Host resistance influences patterns of experimental viral adaptation and virulence evolution

Jason L Kubinak1,2,* and Wayne K Potts2

1Division of Microbiology and Immunology; Department of Pathology; School of Medicine; University of Utah; Salt Lake City, UT USA;
2Department of Biology; University of Utah; Salt Lake City, UT USA

Keywords: host resistance, genetic diversity, viral adaptation, serial passage, experimental evolution

Infectious diseases are major threats to all living systems, so understanding the forces of selection that limit the evolution of more virulent pathogens is of fundamental importance; this includes the practical application of identifying possible mitigation strategies for at-risk host populations. The evolution of more virulent pathogens has been classically understood to be limited by the tradeoff between within-host growth rate and transmissibility. Importantly, heterogeneity among hosts can influence both of these factors. However, despite our substantial understanding of how the immune system operates to control pathogen replication during infection, we have only a limited appreciation of how variability in intrinsic (i.e., genetically determined) levels of host resistance influences patterns of pathogen adaptation and virulence evolution. Here, we describe results from experimental evolution studies using a model host–pathogen (virus–mammal) system; we demonstrate that variability in intrinsic levels of resistance among host genotypes can have significant effects on patterns of pathogen adaptation and virulence evolution during serial passage. Both the magnitude of adaptive response as well as the degree of pathogen specialization was positively correlated with host resistance, while mean overall virulence of post-passage virus was negatively correlated with host resistance. These results are consistent with a model whereby resistant host genotypes impose stronger selection on adapting pathogen populations, which in turn leads to the evolution of more specialized pathogen variants whose overall (i.e., mean) virulence across host genotypes is reduced.

Introduction

To reduce the burden of infectious disease it is essential that we identify the processes that limit the evolution and spread of pathogenic microorganisms. Variability in levels of resistance among hosts is thought to be an important force of selection influencing these phenomena.1-6 Additionally, theoretical considerations regarding the evolution of virulence (here defined as infection-induced host morbidity/mortality) are dominated by the assumption that virulence is an emergent property of pathogen fitness tradeoffs between within-host growth rate and transmissibility.7 This further implicates variability in resistance among hosts as a crucial factor influencing infectious disease dynamics in nature as it represents a direct force of selection capable of acting on both of these pathogen phenotypes. Finally, the multiple studies demonstrating that genetic variation within host populations can limit the burden of infectious disease6,8-15 imply that polymorphisms among individuals (e.g., at resistance loci) are important determinants of the epidemiological and evolutionary outcomes of host–pathogen interactions. However, despite our substantial understanding of how the immune system operates to control pathogen replication during infection,6,8-15 we have only a limited appreciation of how intrinsic (i.e., genetically determined) differences in levels of resistance among hosts influences patterns of pathogen adaptation and virulence evolution. Importantly, resistance variability among hosts can be defined in both qualitative (differences in resistance mechanisms employed) and quantitative terms (difference in intensity of immune response). Here, we make no discrimination and instead take the conservative approach of defining resistance as a host genotype’s capacity to control pathogen replication.

The predicted direction of pathogen adaptation and virulence evolution in response to resistance variability among hosts is controversial. Previous work in experimental host–pathogen systems has shown that artificial enhancement (e.g., through vaccination or drug therapy) of host resistance can favor virulent pathogen genotypes over avirulent competitors during co-infection17-20. Additionally, work from our lab employing serial passage of Cryptococcus neoformans (a generalist fungal pathogen) through different genotypes of inbred mice demonstrated that the host genotype a pathogen stock was serially passaged through had a significant influence on the trajectory of pathogen adaptation...
and virulence evolution. Moreover, in this study serial passage through the most resistant host genotype produced the most broadly virulent (i.e., highest mean virulence across multiple host genotypes) post-passage *C. neoformans* stock, but this stock came from a human isolate and it has been suggested that most of the virulence evolution came from adaptation to “mouse”, rather than adaptation to mouse polymorphisms.21 Studies in wild populations have also found associations between resistant host populations and higher frequencies of virulent strains of pathogens, further supporting the idea that resistant hosts favor more virulent pathogens.1,22,23

However, there is evidence for the opposing view. For example, it has been observed that serial passage of Marek disease virus through susceptible rather than resistant MHC-congenic genotypes of chickens selects for a more virulent pathogen strain,24 and that passage of a plant virus on a susceptible host ecotype led to the evolution of a pathogen strain capable of infecting and causing disease in highly resistant host ecotypes.25 It has also been argued that because within-host competition can favor virulent over avirulent pathogen strains, that resistant hosts may impede virulence evolution by limiting competitive interactions between co-infecting pathogens.26 Finally, it is also possible that hosts with intermediate levels of immunity may provide the optimal environment for adaptation as both the strength of immune-mediated selection and pathogen population size may be optimized in such individuals.5

