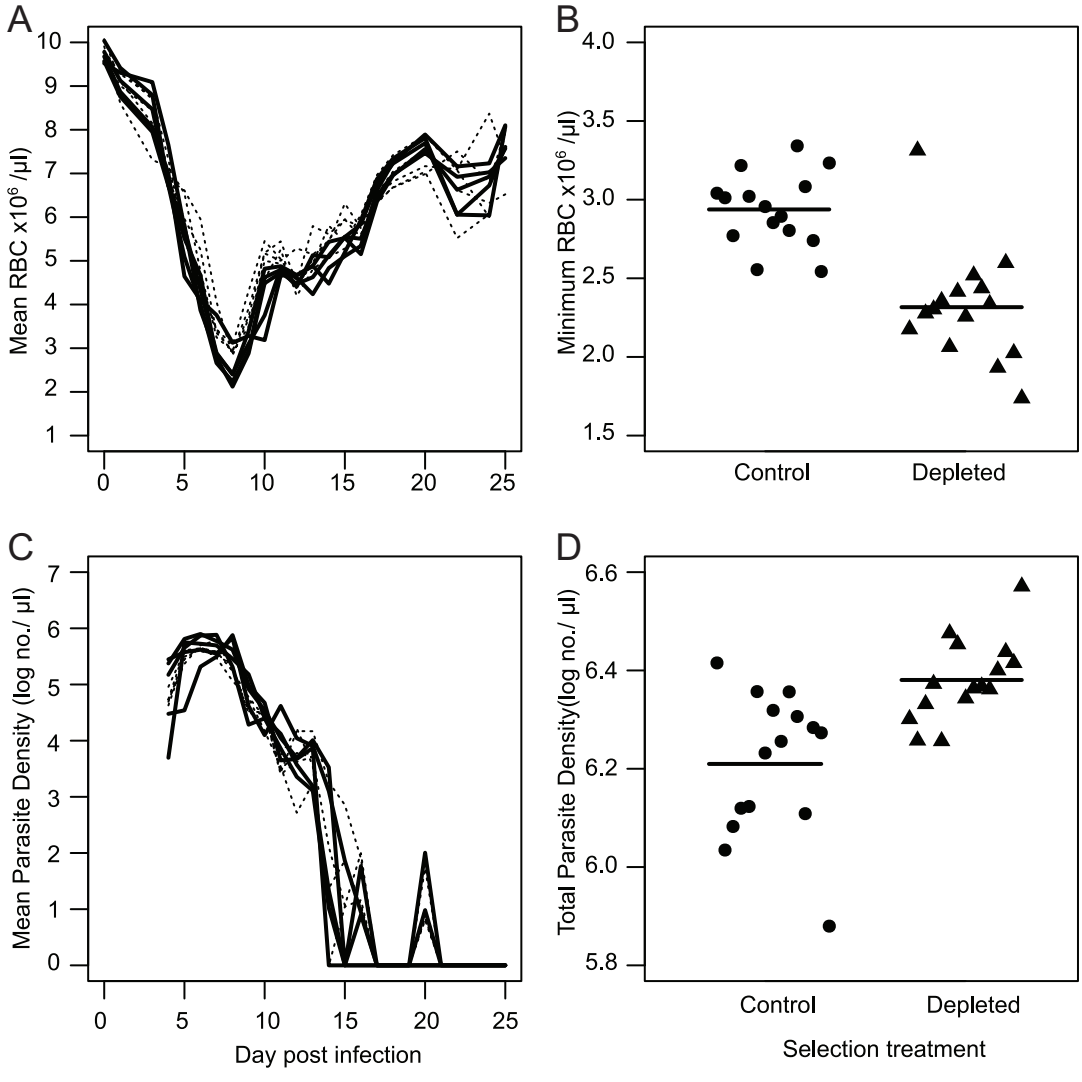

Figure 3: Red blood cell and parasite densities during infection of immunologically normal mice with parasites that had previously been passaged 10 times through control mice or 10 times through $CD4^+$ T cell–depleted mice (evaluation experiment 1). Curves (*A*, *C*) show the red blood cell (*A*) and parasite (*C*) kinetics of five C-lines (dotted) and five D-lines (solid), each assayed in up to 3 mice. Points on the scatterplots show the minimum red blood cell densities (*B*) and the log total parasite densities (*D*) of each individual mouse infected with C-lines (circles) or D-lines (triangles). Horizontal black lines indicate mean values. D-lines induced more severe anemia (*A*, *B*; $F_{1,28} = 31.6$, $P < .001$) and reached higher total parasite densities than their comparator C-lines (*C*, *D*; $F_{1,28} = 15.42$, $P < .001$).

ulence in immunologically normal mice than parasites selected through normal mice.

