Composition of the gut microbiota modulates the severity of malaria

Nicolas F. Villarino[a,1,2], Gary R. LeCleir[a,1], Joshua E. Denny[a,b,1], Stephen P. Dearth[c], Christopher L. Harding[b], Sarah S. Sloan[a], Jennifer L. Gribble[a], Shawn R. Campagna[c,d], Steven W. Wilhelm[a], and Nathan W. Schmidt[a,b,3]

[a]Department of Microbiology, University of Tennessee, Knoxville, TN 37996; [b]Department of Microbiology and Immunology, University of Louisville, Louisville, KY 40202; [c]Department of Chemistry, University of Tennessee, Knoxville, TN 37996; and [d]Biological and Small Molecule Mass Spectrometry Core, University of Tennessee, Knoxville, TN 37996

Edited by Maria M. Mota, Instituto de Medicina Molecular, Lisbon, Portugal, and accepted by the Editorial Board January 4, 2016 (received for review March 10, 2015)

***Plasmodium* infections result in clinical presentations that range from asymptomatic to severe malaria, resulting in ∼1 million deaths annually. Despite this toll on humanity, the factors that determine disease severity remain poorly understood. Here, we show that the gut microbiota of mice influences the pathogenesis of malaria. Genetically similar mice from different commercial vendors, which exhibited differences in their gut bacterial community, had significant differences in parasite burden and mortality after infection with multiple *Plasmodium* species. Germfree mice that received cecal content transplants from "resistant" or "susceptible" mice had low and high parasite burdens, respectively, demonstrating the gut microbiota shaped the severity of malaria. Among differences in the gut flora were increased abundances of *Lactobacillus* and *Bifidobacterium* in resistant mice. Susceptible mice treated with antibiotics followed by yogurt made from these bacterial genera displayed a decreased parasite burden. Consistent with differences in parasite burden, resistant mice exhibited an elevated humoral immune response compared with susceptible mice. Collectively, these results identify the composition of the gut microbiota as a previously unidentified risk factor for severe malaria and modulation of the gut microbiota (e.g., probiotics) as a potential treatment to decrease parasite burden.**

Plasmodium | gut microbiome | severe malaria

Infection by *Plasmodium* species remain a global health burden causing over 200 million cases of malaria and around 1 million deaths annually, with the vast majority of fatalities being children under the age of 5 y living in sub-Saharan Africa (1). Many *Plasmodium* infections are either asymptomatic or cause only mild malaria. However, some infections progress to severe malaria that most often manifests as impaired consciousness (cerebral malaria), respiratory distress, and severe anemia (2). The best correlate of disease severity following *Plasmodium falciparum* infection in humans is parasite density (3, 4).

The gut microbiota has an impact on multiple facets of host physiology (5), including shaping susceptibility to numerous diseases (6–14). The effects of the gut microbiota on the host are strongly influenced by the collective composition of the bacterial populations (15), and commensal florae are known to affect local pathogen burdens and host immunity (16–18). In addition to influencing local gut immunity, the gut microbiome affects host immunity to extragastrointestinal tract viral infections (19).

Recent studies also support that the gut microbiome modulates *Plasmodium* infections in humans. Anti–α-gal Abs, induced by the gut pathobiont *Escherichia coli* O86:B7, cross-react with sporozoites from human and rodent *Plasmodium* species that impair transmission of the parasite between the vector and vertebrate host; however, this cross-reactive immunity did not affect blood stage parasite burden (20). Additionally, the stool bacteria composition of Malian children correlated prospectively with risk of *P. falciparum* infection, but not progression to febrile malaria (21). Importantly, it remains unclear whether the gut microbiome also contributes to the development of severe malaria. Using the murine model of malaria, these data demonstrate that the gut microbiome affects blood stage parasite burden and the subsequent severity of malaria.

Results

Mice from Different Vendors Exhibit Differential Susceptibility to Malaria. Genetically similar inbred strains of mice (C57BL/6) maintained by different vendors [Jackson Laboratory (Jax) and Taconic (Tac)] have differences in their gut bacterial communities (22, 23). To determine whether these differences had any effect on *Plasmodium* infections, C57BL/6 mice from Jax, Tac, National Cancer Institute/Charles River (NCI), and Harlan (Har) were infected with *Plasmodium yoelii*. Following infection, profound differences in parasitemia (the fraction of RBCs infected with *P. yoelii*) were observed between the four groups of mice (Fig. 1 *A* and *B*). Whereas resistant mice (Jax and Tac) exhibited a maximum of ∼10% parasitemia, they had no signs of morbidity (weight loss) or mortality, which was in contrast to the substantial weight loss and mortality observed in susceptible mice (NCI and Har), where parasitemia was >60% (Fig. 1 *C* and *D*). Moreover, NCI and Har mice exhibited more profound and longer lasting anemia

Significance

***Plasmodium* infections cause >200 million cases of malaria and ∼1 million deaths annually. Although these infections result in disease states that range from asymptomatic to life-threatening, factors that contribute to disease severity remain poorly defined. This report demonstrates that the assemblage of microbes in the gut can modulate the severity of malaria. Mice from different vendors with differences in their gut microbiome showed significant differences in pathology after infection with *Plasmodium*. Among the bacterial populations that were different between "resistant" and "susceptible" mice were *Lactobacillus* and *Bifidobacterium*, and treatment of mice with *Lactobacillus* and *Bifidobacterium* resulted in decreased *Plasmodium* burden. These results identify both a previously unidentified risk factor for severe malaria and a potential new avenue of treatment.**

Author contributions: N.F.V., S.R.C., S.W.W., and N.W.S. designed research; N.F.V., G.R.L., J.E.D., S.P.D., C.L.H., S.S.S., J.L.G., and N.W.S. performed research; N.F.V., G.R.L., J.E.D., S.P.D., S.R.C., S.W.W., and N.W.S. analyzed data; G.R.L., J.L.G., and S.W.W. analyzed DNA sequencing; S.P.D. and S.R.C. analyzed the metabolomics data; and N.F.V., G.R.L., S.R.C., S.W.W., and N.W.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.M.M. is a guest editor invited by the Editorial Board.

Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive under Bioproject PRJNA289122.

[1]N.F.V., G.R.L., and J.E.D. contributed equally to this work.

[2]Present address: Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA 99164.

[3]To whom correspondence should be addressed. Email: nathan.schmidt@louisville.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1504887113/-/DCSupplemental.

MICROBIOLOGY

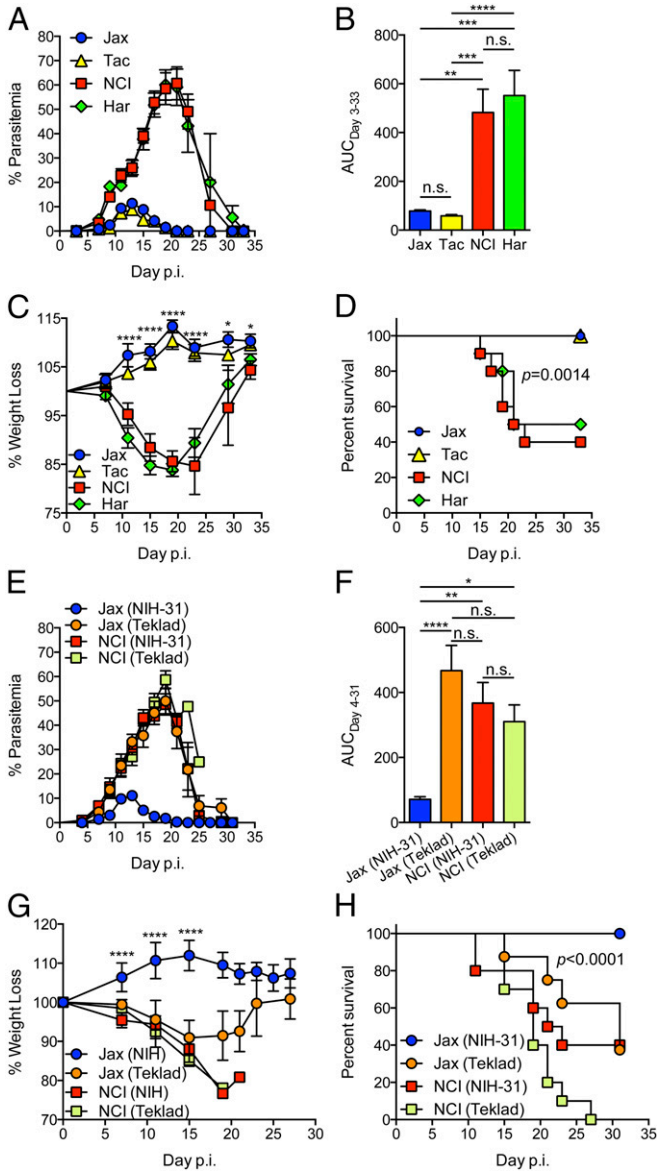

Fig. 1. *Plasmodium* parasite burden, morbidity, and mortality vary by mouse vendor and diet. C57BL/6 mice were infected with *P. yoelii* parasitized RBCs. (*A*) Fraction of RBCs infected with *P. yoelii* (percentage of parasitemia). (*B*) AUC analysis. Data were analyzed by one-way ANOVA and Tukey's multiple comparison posttest. (*C*) Percentage of weight loss following infection. Data were analyzed by one-way ANOVA. (*D*) Survival of mice following infection. Survival curves were analyzed by log-rank (Mantel–Cox) test. (*E–H*) Mice were fed either NIH-31 or Teklad 22/5 diet before and after *P. yoelii* infection. (*E*) Percentage of parasitemia following *P. yoelii* infection. (*F*) AUC analysis. Data were analyzed by one-way ANOVA and Tukey's multiple comparison posttest. (*G*) Percentage of weight loss following infection. Data were analyzed by one-way ANOVA. (*H*) Survival of mice following infection. Survival curves were analyzed by log-rank (Mantel–Cox) test. Data (mean ± SE) in *A–F* and *H* are cumulative results (n = 8–10 mice per group) from two experiments. Data (mean ± SD) in *G* are from four to five mice per group from one experiment. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. n.s., not significant; p.i., postinfection.

(loss of RBCs per milliliter) compared with Jax and Tac mice (*SI Appendix*, Fig. 1*A*). Additionally, when the total number of RBCs per milliliter was used to derive total pathogen burden, similarities were noted between the parasite burden as detected by parasitemia or parasitized RBCs (pRBCs) per milliliter of blood (*SI Appendix*, Fig. 1 *B–E*). Of note, mice infected with different doses of *P. yoelii* pRBCs showed similar parasitemia kinetics between the different doses and susceptibility to infection (*SI Appendix*, Fig. 2), suggesting mice from different vendors are differentially susceptible to progression to severe malaria but not to blood stage infection.

To determine the broader applicability of these data, another mouse strain and two *Plasmodium* species were tested. BALB/c mice from Jax, Tac, Charles River (CR), and Har were infected with *P. yoelii*. Mice were purchased from CR in lieu of NCI. Of note, C57BL/6 mice purchased from CR exhibit similar parasitemia and morbidity as NCI mice following infection with *P. yoelii* (*SI Appendix*, Fig. 3). Consistent with *P. yoelii* infections in C57BL/6 mice (*SI Appendix*, Fig. 3), BALB/c mice from Jax and Tac exhibit reduced *P. yoelii* parasitemia compared with mice from CR and Har (*SI Appendix*, Fig. 4 *A* and *B*). Furthermore, C57BL/6 mice from Jax and Tac exhibited reduced parasitemia compared with mice from CR and Har following *Plasmodium chabaudi* infection (*SI Appendix*, Fig. 4 *C* and *D*). Finally, we assessed the development of experimental cerebral malaria in C57BL/6 mice infected with *Plasmodium berghei* ANKA. Jax and Tac mice trended toward reduced parasitemia compared with NCI and Har mice at early time points; moreover, there was a significant (P = 0.04) difference in survival between these groups of mice (*SI Appendix*, Fig. 4 *E* and *F*). In sum, these data indicate the severity of malaria was dependent on the source of mice.