Recently, our lab used serial passage of Friend virus (FV) complex through distinct genotypes of inbred mice (see Materials and Methods for more detailed description of host–pathogen system) to study how genetic variability among hosts influences patterns of viral adaptation and virulence evolution.27,28 FV complex is a mouse-specific retroviral pathogen that is an established model in retroviral immunology as well as virally-induced oncogenesis.29–31 In these experiments we found that serial passage led to rapid increases in viral fitness, which consequently resulted in significantly more severe disease. More importantly, serial exposure to one host genotype resulted in fitness tradeoffs when adapted viruses were exposed to a host with an unfamiliar genotype, implying that pathogen adaptation is host genotype-specific.27,28

Here, we expand upon these experiments to test multiple predictions that arise from the hypothesis that differences in levels of resistance to infection among host genotypes influences patterns of pathogen adaptation and virulence evolution in a predictable manner. Specifically, we tested the following predictions: (1) that variability in levels of resistance among distinct host genotypes influences the magnitude of a pathogen adaptive response, (2) that variability in levels of resistance influences the degree of pathogen specialization for a given host genotype, and (3) that variability in levels of host resistance influences evolved patterns of overall virulence (i.e., mean virulence across different host genotypes). Data from our experiments also provide insights into how virulence evolution may influence the nature of selection on host resistance, which will be discussed as well. Collectively, results from our experiments have important implications regarding the influence resistance variability among different host genotypes has on the direction, rate, and disease consequences of a rapidly adapting viral pathogen.

**Results**

**Differences in levels of resistance among host genotypes influences the magnitude of pathogen adaptive responses.** Results from two previous studies conducted in our lab demonstrated that viral fitness and virulence both increased dramatically after serial passage of FV complex through a series of hosts from the same genotype.27,28 Here, in order to determine if there were significant differences in baseline resistance to FV complex infection, we compared the mean viral fitness of unpassaged FV complex among cohorts of animals (*n* = 7–12 per cohort) from each of the five different host genotypes used in these previous experiments. Variability in levels of resistance to FV infection was evident as host genotype had a significant effect on infectious virus particle titers among these cohorts (ANOVA [host genotype] , *F* 4,91 = 16.98, *P* < 0.0001). There was no significant effect of host genotype when comparing baseline resistance estimates among A/WySn and BALB/c hosts (ANOVA [host genotype] , *F* 3,42 = 0.33, *P* = 0.80), although all three BALB/c genotypes on average had higher viral loads compared with the A/WySn host genotype. On the other hand, pair-wise comparisons between the DBA/2J host genotype and all other host genotypes demonstrated highly significant differences (Fig. 1A; Table S1). Thus, there are clear differences in baseline resistance to infection among our host genotypes. The A/WySn genotype is the most resistant, the BALB/c genotypes are intermediate in their resistance, and the DBA/2J genotype is the least resistant.

We next estimated the magnitude of the adaptive response made by a given post-passage virus stock. Host-genotype-of-passage had a highly significant effect on the observed magnitudes of viral adaptive responses (ANOVA [host-genotype-of-passage] , *F* 4,91 = 75.21, *P* < 0.0001) (Fig. 1B; Table S2), which supports genetic variation among host genotypes as an important force of selection influencing the magnitude of pathogen adaptation during the course of serial passage. More importantly, and consistent with our hypothesis, we observed a significant correlation between host resistance to infection and the magnitude of adaptive responses made by a given post-passage virus stock such that serial passage through more resistant hosts resulted in larger pathogen adaptive responses (ANOVA [host-genotype-of-passage] , *R* 2 = 0.97, *F* 1,4 = 94.29, *P* = 0.002) (Fig. 1C). Finally, we also found that A/WySn- and BALB/c-passaged stocks also demonstrated significantly larger magnitude increases in virulence after serial passage compared with DBA/2J-passaged virus (Table S3). Hence, serial passage of FV complex through more resistant host genotypes results in proportionally larger adaptive responses in post-passage viruses.