The Experiments

A very general finding from serial passage experiments in a wide range of host-parasite systems, including *Plasmodium chabaudi* in lab mice, is that pathogens become more virulent to the serial passage host (Ebert 1998; Mackinnon and Read 2004*a*; Barclay et al. 2012; Kubinak et al. 2012; Kubinak and Potts 2013; Spence et al. 2013). These patterns are assumed to be a consequence of pathogen adaptation to the serial passage host, since serial passage selects for higher pathogen densities at the time of transfer. We saw an increase in virulence for lines passaged through both immune-deficient and immunologically normal mice (fig. 4*C*, 4*D*). Interesting, our data also show that adaptation is faster in immune-deficient hosts: D-line parasite densities were already higher than C-line parasite densities after 10 passages (fig. 3*D*).

Formally, our data show only that immunosuppression increases the rate of virulence evolution. It is possible that

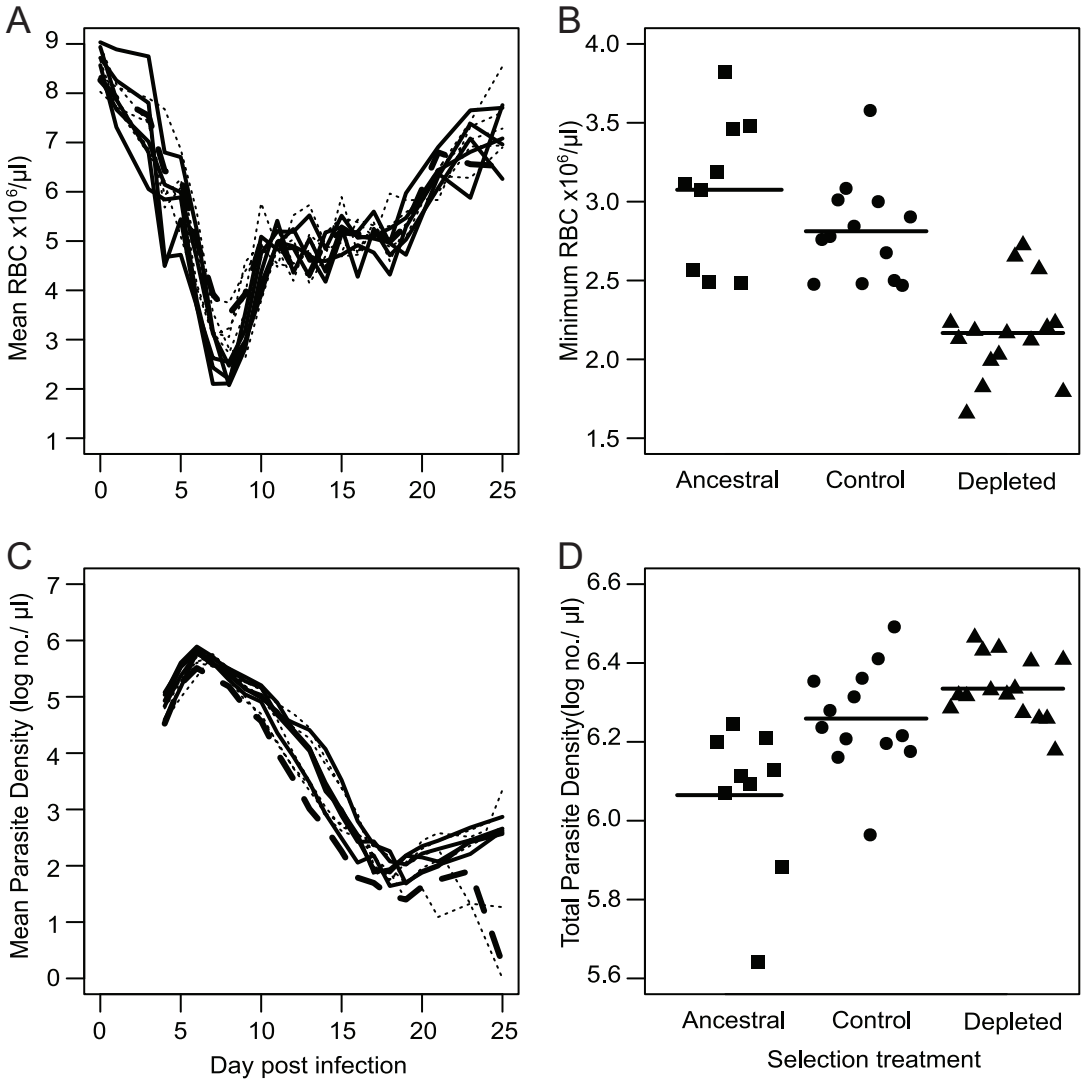

Figure 4: Red blood cell and parasite densities during infection of immunologically normal mice with parasites that had previously been passaged 21 times through control mice or 21 times through $CD4^+$ T cell–depleted mice (evaluation experiment 1). Curves (*A*, *C*) show the red blood cell (*A*) and parasite (*C*) kinetics of five C-lines (thin dotted curves) and five D-lines (solid curves), each assayed in up to 3 mice. The thick dotted curves are the mean of 9 mice infected with the ancestral lineage. Points on the scatterplots show the minimum red blood cell densities (*B*) and the log total parasite densities (*D*) of each individual mouse infected with C-lines (circles), D-lines (triangles), or ancestral parasites (squares). Horizontal black lines indicate mean values. D-lines induced more anemia than C-line parasites and ancestral parasites (*A*, *B*; $F_{1,26} = 30.2$, $P < .001$, and $F_{1,22} = 32.6$, $P < .001$, respectively). Both C-lines and D-lines reached higher total densities than ancestral parasites (*D*; $F_{1,20} = 7.9$, $P = .010$, and $F_{1,22} = 23.6$, $P < .001$ respectively), but the C-lines and D-lines did not differ from each other (*C*, *D*; $F_{1,26} = 3.4$, $P = .075$).