Diet is a strong modulator of organismal health as well as the gut microbiome and its function (24). To determine whether the diet could shape the severity of malaria, Jax and NCI mice were fed one of two commercially available rodent diets, either NIH-31 (used in Fig. 1 *A–D*) or Teklad 22/5. Parasitemia in NCI mice was unaffected; however, Jax mice had high levels of parasitemia when fed Teklad 22/5 (Fig. 1 *E* and *F*). Consistent with the parasitemia data, Jax mice fed Teklad 22/5 also exhibited substantial weight loss and elevated mortality compared with Jax mice fed NIH-31 (Fig. 1 *G* and *H*). Because these diets had no effect on parasite burden in NCI mice, the changes in parasitemia in Jax mice were unlikely due to a direct effect of these diets on the parasite burden. Moreover, high parasite burdens in NCI mice fed NIH-31 suggest this diet supported the proliferative expansion of *P. yoelii*. When Jax and NCI mice were placed on the reciprocal vendor-specific diet and then infected with *P. yoelii*, we noted a modest increase in parasite burden in Jax mice fed the NCI in-house diet but no effect of the Jax in-house diet on NCI mice (*SI Appendix*, Fig. 5). Collectively, these datasets led to the hypothesis that the gut microbiota influenced *Plasmodium* infections.

Gut Bacterial Community Structure and Function Are Different in Resistant and Susceptible Mice. To test directly for differences in the gut microbiome, sections of the gastrointestinal tract from resistant (Jax and Tac) and susceptible (NCI and Har) mice were collected and the bacterial communities were characterized using 16S rRNA gene analysis (*SI Appendix*, Table 1). There was a high degree of similarity between the microbial community assemblages found within the cecum and colon of mice from the same vendor (*SI Appendix*, Fig. 6*A*), whereas there were clear differences between the microbial communities of these regions compared with the distal half of the small intestine in mice from the same vendor. Moreover, significant differences between mice from all vendors were apparent in the nonmetric multidimensional scaling analysis of population structure within the cecum, with the susceptible NCI and Har libraries showing a comparative overlap with each other yet distinct differences compared with the resistant Jax and Tac communities (*SI Appendix*, Fig. 6*B*). Analysis of the cecal bacterial communities at the family level revealed substantial differences, with Clostridiaceae, Erysipelotrichaceae, Lactobacillaceae, and Peptostreptococcaceae (members of the Firmicutes phylum) being proportionally more abundant in resistant (Jax and Tac) mice, whereas Bacteroidaceae and Prevotellaceae (members of Bacteroidetes phylum) and Sutterellaceae (member of Proteobacteria phylum) were proportionally more abundant in susceptible (NCI and Har) mice (Fig. 2 *A* and *B*). Finally, dietary changes are capable of inducing significant changes in the gut microbiome (25) that reach steady state within 3–4 d in mice (26). Consistent with these reports, we observed defined changes in the gut bacterial

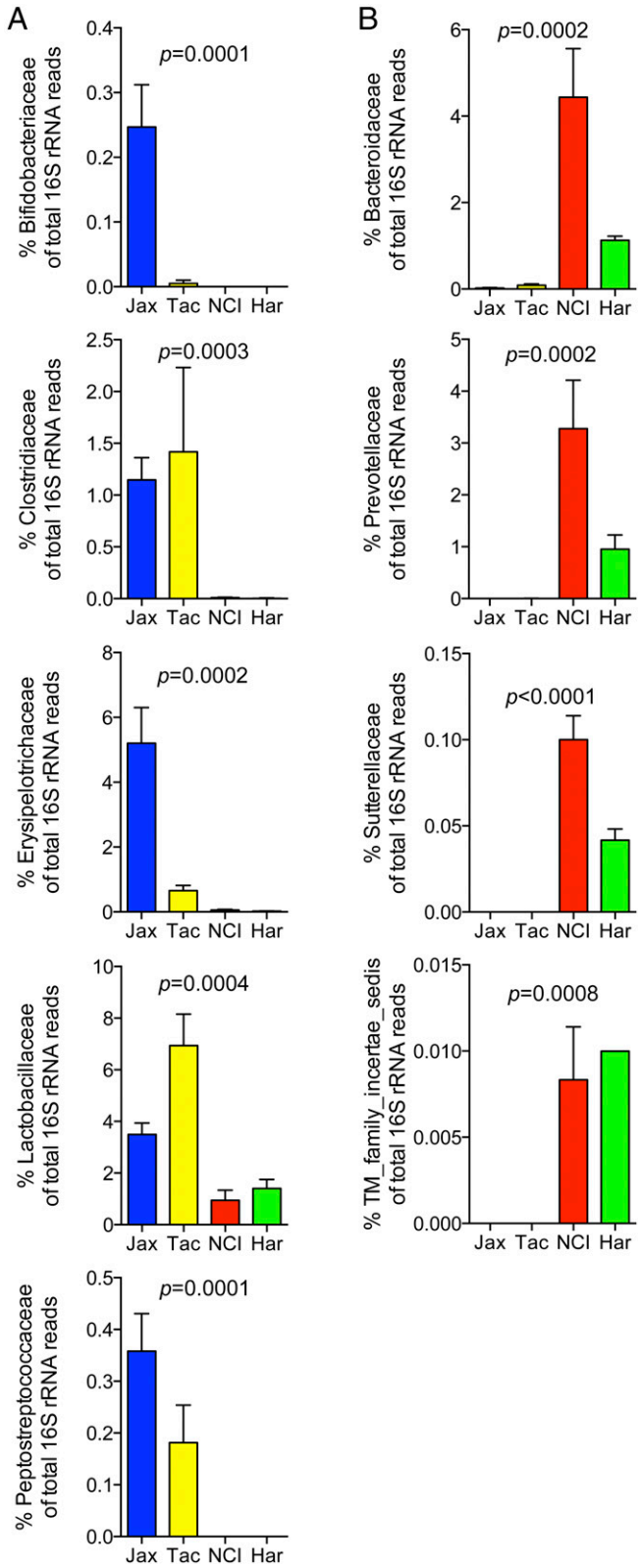

Fig. 2. Susceptibility to malaria correlates with differences in cecal bacteria populations. (*A*) Bacterial families that were identified as being significantly enriched in Jax or Tac mice. (*B*) Bacterial families identified as being significantly enriched in NCI or Har mice. Data (mean ± SE) in *A* and *B* are from six mice per group and extracted from analysis in *SI Appendix*, Fig. 6*C*. Data were analyzed by the Kruskal–Wallis test.