**Differences in levels of resistance among host genotypes influences the degree of pathogen specialization.** Previous work in our lab has shown that FV complex can adapt to unique host genotypes27 i.e., become specialized. Importantly, we are defining specialization here as adaptations that result in increased viral fitness in one host genotype at the cost of viral fitness in a
host with a different genotype (a consequence of host genotype-specific pathogen adaptation). Thus, another independent test of the hypothesis that differences in levels of resistance among host genotypes influences patterns of pathogen adaptation is to compare the magnitude of pathogen specialization (calculation and rationale explained in Materials and Methods) that emerges after serial passage through hosts with varying grades of resistance. As predicted, we observed a significant negative correlation between the mean degree of pathogen specialization and viral fitness in unfamiliar hosts (ANOVA[speciation × fitness], $R^2 = 0.27, F_{1,17} = 5.87, P = 0.03$); i.e., more specialized viruses have lower average fitness when infecting novel host genotypes (Fig. 2A). Similar to the results above, the host genotype a stock was passaged through had a highly significant effect on the observed degree of pathogen specialization (ANOVA[passage history], $F_{4,263} = 4.98, P = 0.0007$). Critically, we also detected a significant positive correlation between host resistance and specialization (ANOVA[resistance × specialization], $R^2 = 0.94, F_{1,4} = 46.68, P = 0.006$) (Fig. 2B). The most resistant host genotype (i.e., A/WySn) produced the most specialized virus stock, the intermediate BALB/c genotypes produced intermediate specialists, and the least resistant DBA/2J genotype produced the least specialized virus (Fig. 2B; Table S4).

Differences in levels of resistance among host genotypes influence patterns of overall virulence. We observed a significant negative correlation between baseline host resistance and the overall virulence (i.e., mean virulence across host genotypes) of a post-passage stock derived from that genotype (ANOVA[resistance × overall virulence], $R^2 = 0.18, F_{1,23} = 4.92, P = 0.04$) (Fig. 3A). Moreover, as our data indicated that more resistant host genotypes produce more specialized viruses (Fig. 2B and C), we predicted that overall virulence of a post-passage stock would also be negatively correlated with its degree of specialization since the fitness of specialized viruses is more sensitive to differences between host genotypes. This assumes that pathogen fitness and virulence are positively correlated, which is the case in our experimental system (Table S5). In agreement with our prediction, we found that the A/WySn-passaged stock (most specialized) was the least generally virulent, the BALB/c-passaged stocks (intermediate specialists) were intermediate in their overall virulence, and the least specialized DBA/2J-passaged stock was the most.

**Figure 1.** Differences in levels of resistance among host genotypes influence the magnitude of pathogen adaptive responses. (A) There is variability in resistance to unpassaged FV complex infection among the five host genotypes used in this study as demonstrated by significant differences in infectious virus particle titers among genotypes. (B) There are significant differences among post-passage virus stocks in the magnitude of adaptive response made during passage through each of the five host genotypes. (C) There is a significant positive correlation between baseline host resistance to infection with FV complex and the magnitude of a pathogen adaptive response. Significance values in (A and B) are based on the results of a Student t test for pair-wise comparisons. The line of fit for the regression analysis in (C) is based on a power law. In (A–C) errors bars represent SEM. See Tables S1 and S2 for a summary of all pair-wise statistical analyses.
generally virulent pathogen (Fig. 3B; Table S6). Additionally, we detected a significant negative correlation between a post-passage stocks’ mean degree of specialization and overall virulence (ANOVA $R^2 = 0.22, F_{1,4} = 7.64, P = 0.02$), which implies a tradeoff between pathogen specialization and virulence (Fig. 3C).

**Virulence and selection along the host resistance-tolerance axis.** Finally, in addition to resistance, tolerance (i.e., the ability of a host genotype to sustain higher infection intensities without more severe disease) is another means by which hosts can protect themselves from the negative fitness consequences associated with infection (i.e., virulence). This is an important distinction because tolerance reduces the cost of virulence for both host and pathogen and can dramatically influence patterns of pathogen adaptation and virulence evolution.32,33 Tolerance can be defined as the rate at which host health declines as a function of pathogen load, and can thus be estimated as the slope of the regression line between pathogen load and virulence for each host genotype (typically referred to as a “reaction norm”).32,33 Using an analysis of covariance (ANCOVA), we found that host genotype significantly influenced the correlation between pathogen load and virulence, indicating that there was significant variability in levels of tolerance among the host genotypes used in this study (ANCOVA $R^2 = 0.94, F_{4,360} = 17.93, P < 0.0001$). More importantly, by comparing how resistance and tolerance co-varies among host genotypes as a function of disease severity we can infer in what direction virulence evolution may skew selection along the resistance–tolerance axis. Interestingly, our data imply that there is a fitness tradeoff between resistance and tolerance such that more resistant and less tolerant genotypes suffer disproportionately from disease vs. less resistant and more tolerant host genotypes (Fig. 4). Thus, results from our experimental host–pathogen system imply that selection (at least in the short-term) may favor less resistant, and more tolerant, host genotypes.