the C-line and D-line parasites eventually would have become similarly virulent if we had continued to passage the C-lines. There is some evidence of an upper bound to the densities and virulence that can occur following serial passage (Mackinnon and Read 2004*a*; Spence et al. 2013). Limitation in resources such as red blood cell availability could be responsible, because malaria parasites require red blood cells to replicate. Such an upper bound might explain why we observed no increases in density on the day of transfer in the immunodeficient animals with successive passage (fig. 2), an expected signature of adaptation. Presumably, alterations in growth rates did occur, but we did not assay infection kinetics in the serial passage animals before day 7.

Why would serial passage in immunodeficient mice result in parasites better adapted to growth in normal mice

than parasites serially passaged in normal mice? Throughout our serial passage study we found that $CD4^+$ T cell–depleted mice had higher parasite densities than control mice (fig. 2). Higher densities in immunodeficient mice could lead to evolution of higher virulence through two mechanisms. First, the total number of de novo mutations, the generator of heritable variation, increases as population sizes increase, and the rate of adaptation increases with heritable variation (Maynard Smith 1989). Thus, the larger pathogen population sizes in $CD4^+$ T cell–depleted mice compared to control mice may explain the observed higher virulence at the end of our experiment. Second, the larger densities and hence mutational diversity in $CD4^+$ T cell–depleted mice could result in enhanced competition between parasite variants. Experiments in *P. chabaudi* have shown that competitive suppression of less virulent strains to be enhanced in the denser parasite populations found in $CD4^+$ T cell–depleted mice compared to control mice (Barclay et al. 2008), in part due to resource limitation as more competitive strains can grow unchecked by immunity (Hellriegel 1992; Hetzel and Anderson 1996; Haydon et al. 2003; Barclay et al. 2008; Mideo et al. 2008). Thus, if large parasite populations generate more variants which compete, virulence could be driven upward.