communities in Jax mice fed Teklad 22/5 or NIH-31 (*SI Appendix*, Figs. 7 and 8). In Jax mice fed the Teklad diet, there was a noted decrease in Peptostreptococcaceae below the levels observed in either Jax or Tac mice, resulting in an increased similarity to the susceptible NCI and Har mice (*SI Appendix*, Fig. 7*C*). These changes coincide with a shift in the severity of malaria between these two groups of mice (Fig. 1 *E–H*).

Consistent with changes in the gut bacterial community, analysis of metabolites in the small intestine, cecum, and plasma of Jax and NCI mice revealed differential expression between each tissue (*SI Appendix*, Fig. 9*A*). An *F* test of partial least squares discriminant analysis (27) used to probe variation between metabolite profiles in Jax and NCI mice on a per tissue basis confirmed that the means of the variate-1 (component 1), which differentiated Jax from NCI mice in all tissues, were significantly different ($P \leq 0.0003$, $P \leq 0.0001$, $P \leq 0.0001$) for the small intestine, cecum, and plasma, respectively, (*SI Appendix*, Fig. 9 *B–D* and Table 2). Several metabolites exhibited large (≥1.5 fold) and statistically significant ($P \leq 0.1$) differences between Jax and NCI mice, with the top 25% of metabolites associated with distinct metabolic pathways (*SI Appendix*, Fig. 9 *E* and *F* and Tables 3–5). Therefore, differences in the gut bacterial populations and metabolites support the hypothesis that the severity of malaria was modulated by differences in gut bacterial communities.

Differences in the Gut Microbiome Shape Susceptibility to Malaria. To test this hypothesis directly, genetically identical germfree (GF) C57BL/6 mice received cecal content transplants from either Jax or NCI mice. Of note, GF C57BL/6J mice exhibited no difference in parasitemia compared with conventional C57BL/6J mice following infection with *P. yoelii nigeriensis* (28). Sequence analyses demonstrated the bacterial communities in colonized, GF mice reflected the bacterial communities of the donor communities and were different from the communities in GF mice exposed to only environmental microbes (Fig. 3*A*). Furthermore, there was only a slight decrease in community diversity between the respective donor and colonized GF mice (*SI Appendix*, Fig. 10). Following *P. yoelii* infection, GF mice that received either Jax or NCI cecal transplants had parasite burdens similar to control Jax and NCI mice (Fig. 3 *B* and *C*). Both NCI control mice and GF mice that received NCI cecal transplants also had decreased survival compared with the Jax control mice and GF mice that received Jax cecal transplants (Fig. 3*D*). Collectively, these data provided a direct demonstration that the severity of malaria was modulated by the gut microbiota.

Decreased Parasite Burden in Mice Treated with *Lactobacillus* and *Bifidobacterium*. To identify individual microbial phylotypes that may shape the severity of malaria, a deeper analysis was performed on the bacterial communities in the cecum. When pooled by resistance (Jax/Tac) or susceptibility (NCI/Har) to *P. yoelii*, several phylotypes (referred to here as operational taxonomic units) emerged from a linear discriminant analysis effect size (LEfSe)-driven analysis (29) as biomarkers of the resistant or susceptible phenotype. Among those differences, *Lactobacillus* and *Bifidobacterium* were overly abundant in the resistant mice compared with susceptible mice (*SI Appendix*, Fig. 11), with differences in *Lactobacillus* being the greatest driver of the differential community structure between resistant and susceptible mice (*SI Appendix*, Fig. 11*B*).

To evaluate the linkage between *Lactobacillus* and *Bifidobacterium* toward resistance to severe malaria, Jax and NCI mice were treated with laboratory-cultured yogurt supplemented with probiotics that contained *Lactobacillus* and *Bifidobacterium* species before and following infection with *P. yoelii*. DNA sequencing of *Lactobacillus* isolated from fecal pellets from Jax and NCI mice or laboratory-cultured yogurt demonstrated phylogenetic congruence (*SI Appendix*, Fig. 12). Consumption of *Lactobacillus* and *Bifidobacterium* can modulate the gut microbial community structure (30) or function (31). Following infection with *P. yoelii*, both Jax and NCI mice treated with yogurt had a modest, but significant (Jax: $P < 0.0001$, NCI: $P = 0.0418$), decrease in parasite burden compared with control untreated mice (*SI Appendix*, Fig. 13). Jax and NCI mice treated with