**Discussion**

Here, we have described a set of experiments using a model host–pathogen system demonstrating that variability in intrinsic levels of resistance among host genotypes can have profound effects on pathogen adaptation and virulence evolution. We observed that the magnitude of a pathogen’s adaptive response as well as...
the degree of pathogen specialization was positively correlated with host resistance. However, the mean virulence of a post-passage stock was negatively correlated with host resistance and the degree of pathogen specialization. These results are consistent with a model whereby resistant host genotypes impose stronger selection on pathogen populations to adapt, which in turn leads to the evolution of more specialized pathogen variants whose mean virulence across host genotypes is reduced. To our knowledge, this is one of the first studies to suggest that resistant hosts can inhibit the overall virulence of a pathogen species by promoting specialization.

Host–pathogen antagonistic coevolution theory is based on the assumption that reciprocal forces of selection between hosts and their pathogens promotes cycles of adaptation and counter-adaptation, and recent studies have demonstrated that such co-evolutionary dynamics can indeed play a major role in determining rates of molecular evolution in both hosts and their pathogens. In this model, host resistance is thought to be the primary driver of pathogen adaptation, and virulence associated with infection is predicted to be the primary driver of host adaptation. Thus, given the intimate relationship predicted to exist between these variables it is surprising that few studies have focused on experimentally defining the relationship between host resistance, pathogen adaptation, and virulence evolution. In fact, most of the previous work on the subject in vertebrates has been performed using a single host–pathogen model, the well-developed mouse–malaria

Figure 3. There is an inverse correlation between the degree of specialization of a post-passage stock and its overall virulence. (A) There is a significant negative correlation between baseline resistance and overall virulence (i.e., mean virulence across host genotypes) of a post-passage virus stock. Mean virulence estimates from each genotype a post-passage virus was tested in are shown in (A). (B) The mean virulence associated with infection by each post-passage stock was compared. A highly specialized virus (A/WySn-passaged virus) is the least generally virulent, intermediate specialists (the BALB/c-passaged viruses) are intermediate in virulence, and the least specialized (DBA/2J-passaged virus) was the most generally virulent. (C) There is a significant negative correlation between the degree of pathogen specialization and overall virulence of a post-passage virus stock. Again, mean virulence estimates from each genotype a post-passage virus was tested in are shown in (C). Significance values in (B) are based on the results of a Student t test for pair-wise comparisons. The lines of fit in (A and C) are based on linear regression. Error bars in (B) represent SEM. See Table S5 for a summary of all pair-wise statistical analyses.
system. Most recently, Barclay et al.\textsuperscript{17} demonstrated that serial passage of the malaria parasite \textit{Plasmodium chabaudi} through vaccinated vs. unvaccinated mice resulted in the emergence of a significantly more virulent pathogen stock. Another recent study has also demonstrated that virulent vs. avirulent malaria clones had a selective advantage during infection of animals treated with antimalarial drugs (during both single and mixed infection).\textsuperscript{19} These studies suggest that virulence evolution may be an unintended consequence of artificial enhancement of host resistance through vaccination or drug therapy. Additionally, Mackinnon et al.\textsuperscript{18} used serial passage of rodent malaria through immunized vs. non-immunized mice to show that passage through immunized animals selects for more virulent malaria parasites.\textsuperscript{18} Results from our experiments are in contrast to these previous studies and suggest that resistant host genotypes can favor the evolution of less generally virulent pathogens through enhanced selection for specialization in a pathogen population.

We show that viruses emerging from more resistant hosts have lower overall virulence than viruses emerging from more susceptible hosts. This might seem to be in conflict with the above results. However, there are numerous differences between these studies and ours that could account for the apparent inconsistencies. First, and probably foremost, the primary effector in vaccinated hosts are antibodies,\textsuperscript{17} whereas the primary effector in our 12-d infections are T cells.\textsuperscript{37,38} Plasmodium escape from the initial humoral response could cause subsequent infections in both vaccinated and unvaccinated mice to be more virulent because critical B-cell epitopes have been eliminated. Second, all of these malaria studies operate on a very different experimental paradigm than our study. The malaria studies described above evaluate virulence among cohorts of animals from the same host genotype, whereas in our study viruses adapted to one host genotype are tested in other host genotypes where virulence is generally reduced. This is likely due to adaptations to one host genotype being costly or at least neutral in other host genotypes.\textsuperscript{27,28} Finally, the differences between a retroviral infection and a malaria (protozoan) infection are vast and many of these variables could contribute to the observed differences. It will be important to conduct these kinds of experiments in various host-parasite systems to evaluate the general patterns that emerge.