Mathematical models representing the influence of immune suppression on pathogen adaptation could guide further measurements to clarify the crucial mechanisms at play. For instance, the first explanation above could hold only if the appearance of mutations is rate limiting and subsequent selection is strong enough for new variants to displace established strains in the short time available (in the limit, this corresponds to the strong selection, weak mutation regime explored in Gillespie 1984). Models could be used to compute quantitative bounds on what mutation rates and selection coefficients are consistent with the observed data, which in turn could be tested with targeted experiments or genome sequencing of stored samples.

A different explanation, and unrelated to differences in densities, is that by depleting $CD4^+$ T cells we might have reduced the selective advantage of slower-growing, less virulent strains (i.e., delayed immune clearance), allowing more virulent strains to expand in numbers and persist until onward transmission to a new host. This theory, first proposed by Antia et al. (1994) and subsequently developed by King et al. (2009), is based on the assumption that the adaptive immune response targets faster-growing, more virulent strains over slower-growing, less virulent strains. By depleting part of the cell-mediated immune response, could we have shifted evolution toward higher rates of virulence? We doubt this explanation for our data because in *P. chabaudi*, virulent strains are cleared more slowly by immunity (Gandon et al. 2003; Mackinnon and Read 2004*b*; Barclay et al. 2008). Thus, the biology of our system violates a key assumption of the models of Antia et al. (1994) and King et al. (2009).

Another possibility is that selection and adaptation are not involved, but instead the larger population sizes in the D-lines have allowed greater antigenic diversity to accumulate, either through mutation or through epigenetic mechanisms responsible for antigenic switching in *Plasmodium* (Jiang et al. 2013). Antigenically more diverse infections might be harder to control immunologically (Taylor et al. 1997), resulting in more parasites and greater virulence. Whether sufficient antigenic diversity can accumulate through our serial passage process is unclear. Comparative whole transcriptome analysis of D- with C-line parasites, as well as the ancestral lineage, could reveal any changes related to virulence and are the focus of ongoing investigations.

Interestingly, serial passage of parasites through mice immunized with either whole parasite preparation (Mackinnon and Read 2004*a*) or a candidate blood-stage malaria vaccine (Barclay et al. 2012) also results in parasites that are more virulent to immunologically normal, naive hosts. Presumably, immunization promotes the evolution of virulence in this system because low-virulence strains are rapidly cleared by immunity relative to more virulent strains (Mackinnon et al. 2008). The data we present here suggest that faster-growing high virulence strains will also be selectively favored in an immune-suppressed host. Thus, passage through immune-suppressed and immune-enhanced selected parasites did not result in any trade-offs detectable during infection of immune intact mice. Together, these data suggest that virulence is maintained at an intermediate level under "normal" host conditions. If those normal conditions are perturbed in either direction by vaccination or host immune suppression, virulence evidently evolves to increase.

Ultimately, it will be essential to consider parasite density, virulence, and transmissibility over the full duration of infection, across a whole range of immune-enhanced and immune-suppressed host types, which will help determine the consequences for selection in natural transmission settings. Simple models show that the chronic infections often associated with immune compromise can play an important facilitating role in pathogen evolutionary emergence (J. O. Lloyd-Smith et al., unpublished manuscript).

Wallace's Question

Our experimental data are consistent with the possibility that immunosuppressed people might set in play evolution that increases disease burdens, even in immunologically normally people. We are of course a long way from being able to assess the likelihood that this will happen. Most

obviously, generalizing from animal models is notoriously difficult, not least in malaria (reviewed in this context by Råberg et al. 2006; Wargo et al. 2007; Barclay et al. 2012). We have also bypassed mosquito transmission, a possible source of selection against virulence in this system (Ebert 1998), though we note that virulence differences generated by protocols very similar to ours are not eliminated by mosquito transmission (Mackinnon and Read 2004*a*; Mackinnon et al. 2005). If the directions of within- and between-host selection on virulence are in opposition, the question is how evolution would play out in nature (Park et al. 2013). Our data are consistent with positive within-host selection for virulence generated by immunodeficiency.