MICROBIOLOGY

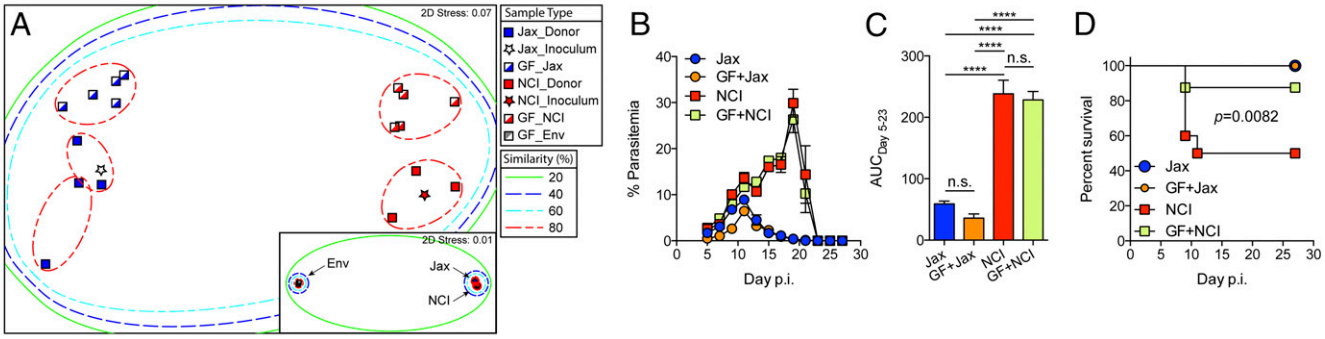

Fig. 3. Gut microbiome shapes susceptibility to severe malaria. GF mice were colonized with cecal contents from Jax or NCI mice. (*A*) Bacterial population analysis was performed using nonmetric multidimensional scaling, as described in *SI Appendix*, Fig. 6. (*B–D*) Colonized GF mice and control Jax and NCI mice were infected with *P. yoelii*. (*B*) Percentage of parasitemia following *P. yoelii* infection. (*C*) AUC analysis. Data (mean ± SE) in *B* and *C* from four to five mice per group are representative of two experiments. Data were analyzed by one-way ANOVA and Tukey's multiple comparison posttest. (*D*) Survival of mice following infection. Data are cumulative results (n = 8–10 mice per group) from two experiments. Survival curves were analyzed by log-rank (Mantel–Cox) test. ****$P < 0.0001$. Env, environment.

milk used to make the yogurt showed a similar parasite burden as control Jax and NCI mice [mean area under the parasitemia curve (AUC) on days 5–34 ($AUC_{Day\ 5–34}$): Jax control (n = 4): 107.2 ± 11.39 (SD) vs. Jax milk (n = 4) 83.55 ± 24.83 (SD), P = 0.13; NCI control (n = 4): 447.1 ± 85.65 (SD) vs. NCI milk (n = 3) 384.8 ± 73.08 (SD), P = 0.36]. However, when mice were treated with antibiotics before yogurt treatment, we observed a profound decrease (14-fold) in parasite burden in the susceptible NCI mice (Fig. 4 *A* and *B*), and no weight loss in those mice was noted compared with the other NCI groups (Fig. 4*C*). These data support the ability of *Lactobacillus* and *Bifidobacterium* to contribute toward the modulation of *Plasmodium* parasite burden, yet other constituents of the gut microbiota may also contribute to regulating the severity of malaria.

Severity of Malaria Correlates with the Magnitude of the Host Immune Response. The gut microbiota can shape host immunity to systemic viral infections (19), and T follicular helper (Tfh)-produced IL-21 is required for germinal center (GC) B-cell help and clearance of murine *Plasmodium* infections (32). Consistent with these observations, resistant Jax mice exhibited elevated *P. yoelii*-specific $CD4^+$ T-cell [$CD49d^{hi}CD11a^{hi}$ (33)], Tfh cell, and GC B-cell responses compared with susceptible NCI mice (Fig. 5 *A–C* and *SI Appendix*, Fig. 14). Jax and NCI mice had similar titers of IgM specific for the 19-kDa fragment of merozoite surface protein 1 ($MSP1_{19}$) from *P. yoelii* (Fig. 5*D*), suggesting similar activation of B cells in both groups. In contrast, Jax mice exhibited accelerated Ab class switching from $MSP1_{19}$-specific IgM to IgG isotypes, four- to 10-fold higher titers at day 14 postinfection, compared with NCI mice (Fig. 5*D*). Thus, one mechanism by which the gut microbiome shapes the severity of malaria following *P. yoelii* infection may be through modulation of the host immune response.

Discussion

This study demonstrates that the murine gut microbiome influences the parasite burden of *Plasmodium* rodent species and modulates the severity of malaria in mice. Importantly, parasite burden is currently the best-known correlate of disease severity following *P. falciparum* infection in humans (3, 4). An association between the gut microbial community and *Plasmodium* parasites has been previously recognized in the mosquito vector (34–38). Interestingly, the unique assemblage of skin bacteria on human skin has also been shown to have an impact on the attractiveness of *Anopheles* mosquitoes to particular individuals (39, 40).

Two recent publications further support that the gut microbiota affects mammalian stages of the *Plasmodium* life cycle. The first study demonstrated that specific gut bacteria could have an impact on the transmission of *P. berghei* sporozoites from mosquitos to mice (20). The authors showed that the gut pathobiont *E. coli* O86:B7 induced the production of anti–α-gal Abs. When *Plasmodium*-infected mosquitoes injected sporozoites into the dermal tissue during a blood meal, the anti–α-gal Abs bound to the *Plasmodium* sporozoites, which prevented their migration to the liver (20). These results also extended to humans, where the presence of anti–α-gal IgM Abs correlated with protection against *P. falciparum* infection. The effect of *E. coli* O86:B7 on *Plasmodium* infection was limited to transmission of sporozoites because there was no effect of the anti–α-gal Abs on the symptomatic blood stage of the infection. Consistent with these findings, a second report