Studies in wild populations have also provided correlative evidence supporting the link between host resistance and virulence evolution. For example, Thrall et al.\textsuperscript{22} found that virulent strains of the fungal rust pathogen \textit{Melampsora lini} were found more frequently within resistant populations of the host plant \textit{Linum marginale}, while avirulent strains were isolated more frequently from susceptible populations. Moreover, increased resistance in rabbit populations in both Europe and Australia to myxoma virus infection was observed to be associated with increased prevalence of virulent myxoma virus strains in those regions (see Gandon and Michalakis\textsuperscript{1} [and references therein]). Results from our experiments are consistent with these previous studies. We demonstrate that serial passage through resistant vs. susceptible host genotypes produces the largest adaptive responses in a viral pathogen, which in turn was also associated with the most dramatic increases in virulence. These results are significant for two reasons. First, whereas previous work has focused on artificially manipulating levels of resistance in a single genotype, differences we observe in levels of infectivity and virulence are a response to intrinsic differences in the relative level of resistance to infection among our different host genotypes. Second, as we now discuss, our results also provide novel insight into how differences in levels of resistance to infection among host genotypes influence the relationship between pathogen specialization and virulence evolution.

Theory suggests that the degree of host specialization can dramatically alter the direction of virulence evolution in pathogen populations,\textsuperscript{39} and numerous serial passage experiments have demonstrated that host specialization is a recurring phenomenon across a wide range of parasitic taxa.\textsuperscript{40-43} Importantly, host specialization necessarily assumes tradeoffs in pathogen fitness among different host genotypes. In situations where virulence is positively correlated with pathogen load (often, but not always
the case\textsuperscript{22,44}) host specialization could theoretically limit the evolution of more broadly virulent pathogens (i.e., pathogens whose intrinsic virulence is not influenced by the host environment). Consistent with this argument, our experiments demonstrate that specialization rapidly emerges during serial passage and that more specialized pathogens have reduced fitness and virulence when exposed to novel host genotypes. This result is all the more striking when we consider that serial passage through resistant host genotypes produced the largest magnitude increases in pathogen virulence, which provides further evidence in favor of the notion that specialization reduces overall virulence of a pathogen. Our work provides novel insight into underlying factors determining this tradeoff by demonstrating that the level of host resistance determines the magnitude of these tradeoffs between pathogen specialization and overall virulence. Moreover, this is the first time such effects have been demonstrated in a vertebrate host–pathogen experimental system.

Tolerance mechanisms limit the burden of infectious disease by reducing the rate at which host fitness declines as a function of infection intensity. In contrast, resistance mechanisms directly limit pathogen replication and its associated damage to host tissues. Results from our experiments provide evidence of genetic variation for tolerance in a mammalian host. Moreover, we also observed an inverse correlation between resistance and tolerance among host genotypes with the most resistant genotypes being the least tolerant and vice versa, which implies an antagonistic pleiotropic relationship between these two sets of mechanisms. This is consistent with theoretical predictions regarding the evolution of host resistance vs. tolerance.\textsuperscript{33,45,46} One possible explanation for this observation could be that there is a cumulative cost of mounting a more aggressive immune response either through increased immunopathology (i.e., immune-mediated damage to host tissues) or selection for more destructive (e.g., cytolytic) virus variants. However, two pieces of evidence suggest the former is likely to be the more important cost in our system. First, the most initially resistant host genotypes selected for the most specialized and least generally virulent viruses. Second, resistance against post-passage virus stocks is associated with more severe disease. These data imply that increasing immunopathology associated with an immune response against a more virulent pathogen carries a fitness cost that may favor tolerant over resistant host genotypes. Our lab is now working on characterizing the nature of the immune response among host genotypes to our adapted strains of FV complex in order to address this hypothesis.

It is widely assumed that virulence evolution is mediated by a tradeoff between within-host growth rate and transmissibility.\textsuperscript{7} In this model, selection favors more effective replicators (higher intrinsic growth rates, better competitors, etc.) but at the cost of increased harm to the host. This consequently reduces the likelihood of transmission due to infection-induced host morbidity/mortality. Thus an optimal level of virulence is predicted.\textsuperscript{47,48} However, this model ignores the role of host–pathogen genotype × genotype interactions in determining virulence phenotypes despite the growing number of theoretical and empirical studies highlighting the importance of such effects.\textsuperscript{27,49-55} Here, we show that genetically determined differences in levels of host resistance influences both the magnitude of pathogen adaptation and the degree of host specialization (which is a consequence of genotype × genotype interactions that emerged during serial passage), and this in turn influences patterns of virulence evolution. Thus, our data implies that resistance variability among hosts is likely to be an important factor influencing the spread and severity of disease associated with infectious agents in natural populations, which is in agreement with recent theoretical treatments on the topic.\textsuperscript{2,9,56} Future studies should expand upon this work to include more host genotypes and different pathogens in order to assess the generality of these findings.