There are several other reasons to be cautious about generalizing from our results. First, there are many different ways in which a host can be immune suppressed (malnutrition, drugs, cancer, viruses, etc.), which lead to many different forms of immune suppression. Here we test only the effect of immune suppression using a model of $CD4^+$ T cell depletion in mice. $CD4^+$ T cells are fundamental in cellular immune responses, and their function is severely compromised during HIV infection, when the virus utilises $CD4^+$ T cells for replication (Sousa et al. 2002). Thus, although we do not directly test the effects of HIV infection on virulence evolution, our $CD4^+$ T cell depletion model mimics some of the processes that occur during HIV infection. Whether similar patterns would be observed during infection with HIV, or under another model of immune suppression, remains to be determined.

Second, the results may vary depending on the timing of passages. We chose to do our passages every 7 days, partly for convenience and partly because after 7 days naive mice begin mounting a strong acquired immune response against malaria (Druilhe and Khusmith 1987; Langhorne et al. 1989; Stevenson et al. 1992; von der Weld and Langhorne 1993; Mota et al.1998; Metcalf et al. 2011). Given that serial passage in both immunized (Mackinnon and Read 2004*a*; Barclay et al. 2012) and immunodeficient mice (this article) generated parasites more virulent to normal mice than did parasites evolved in control mice, the question becomes what retards adaptation and virulence evolution in the control lines. Without further experimentation, it is difficult to know whether passage earlier or later in the infections would result in accelerated rates of adaptation in control lines.

Finally we offer two other thoughts on the likelihood that immunosuppressed people could act as incubators of more virulent human pathogens. Immune heterogeneities are already rife in human populations. It would be an interesting question to determine the extent to which modern medical practices, or even the HIV epidemic, are of a sufficient scale to impact pathogen adaptation and virulence evolution. It is also important to know whether there is an interaction between externally imposed sources of immunosuppression, such as HIV infection and medical practices, and other sources of heterogeneity, such as host and pathogen genetic background.

In summary, our data are consistent with selection for increased virulence under host immune suppression. This is a strong call for additional research, in this system and in others, to determine the general nature of our results and the relevance of our findings to human diseases.

Acknowledgments

For discussion during the very long incubation of this project, we thank A. August, M. Boots, M. Cantorna, M. Ferrari, M. Mackinnon, M. Poss, members of the Read-Thomas lab group, and the Research and Policy in Infectious Disease Dynamics program of the Science and Technology Directorate, Department of Homeland Security, and the Fogarty International Center, National Institutes of Health. We thank M. Rabba for the configuration of figure 1. The work was funded by Pennsylvania State University (start-up funds to A.F.R.).

Literature Cited

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

Anderson, R. M, and R. M. May. 1982. Coevolution of hosts and parasites. Parasitology 85:411–426. doi:10.1017/S00311820000-55360.

Antia, R., B. R. Levin, and R. M. May. 1994. Within-host population dynamics and evolution and maintenance of microparasite virulence. American Naturalist 144:457–474.

Barclay, V. C., L. Råberg, B. H. K. Chan, S. Brown, D. Gray, and A. F. Read. 2008. CD4+T cells do not mediate within-host competition between genetically diverse malaria parasites. Proceedings of the Royal Society B: Biological Sciences 275:1171–1179. doi:10.1098/rspb.2007.1713.

Barclay, V. C., B. H. K. Chan, R. F. Anders, and A. F. Read. 2008. Mixed allele malaria vaccines: host protection and within-host selection. Vaccine 26:6099–6107. doi:10.1016/j.vaccine.2008.09.004.

Barclay, V. C., D. Sim, B. H. K. Chan, L. A. Nell, M. A. Rabaa, A. S. Bell, R. F. Anders, and A. F. Read. 2012. The evolutionary consequences of blood-stage vaccination on the rodent malaria *Plasmodium chabaudi*. PLoS Biology 10:e1001368. doi:10.1371/journal.pbio.1001368.

Bell, A. S., J. C. de Roode, D. Sim, and A. F. Read. 2006. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution 60:1358–1371.

Best, S. M, and P. J. Kerr. 2000. Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus

in resistant and susceptible European rabbits. Virology 267:36–48. doi:10.1006/viro.1999.0104.

Borkow, G, and Z. Bentwich. 2004. Chronic immune activation associated with chronic helminthic and human immunodeficiency virus infections: role of hyporesponsiveness and energy. Clinical Microbiology Reviews 17:1012–1030. doi:10.1128/CMR.17.4.1012-1030.2004.