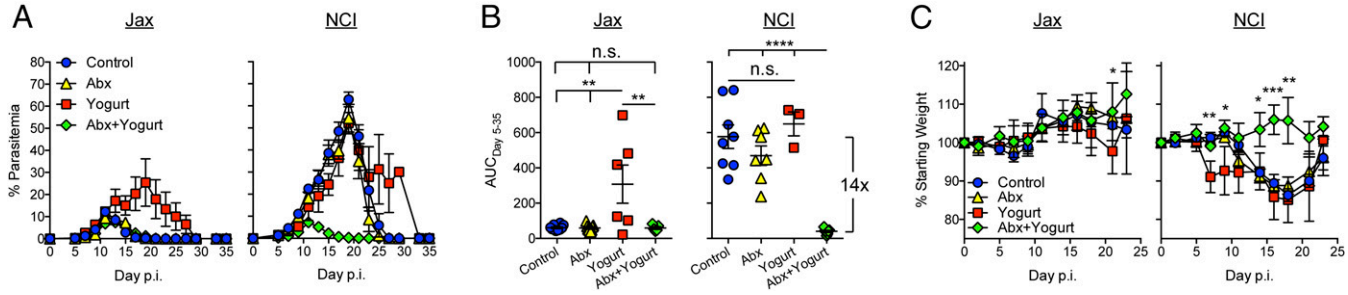

Fig. 4. Susceptible mice treated with yogurt have decreased parasitemia and morbidity. Jax and NCI mice were left untreated (control), treated with antibiotics for 3 wk and then left untreated for an additional 3 wk (Abx), left untreated for 3 wk followed by treatment with yogurt five times per week for 3 wk (Yogurt), or treated with antibiotics for 3 wk followed by treatment with yogurt five times per week for 3 wk (Abx+Yogurt). Mice were then infected with *P. yoelii*. Yogurt-treated mice continued to receive yogurt five times per week following infection. (*A*) Percentage of parasitemia following *P. yoelii* infection. (*B*) AUC analysis. Data were analyzed by one-way ANOVA and Tukey's multiple comparison posttest. (*C*) Percentage of weight loss following infection. Data were analyzed by one-way ANOVA. Data (mean ± SE) in *A–C* are cumulative results (n = 3–10 mice per group) from two experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

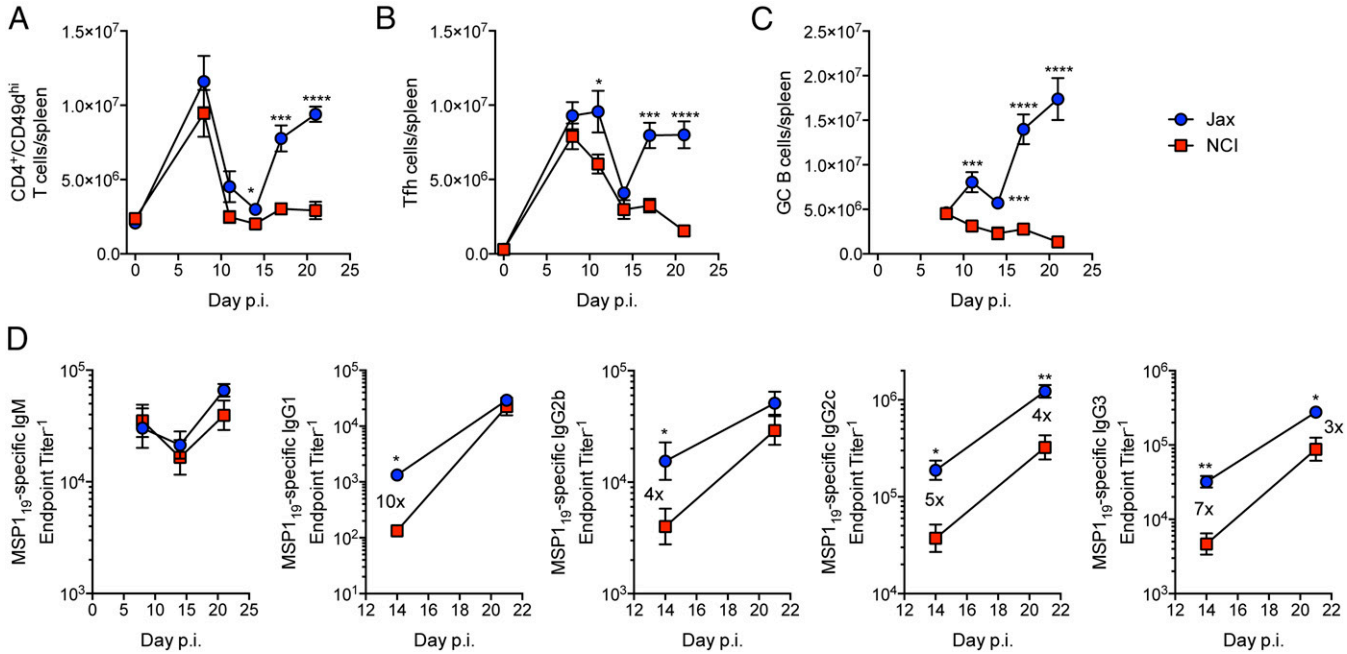

Fig. 5. Resistant Jax mice have an elevated cellular and humoral immune response to *Plasmodium*. Jax and NCI mice were infected with *P. yoelii*. Total number of $CD4^+CD11a^{hi}CD49d^{hi}$ cells (*A*), Tfh cells (*B*), and germinal center (GC) B cells (*C*) per spleen on the indicated day. Data (mean ± SE) are cumulative results (n = 5–10 mice per data point) from three experiments. (*D*) Serum $MSP1_{19}$-specific Ab end-point titers. Data (mean ± SE) are cumulative results (n = 3–7 mice per data point) from two experiments. Numbers in the panels represent the fold difference between the means of the Jax and NCI mice. Data were analyzed by an unpaired two-tailed t test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

demonstrated that the unique composition of stool bacteria in Malian children correlated with prospective risk of *P. falciparum* infection, although not progression to febrile malaria (21). Although the mechanism responsible for this observation is unknown, the similarities between these two studies (i.e., susceptibility to infection but not severity of blood stage infection) suggest the prospective risk of *P. falciparum* infection differentiated by stool bacteria composition may be attributed to differences in anti–α-gal IgM Abs. In contrast to these two publications, we show that the gut microbiota modulates the severity of *P. yoelii* blood stage infections in mice, implying a different mechanism. Moreover, our findings show that the influence of the gut microbiome on *Plasmodium* infections is broad and not limited to the transmission of the parasite. Taken together, our observations and the findings of Yilmaz et al. (20) result in the intriguing speculation that the human intestinal microbiota might have an impact on different stages of the *Plasmodium* life cycle in humans. Clearly, this area is ripe for future research.