**Materials and Methods**

**Animals and virus stocks.** Five different genotypes of inbred mice were used in this study. A/WySn, three BALB/c MHC-congenic strains (BALB/c-H\textsuperscript{2}k, BALB/c-H\textsuperscript{2}b, and BALB/c-H\textsuperscript{2}d), and DBA/2J mice were purchased from Jackson Laboratories and bred under specific-pathogen-free conditions at the University of Utah. Female, 8–16 weeks old animals were used in all experimental infections. All animal use was in accordance with University and Federal guidelines. Friend virus (FV) complex was used as our pathogen model. Friend virus complex consists of two viruses, the replication competent Friend murine leukemia virus (F-MuLV) and the replication defective spleen focus forming virus (SFFV). Both of these viruses operate in a synergistic fashion to promote infection and disease. A cell line containing a biological clone of FV complex (i.e., possessing the integrated genomes of both F-MuLV and SFFV) was kindly provided by Dr Sandra Ruscetti (NIAID). Supernatant from tissue culture of these cells was used as our initial source of virus and consequently represents the ancestor of our adapted virus stocks. Thus, throughout this manuscript we refer to this biological clone stock as “unpassaged” virus. Post-passage FV stocks were derived by repeated (ten rounds of 12 d infections) serial passage of FV complex via intraperitoneal injection of virus-laden spleen supernatants through a series of animals from each of the host genotypes mentioned above (see Kubinak et al.\textsuperscript{27} for a more detailed description of serial passage methodology).

**Fitness estimates.** The spleen is a primary site of FV complex replication. A culture-based assay for measuring infectious virus particles from the spleens of infected animals was used to estimate viral fitness. A more detailed description of assay methodology can be found in a previously published protocol.\textsuperscript{27}

**Virulence estimates.** FV complex replication within infected animals causes an acute myeloproliferative disease characterized by gross enlargement of the spleen due to clonal expansion of virally infected cells.\textsuperscript{50} Thus, we measured spleen weight as an estimate of virulence associated with acute infection. “Overall” virulence refers to the virulence of a post-passage stock averaged across all host genotypes tested in.

**Statistical analyses.** Data was analyzed using JMP Start statistical software (SAS), and graphically represented using JMP software and Microsoft Excel. One-way ANOVAs are reported when testing significance among groups and the two-tailed Student \( t \) tests are reported for pair-wise statistics provided in
Analyses comparing the magnitude of fitness and virulence increases between post-passage stocks are based on calculated fold differences in the fitness and virulence of post-passage viruses infecting their host-of-passage relative to the average fitness of unpassaged virus in a cohort of animals from the same host genotype. Statistical comparisons for viral fitness and virulence between unpassaged and post-passage viruses for each genotype are reported elsewhere (Kubinak et al.27 and Kubinak et al., in press).

The degree of pathogen specialization (defined here as the extent to which viral fitness declines as a consequence of exposure to novel host genotypes) was estimated by calculating the fold reduction in viral fitness when a post-passage virus infected animals of a novel host genotype (i.e., a genotype the virus hadn’t been passaged through) compared with the mean fitness of that post-passage virus stock tested in a cohort of animals from its respective host genotype-of-passage. Because host genotypes varied considerably in their relative levels of resistance to infection it was necessary to control against this confounding variable statistically prior to estimating a post-passage viruses degree of specialization (because we are comparing estimates of viral fitness among genotypes). To do this we employed generalized linear modeling (GLM) as a post hoc statistical method for removing variation in our data associated with the main effect of host genotype. The term “main effect” refers to the effect of an independent variable (host genotype) averaged across different levels of a dependent variable (viral fitness). Specifically, during the GLM analysis, variation in our data set explained by the main effect of host genotype is removed leaving behind “residual” variation (i.e., variation in the data not explained by host genotype). These residual values were then used to calculate the magnitude of pathogen specialization (− residual fitness in unfamiliar host − mean fitness in host-of-passage + mean fitness in host-of-passage = magnitude of specialization). Larger “specialization” values signify that a post-passage virus suffers greater fitness tradeoffs as a consequence of exposure to unfamiliar host genotypes (i.e., they are more specialized).