Brown, M., P. A. Mawa, P. Kaleebu, and A. M. Elliott. 2006. Helminths and HIV infection: epidemiological observations on immunological hypotheses. Parasite Immunology 28:613–623. doi:10.1111/j.1365–3024.2006.00904.

Carter, R., and D. Walliker. 1976. Malaria parasites of rodents of the Congo (Brazzaville): *Plasmodium chabaudi adami* subsp. nov. and *Plasmodium vinckei lentum* Landau, Michel, Adam and Boulard, 1970. Annales de parasitologie humaine et comparée 51:637–646.

Casadevall, A., and L. Pirofski. 2001. Host-pathogen interactions: the attributes of virulence. Journal of Infectious Disease 184:337–344. doi:10.1086/322044.

Day, T., A. L. Graham, and A. F. Read. 2007. Evolution of parasite virulence when host responses cause disease. Proceedings of the Royal Society B: Biological Sciences 274:2685–2692. doi:10.1098/rspb.2007.0809.

de Roode, J C., M. E. H. Helinski, M. A. Anwar, and A. F. Read. 2005*a*. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. American Naturalist 166:531–542. doi:10.1086/491659.

de Roode, J. C., P. Pansini, S. J. Cheesman, M. E. H. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, B. H. K. Chan, D. Walliker, and A. F. Read. 2005*b*. Virulence and competitive ability in genetically diverse malaria infections. Proceedings of the National Academy of Sciences of the USA 102:7624–7628. doi:10.1073/pnas.0500078102.

Druilhe, P., and S. Khusmith. 1987. Epidemiological correlation between levels of antibodies promoting merozoite phagocytosis of *Plasmodium falciparum* and malaria-immune status. Infection and Immunity 55:888–891.

Ebert, D. 1998. Experimental evolution of parasites. Science 282:1432–1436. doi:10.1126/science.282.5393.1432.

Ezenwa, V. O., R. S. Etienne, G. Luikart, A. Beja-Pereira, and A. E. Jolles. 2010. Hidden consequences of living in a wormy world: nematode-induced immune suppression facilitates tuberculosis invasion in African buffalo. American Naturalist 176:613–624. doi:10.1086/656496.

Ezenwa, V. O., and A. E. Jolles. 2011. From host immunity to pathogen invasion: the effects of helminth coinfection on the dynamics of microparasites. Integrative and Comparative Biology 51:540–551. doi:10.1093/icb/icr058.

Fenner, F., and B. Fantini. 1999. Biological control of vertebrate pests: the history of myxomatosis, an experiment in evolution. CABI, Wallingford.

Gandon, S, M., J. Mackinnon, S. A. Nee, and F. Read. 2001. Imperfect vaccines and the evolution of pathogen virulence. Nature 414:751–756. doi:10.1038/414751a.

Gandon, S, Mackinnon, M. J., Nee, S., and A. F. Read. 2003. Imperfect vaccination: some epidemiological and evolutionary consequences. Proceedings of the Royal Society B: Biological Sciences 270:1129–1136. doi:10.1098/rspb.2003.2370.

Gandon, S., and Y. Michalakis. 2000. Evolution of parasite virulence against qualitative or quantitative host resistance. Proceedings of the Royal Society B: Biological Sciences 267:985–990. doi:10.1098/rspb.2000.1100.

Gillespie, J. H. 1984 Molecular evolution over the mutational landscape. Evolution 38:1116–1129.

Good, M. F., J. M. Reiman, I. B. Rodriguez, K. Ito, S. K. Yanow, I. M. El-Deeb, M. R. Batzloff, et al. 2013. Cross-species malaria immunity induced by chemically attenuated parasites. Journal of Clinical Investigation. doi:10.1172/JCI66634.

Haydon, D. T., L. Matthews, R. Timms, and N. Colegrave. 2003. Top-down or bottom-up regulation of intra-host blood-stage malaria: do malaria parasites most resemble the dynamics of prey or predator? Proceedings of the Royal Society B: Biological Sciences 270:289–298. doi:10.1098/rspb.2002.2203.