One potential mechanism by which the gut microbiota regulates the severity of malaria is a direct effect on the parasite itself, where gut microbiota-derived products either promote or inhibit its growth. Although this possibility has not been formally excluded, we observe similar parasitemia expansion kinetics, when plotted on a log scale, between days 5 and 11 postinfection in both resistant and susceptible mice. This observation suggests that the gut microbiota does not have a direct effect on the parasite. Consequently, it is more likely that the gut microbiota has an impact on the severity of malaria by modulating the host immune response to *Plasmodium*. Consistent with this possibility, resistant Jax mice exhibited an elevated anti-*Plasmodium* immune response compared with susceptible NCI mice. Although these data correlate with the parasite burden in these mice, further experiments will be necessary to demonstrate whether the differential immune response is responsible for the difference in severity, and if so, how the gut microbiota modulates the host immune response to this extragastrointestinal infection. It has been previously shown that the gut microbiome provides signals to monocytes/macrophages that primed those cells to respond to and help control systemic lymphocytic choriomeningitis virus infections (19). Whether the gut microbiome modulates host immunity to *Plasmodium* through similar or different effects on the host immune system remains to be determined.

As mentioned above, diet has a major role in shaping the composition and activity of the gut microbiota (25, 41, 42). Consequently, manipulating the structure and function of these complex communities through the diet provides an opportunity to manipulate the host immune system (41). In our study, we identified that *Lactobacillus* and *Bifidobacterium* species in cecal content could have a protective role by modulating the parasite burden and attenuating the severity of the disease. It is also possible that these bacterial genera correlate with decreased parasitemia through niche competition that decreases the abundance of bacterial genera that cause elevated parasitemia. Because antibiotic treatment followed by yogurt treatment triggered a 14-fold reduction in parasite burden in susceptible mice, the results suggest that through optimization (e.g., identifying and treating with the most effective "protective" bacterial species or eliminating bacteria that contribute to high parasitemia), modulating the gut microbiome has the potential to be a novel prophylaxis to prevent severe malaria. Consistent with this possibility, prior work has shown that children in a rural African village in Burkina Faso have an enrichment of the Bacteroidetes phylum and a depletion of the Firmicutes phylum, which contains *Lactobacillus*, compared with European children (43). This bacterial assemblage resembles the community structure in susceptible mice that have increased Bacteroidetes and reduced Firmicutes compared with resistant mice (*SI Appendix*, Fig. 11). Therefore, the commonality between the bacterial community structure in African children and *Plasmodium*-susceptible mice suggests the possibility that probiotic modulation of the gut microbiota in mice to control severe malaria may work in humans.

This report demonstrates that the severity of malaria in mice is profoundly affected by the composition of the gut microbiota. The data lead to the hypothesis that differences in the gut microbiota may explain why some humans infected with *Plasmodium* progress to severe disease and others do not. The results also support the possibility that manipulating the gut microbiota has the potential to control the severity of malaria in humans. Whereas modulating the

MICROBIOLOGY

gut microbiota may not prevent *Plasmodium* infections, altering the gut microbiome has the potential to ameliorate severe disease and save thousands of lives annually.

Materials and Methods

Mice and Infections. Conventionally housed mice were purchased from Jax, NCI, CR, Har, and Tac. GF mice were purchased from the National Gnotobiotic Rodent Resource Center at the University of North Carolina at Chapel Hill. The University of Tennessee and University of Louisville Institutional Animal Care and Use Committees reviewed and approved animal experiments. Mice were fed NIH-31 Modified Open Formula Mouse/Rat Irradiated Diet (Harlan 7913), unless otherwise noted, in which case mice were fed Teklad 22/5 Rodent Diet (Harlan 8640), Jax in-house diet (5K67; Cincinnati Lab & Pet Supply, Inc.), or NCI in-house diet (5L79 Cincinnati Lab & Pet Supply, Inc.). GF mice received diluted cecum material administered by oral gavage. After transplants, mice were housed using conventional conditions. Mice were infected with *P. yoelii* 17XNL, *P. chabaudi* AS, and *P. berghei* ANKA. Blood samples were taken from the tail at regular intervals from 3 to 35 d postinfection. Parasitemia, percentage of RBCs infected with *Plasmodium*, was assessed by evaluation of thin blood smears or flow cytometry. Yogurt was made using a starter culture (Yogurt Starter Culture no. 2; Custom Probiotics) enriched with a probiotic powder supplement containing numerous *Lactobacillus* and *Bifidobacterium* species (11 Strain Probiotic Powder; Custom Probiotics). Mice were treated with an oral antibiotic mixture consisting of ampicillin, vancomycin, metronidazole, neomycin sulfate, and gentamycin sulfate. Cellular immune response was measured by flow cytometry, and $MSP1_{19}$-specific Abs were measured by ELISA.

Gut Microbiota Analysis. The distal half of the small intestine, cecum, and colon was excised from mice and flash-frozen in liquid nitrogen. DNA was extracted from samples using the MoBio PowerSoil DNA Isolation Kit. Bacterial 16S rRNA genes were amplified using bacteria-specific PCR primers targeting the V4 region. DNA sequencing was completed using the MiSeq (Illumina) platform at Hudson Alpha Institute for Biotechnology, Huntsville, AL. Sequences have been deposited in the NCBI Sequence Read Archive under Bioproject PRJNA289122. The Mothur software package was used to process sequences, to cluster sequences for phylogenetic classification, and to sort sequences into groups based on digestive tract regions. The PRIMER-E software package was used to interrogate the relationships between phylotypes across samples and to derive correlations between phylotype presence/abundance and other parameters. Detection of "biomarker" sequences was performed using the software package LEfSe (huttenhower.sph.harvard.edu/galaxy/).