“Baseline resistance” of a given genotype is estimated as the average viral load obtained from cohorts of animals infected with unpassaged virus. “Resistance” estimates provided in Figure 4 represent the average viral load across cohorts of animals from the same genotype infected with different post-passage viruses (their own and others). “Tolerance” is defined as the degree to which host health declines as a function of viral load, and was estimated as the slope of the regression line defining the relationship between viral load and splenomegaly. (See Raberg et al.23 for a detailed description of rationale and methodology). Analysis of covariance (ANCOVA) is a statistical approach used to assess whether the relationship between two variables is equivalent across treatments. We employed ANCOVA to test for differences among host genotypes in the relationship between viral fitness and splenomegaly. A significant effect provides support for the hypothesis that there is variation in tolerance among host genotypes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This work was supported by National Science Foundation Grant DEB 0918969 (to WKP and Dr Fred Adler); National Science Foundation Doctoral Dissertation Improvement Grant DEB 0910052 (to JLK and WKP); and National Science Foundation Educational Outreach Grant DGE 08-41233 (to JLK). We would like to thank two anonymous reviewers for their constructive comments during manuscript preparation.

Supplemental Materials
Supplemental materials may be found here:
www.landesbioscience.com/journals/virulence/article/24724

References
1. Gandon S, Michalakis Y. Evolution of parasite virulence against qualitative or quantitative host resistance. Proc Natl Acad Sci USA 2000; 97:863-867; PMID:10713751; http://dx.doi.org/10.1073/pnas.97.16.863
2. Regoes RR, Nowak MA, Bonhoeffer S. Evolution of virulence in a heterogeneous host population. Evolution 2000; 54:64-71; PMID:10937184.
3. André JB, Ferdy JB, Godelle B. Within-host parasite population dynamics and the evolution of micro-parasites in a heterogeneous host population. Evolution 2003; 57:1489-97; PMID:12940354.
4. Ganz HH, Ebert D. Benefits of host genetic diversity for resistance to infection depend on parasite diversity. Ecology 2010; 91:1263-8; PMID:20503859; http://dx.doi.org/10.1890/09-1243.1
5. Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, et al. Unifying the epidemiological and evolutionary dynamics of pathogens. Science 2004; 303:327-32; PMID:14726583; http://dx.doi.org/10.1126/science.1090727
6. Ganousov VV, Bergstrom CT, Antia R. Within-host parasite population dynamics and the evolution of micro-parasites in a heterogeneous host population. Evolution 2006; 59:218; PMID:16296490.
7. Allison S, Hurford A, Mideo N, Van Baalen M. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. J Evol Biol 2009; 22:245-59; PMID:19196383; http://dx.doi.org/10.1111/j.1420-9101.2008.01658.x
8. King KC, Lively CM. Does genetic diversity limit disease spread in natural host populations? Heredity (Edinb) 2012; 109:199-203; PMID:22773998; http://dx.doi.org/10.1038/hdy.2012.33
9. Lively CM. The effect of host genetic diversity on disease spread. Am Nat 2010; 175:E149-52; PMID:20388005; http://dx.doi.org/10.1086/652430
10. Albertsen F, Ebert D. Genetic diversity of Daphnia magna populations enhances resistance to parasites. Ecol Lett 2008; 11:918-28; PMID:18479453; http://dx.doi.org/10.1111/j.1461-0248.2008.01203.x
11. Reber A, Castella G, Christe P, Chapuisat M. Experimentally increased group diversity improves disease resistance in an ant species. Ecol Lett 2008; 11:682-9; PMID:18371089; http://dx.doi.org/10.1111/j.1461-0248.2008.01177.x
12. Tarpy DR. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proc Biol Sci 2003; 270:99-103; PMID:12596763; http://dx.doi.org/10.1098/rspb.2002.2199
13. Hughes WO, Boomsma JJ. Genetic diversity and disease resistance in leaf-cutting ant societies. Evolution 2004; 58:1251-60; PMID:15266974.
14. Ostfeld RS, Keesing F. Effects of host diversity on infectious disease. Annu Rev Ecol Evol Syst 2012; 43:157-82; http://dx.doi.org/10.1146/annurev-ecolsys-102710-145022
15. Murphy K. Janeway’s Immunobiology. New York: Garland Science (Taylor and Francis, Inc.), 2011.
16. Barclay VC, Sim D, Chan BH, Nell LA, Raha MA, Bell AS, et al. The evolutionary consequences of blood-stage vaccination on the rodent malaria Plasmodium chabaudi. PLoS Biol 2012; 10:e1001368; PMID:22870063; http://dx.doi.org/10.1371/journal.pbio.1001368
17. Mackinnon MJ, Read AF. Immunity promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
18. Schneider P, Bell AS, Sim DG, O’Donnell AJ, Blanford S, Paaijmans KP, et al. Virulence, drug sensitivity and transmission success in the rodent malaria, Plasmodium chabaudi. Proc Biol Sci 2012; 279:4677-85; PMID:23015626; http://dx.doi.org/10.1098/rspb.2012.1792
19. Mackinnon MJ, Read AF. Immunology promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
20. Mackinnon MJ, Read AF. Immunology promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
21. Mackinnon MJ, Read AF. Immunology promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
22. Mackinnon MJ, Read AF. Immunology promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
23. Mackinnon MJ, Read AF. Immunology promotes virulence evolution in a malaria model. PLoS Biol 2004; 2:E230; PMID:15221031; http://dx.doi.org/10.1371/journal.pbio.0020230
20. Raber L, de Roode JC, Bell AS, Stawou P, Gray D. Read AF. The role of immune-mediated apparent competition in genetically diverse malaria infections. Am Nat 2006; 168:41-53; PMID:16874614; http://dx.doi.org/10.1086/492878