Hellriegel, B. 1992. Modelling the immune response to malaria with ecological concepts: short-term behaviour against long-term equilibrium. Proceedings of the Royal Society B: Biological Sciences 250:249–256. doi:10.1098/rspb.1992.0156.

Hetzel, C., and R. M. Anderson. 1996. The within-host cellular dynamics of bloodstage malaria: theoretical and experimental studies. Parasitology 113:25–38. doi:10.1017/S0031182000066245.

Jiang, L., J. Mu, Q. Zhang, T. Ni, P. Srinivasan, K. Rayavara, W. Yang, et al. 2013. PfSETvs methylation of histone H3K36 represses virulence genes in *Plasmodium falciparum*. Nature 499:223–227. doi:10.1038/nature12361.

Ebert, D., and J. J. Bull. The evolution and expression of virulence. Pages 153-167 *in* S. C. Stearns and J. C. Koella, eds. Evolution in health and disease. 2nd. ed. Oxford University Press, Oxford.

Kerr, P. J. 2012. Myxomatosis in Australia and Europe: a model for emerging infectious diseases. Antiviral Research 93:387–415. doi:10.1016/j.antiviral.2012.01.009.

Kerr, P. J, and G. McFadden. 2002. Immune responses to myxoma virus. Viral Immunology 15:229–246. doi:10.1089/08828240260066198.

King, A. A., S. Shrestha, E. T. Harvill, and O. N. Bjørnstad. 2009. Evolution of acute infections and the invasion-persistence tradeoff. American Naturalist 173:446–455. doi:10.1086/597217.

Kubinak, J. L., J. S. Ruff, C. W. Hyzer, P. R. Slev, and W. K. Potts. 2012. Experimental viral evolution to specific host MHC genotypes reveals fitness and virulence trade-offs in alternative MHC types. Proceedings of the National Academy of Sciences of the USA 109:3422–3427

Kubinak, J. L., and W. K. Potts. 2013. Host resistance influences patterns of experimental viral adaptation and virulence evolution. Virulence 4:410–418.

Landau, I. 1965. Description of *Plasmodium chabaudi*. Parasite of African rodents. Comptes rendus hebdomadaires des séances de l'Académie des sciences 260:3758–3761.

Langhorne, J., S. Gillard, B. Simon, S. Slade, and K. Eichmann. 1989. Frequencies of CD4+ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. International Immunology 1:416–424.

Lloyd-Smith, J. O., M. Poss, and B. T. Grenfell. 2008. HIV-1/parasite co-infection and the emergence of new parasite strains. Parasitology 135:795–806. doi:10.1017/S0031182008000292.

Janeway C. A., P. Travers, M. Walport, and M. Shlomchik. 2001. Immunobiology 5. Garland, New York.

Mackinnon, M. J., D. J. Gaffney, and A. F. Read. 2002. Virulence in malaria: host genotype by parasite genotype interactions. Infection, Genetics and Evolution 1:287–296.

Mackinnon, M. J., and A. F. Read. 1999. Selection for high and low virulence in the malaria parasite. Proceedings of the Royal Society B: Biological Sciences 266:741–748. doi:10.1098/rspb.1999.0699.

———. 2004*a*. Immunity promotes virulence evolution in a malaria model. PLoS Biology 2:E230. doi:10.1371/journal.pbio.0020230.

———. 2004*b*. Virulence in malaria: an evolutionary viewpoint. Philosophical Transactions of the Royal Society B: Biological Sciences 359:965–986. doi:10.1098/rstb.2003.1414.

Mackinnon, M. J., A. S. Bell, and A. F. Read. 2005.The effects of mosquito transmission and population bottlenecking on virulence, multiplication rate and rosetting in rodent malaria. International Journal for Parasitology 35:145–153.

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

Maynard Smith, J. 1989. Evolutionary genetics. Oxford University Press, Oxford.

Metcalf, C. J. E., A. L. Graham, S. Huijben, V. C. Barclay, G. H. Long, B. T. Grenfell, A. F. Read, and O. N. Bjørnstad. 2011. Partitioning regulatory mechanisms of within-host malaria dynamics using the effective propagation number. Science 333: 984–988. doi: 10.1126/science.1204588.