Statistical Analysis. Descriptive and comparative statistical analyses of data, except the gut microbiota and metabolomics data, were done using GraphPad Software (Prism, version 6). The AUC was estimated for each group following the trapezoidal rule with the following equation:

$$AUC_{t1-t-last} = 0.5\sum(Y_i + Y_{i+1}) * (t_{i+1} - t_i),$$

where "t" is sampling time and "Y" is the observed outcome (e.g., percentage of parasitemia).

Additional details are available in *SI Appendix, Materials and Methods*.

ACKNOWLEDGMENTS. We thank Bruce Applegate and Whitney Powell for technical assistance. We thank Dr. Sarah Lebeis and Dr. Yousef Abu Kwaik for reviewing the manuscript. This work was supported by NIH Grant 1R21AI113386 (to N.W.S.) and American Cancer Society Research Scholar Grant RSG-14-057-01-MPC (to N.W.S.), and by the Kenneth & Blair Mossman Professorship (to S.W.W.). The National Gnotobiotic Rodent Resource Center at the University of North Carolina at Chapel Hill was supported by Grants 5-P39-DK034987 and 5-P40-OD010995.

1. Murray CJ, et al. (2012) Global malaria mortality between 1980 and 2010: A systematic analysis. *Lancet* 379(9814):413–431.
2. Crompton PD, et al. (2014) Malaria immunity in man and mosquito: Insights into unsolved mysteries of a deadly infectious disease. *Annu Rev Immunol* 32:157–187.
3. Dondorp AM, et al. (2005) Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med* 2(8):e204.
4. Hanson J, et al. (2012) Relative contributions of macrovascular and microvascular dysfunction to disease severity in falciparum malaria. *J Infect Dis* 206(4):571–579.
5. Honda K, Littman DR (2012) The microbiome in infectious disease and inflammation. *Annu Rev Immunol* 30:759–795.
6. Turnbaugh PJ, et al. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444(7122):1027–1031.
7. Wen L, et al. (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455(7216):1109–1113.
8. Hsiao EY, et al. (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155(7):1451–1463.
9. Johnson CC, et al. (2005) Antibiotic exposure in early infancy and risk for childhood atopy. *J Allergy Clin Immunol* 115(6):1218–1224.
10. Joffe TH, Simpson NA (2009) Cesarean section and risk of asthma. The role of intrapartum antibiotics: A missing piece? *J Pediatr* 154(1):154.
11. Fujimura KE, et al. (2014) House dust exposure mediates gut microbiome Lactobacillus enrichment and airway immune defense against allergens and virus infection. *Proc Natl Acad Sci USA* 111(2):805–810.
12. Manichanh C, et al. (2006) Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55(2):205–211.
13. Frank DN, et al. (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 104(34):13780–13785.
14. Peterson DA, Frank DN, Pace NR, Gordon JI (2008) Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. *Cell Host Microbe* 3(6):417–427.
15. Nicholson JK, et al. (2012) Host-gut microbiota metabolic interactions. *Science* 336(6086):1262–1267.
16. Kamada N, Chen GY, Inohara N, Núñez G (2013) Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol* 14(7):685–690.
17. Benson A, Pifer R, Behrendt CL, Hooper LV, Yarovinsky F (2009) Gut commensal bacteria direct a protective immune response against Toxoplasma gondii. *Cell Host Microbe* 6(2):187–196.
18. Naik S, et al. (2012) Compartmentalized control of skin immunity by resident commensals. *Science* 337(6098):1115–1119.
19. Abt MC, et al. (2012) Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37(1):158–170.
20. Yilmaz B, et al. (2014) Gut microbiota elicits a protective immune response against malaria transmission. *Cell* 159(6):1277–1289.
21. Yooseph S, et al. (2015) Stool microbiota composition is associated with the prospective risk of Plasmodium falciparum infection. *BMC Genomics* 16(1):631.
22. Ivanov II, et al. (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4(4):337–349.
23. Ivanov II, et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139(3):485–498.
24. David LA, et al. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505(7484):559–563.
25. Turnbaugh PJ, et al. (2009) The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 1(6):6ra14.
26. Carmody RN, et al. (2015) Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe* 17(1):72–84.
27. Pérez-Enciso M, Tenenhaus M (2003) Prediction of clinical outcome with microarray data: A partial least squares discriminant analysis (PLS-DA) approach. *Hum Genet* 112(5-6):581–592.
28. Mooney JP, et al. (2015) Inflammation-associated alterations to the intestinal microbiota reduce colonization resistance against non-typhoidal Salmonella during concurrent malaria parasite infection. *Sci Rep* 5:14603.
29. Segata N, et al. (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* 12(6):R60.
30. Cox MJ, et al. (2010) Lactobacillus casei abundance is associated with profound shifts in the infant gut microbiome. *PLoS One* 5(1):e8745.
31. McNulty NP, et al. (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci Transl Med* 3(106):106ra106.
32. Pérez-Mazliah D, et al. (2015) Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria. *PLoS Pathog* 11(3):e1004715.
33. Butler NS, et al. (2012) Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol* 13(2):188–195.
34. Azambuja P, Garcia ES, Ratcliffe NA (2005) Gut microbiota and parasite transmission by insect vectors. *Trends Parasitol* 21(12):568–572.
35. Dong Y, Manfredini F, Dimopoulos G (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog* 5(5):e1000423.
36. Cirimotich CM, et al. (2011) Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae. *Science* 332(6031):855–858.
37. Boissière A, et al. (2012) Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection. *PLoS Pathog* 8(5):e1002742.
38. Bahia AC, et al. (2014) Exploring Anopheles gut bacteria for Plasmodium blocking activity. *Environ Microbiol* 16(9):2980–2994.
39. Verhulst NO, et al. (2009) Cultured skin microbiota attracts malaria mosquitoes. *Malar J* 8:302.
40. Verhulst NO, et al. (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PLoS One* 6(12):e28991.
41. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI (2011) Human nutrition, the gut microbiome and the immune system. *Nature* 474(7351):327–336.
42. Brestoff JR, Artis D (2013) Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* 14(7):676–684.
43. De Filippo C, et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 107(33):14691–14696.