21. McClelland EE, Adler FR, Granger DL, Ports WK. Major histocompatibility complex controls the trajectory but not host-specific adaptation during virulence evolution of the pathogenic fungus Cryptococcus neoformans. Proc Biol Sci 2004; 271:1557-64; PMID:15580630; http://dx.doi.org/10.1098/rspb.2004.2736

22. Thrall PH, Burdon J. Evolution of virulence in a plant host-pathogen metapopulation. Science 2003; 299:1735-7; PMID:12637745; http://dx.doi.org/10.1126/science.1080870

23. Thrall PH, Burdon JV, Rever JD. Local adaptation in the Linum magniflorum-Melampyrum linum host-pathogen interaction. Evolution 2002; 56:1340-51; PMID:12362236.

24. Hunt HD, Duan JR. Mareki disease virus evolution in specific MHC haplotypes. 9th International Symposium on Mareki’s Disease and Avian Herpesviruses. Freie Universitat Berlin, 2012-41.

25. Lalic J, Agudelo-Romero P, Carrasco P, Elena SF. Experimental evolution of specificity in evolvable parasites: Arabidopsis thaliana capacitates it for systemic infection of resistant ecotypes. Philos Trans R Soc Lond B Biol Sci 2010; 365:1997-2007; PMID:20478894; http://dx.doi.org/10.1098/rstb.2010.0044

26. Gandon S, van Baalen M, Jansen VA. The evolution of alternative MHC types. Proc Natl Acad Sci U S A 1997; 94:7811-6; PMID:9223268; http://dx.doi.org/10.1073/pnas.94.15.7811

27. Kubinak JL, Ruff JS, Hyzer CW, Luijkx P, Bérentin C, Schmid-Hempel P, Wegener KM. Experimental evolution of virulence in a parasite increases host recombination frequency. BMC Evol Biol 2012; 12:18; PMID:22336015; http://dx.doi.org/10.1186/1471-2148-12-18

28. Pike R, Filby A, Ploquin MJ, Ekoumon U, Marques R, Antunes I, et al. Race between retroviral spread and CD4+ T-cell response determines the outcome of acute HIV infection. Eur J Immunol 2006; 36:2658-70; PMID:16981182; http://dx.doi.org/10.1002/eji.200636059

29. Zelinsky G, Kraft AR, Schimmer S, Arndt T, Dittmer T, U. Kinetics of CD8+ effector T cell responses and induced CD4+ regulatory T cell responses during Friend retrovirus infection. Eur J Immunol 2006; 36:2658-70; PMID:16981182; http://dx.doi.org/10.1002/eji.200636059

30. Paterson S, Vogwill T, Buckling A, Benmayor R, Spiers AJ, Thomson NR, et al. Antagonistic coevolution accumulates molecular evolution. Nature 2010; 464:275-8; PMID:20182425; http://dx.doi.org/10.1038/nature08798

31. Schulte RD, Makus C, Hasert B, Michels NK, Schulenburg H. Multiple reciprocal adaptations and rapid genetic change upon experimental coevolution of an animal host and its microbial parasite. Proc Natl Acad Sci U S A 2010; 107:7539-44; PMID:20638449; http://dx.doi.org/10.1073/pnas.1003131107

32. Miller MR, White A, Boots M. Evolution of parasitism in response to resistance in their hosts: the good, the bad, and apparent commensalism. Evolution 2006; 60:945-56; PMID:16817535.