Mideo, N., V. C. Barclay, B. H. K. Chan, N. J. Savill, A. F. Read, and T. Day. 2008. Understanding and predicting strain-specific patterns of pathogenesis in the rodent malaria *Plasmodium chabaudi*. American Naturalist 172:214–238. doi:10.1086/591684.

Morris, J. G, and M. Potter. 1997. Emergence of new pathogens as a function of changes in host susceptibility. Emerging Infectious Diseases 3:435–441.

Mota, M. M., K. N. Brown, A. A. Holder, and W. Jarra. 1998. Acute *Plasmodium chabaudi chabaudi* malaria infection induces antibodies which bind to the surfaces of parasitized erythrocytes and promote their phagocytosis by macrophages in vitro. Infection and Immunity 66:4080–4086.

Nga, T. V. T., C. M. Parry, T. Le, N. P. H. Lan, T. S. Diep, J. I. Campbell, N. V. M. Hoang, et al. 2012. The decline of typhoid and the rise of non-typhoid salmonellae and fungal infections in a changing HIV landscape: bloodstream infection trends over 15 years in southern Vietnam. Transactions of the Royal Society of Tropical Medicine and Hygiene 106:26–34. doi:10.1016/j.trstmh .2011.10.004

Nowak, M. A, and R. M. May. 1994. Superinfection and the evolution of parasite virulence. Proceedings of the Royal Society B: Biological Sciences 255:81–89. doi:10.1098/rspb.1994.0012.

Park, M. H., C. Loverdo, S. J. Schreiber, and J. O. Lloyd-Smith. 2013. Multiple scales of selection influence the evolutionary emergence of novel pathogens. Philosophical Transactions of the Royal Society B: Biological Sciences. 368:20120333.

Pinheiro, J. C., and D. M. Bates. 2000. Fitting linear mixed-effects models. *In* Statistics and computing: mixed-effects models in S and S-plus. Springer, New York.

Potter, J. G., and M. Morris Jr. 1997. Emergence of new pathogens as a function of changes in host susceptibility. Emerging Infectious Diseases 3:433–441.

R Development Core Team. 2009. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org.

Råberg, L., J. C. de Roode, A. S. Bell, P. Stamou, D. Gray, and A. F. Read. 2006. The role of immune-mediated apparent competition in genetically diverse malaria infections. American Naturalist 168: 41–53. doi:10.1086/505160.

Read, A. F., and L. H. Taylor. 2001. The ecology of genetically diverse infections. Science 292:1099–1102. doi:10.1126/science.1059410.

Sousa, A. E., J. Carneiro, M. Meier-Schellersheim, Z. Grossman, and R. M. M. Victorino. 2002. CD4 T cell depletion Is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. Journal of Immunology 169: 34003406.

Spence, P. J., W. Jarra, P. Lévy, A. J. Reid, L. Chappell, T. Brugat, M. Sanders, M. Berriman, and J. Langhorne. 2013. Vector transmission regulates immune control of *Plasmodium* virulence. Nature 498:228–231. doi:10.1038/nature12231.

Stephens, R., F. R. Albano, S. Quin, B. J. Pascal, V. Harrison, B. Stockinger, D. Kioussis, H. U. Weltzien, and J. Langhorne. 2005. Malaria-specific transgenic $CD4^+$ T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. Blood 106:1676–1684.

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

Taylor, L. H., D. Walliker, and A. F. Read. 1997. Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. Proceedings of the Royal Society B: Biological Sciences 264:927–935. doi:10.1098 /rspb.1997.0128.

von der Weld, T., and J. Langhorne. 1993. Altered response of CD4 +T cell subsets to *Plasmodium chabaudi chabaudi* in B cell–deficient mice. International Immunology 5:1343–1348. doi:10.1093 /intimm/5.10.1343.

Wallace, B. Can "stepping stones" form stairways? 1989. American Naturalist 133:578–579.

Wargo, A. R., S. Huijben, J. C. de Roode, J. Shepherd, and A. F Read. 2007. Competitive release and facilitation of drug-resistant parasites after therapeutic chemotherapy in a rodent malaria model. Proceedings of the National Academy of Sciences of the USA 104: 19914–19919. doi:10.1073/pnas.0707766104.

Symposium Editor: Curtis M. Lively