Molecular Mechanism for Switching of *P. falciparum* Invasion Pathways into Human Erythrocytes

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The malaria parasite, *Plasmodium falciparum*, exploits multiple ligand-receptor interactions, called invasion pathways, to invade the host erythrocyte. Strains of *P. falciparum* vary in their dependency on sialated red cell receptors for invasion. We show that switching from sialic acid–dependent to –independent invasion is reversible and depends on parasite ligand use. Expression of *P. falciparum* reticulocyte–binding like homolog 4 (PfRh4) correlates with sialic acid–independent invasion, and PfRh4 is essential for switching invasion pathways. Differential activation of PfRh4 represents a previously unknown mechanism to switch invasion pathways and provides *P. falciparum* with exquisite adaptability in the face of erythrocyte receptor polymorphisms and host immune responses.

*Plasmodium falciparum* causes the most lethal form of malaria, a devastating disease responsible for vast morbidity and loss of life. Invasion of erythrocytes by malaria parasites involves complex interactions between multiple ligands and receptors (1). Some *P. fal-

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**Fig. 4.** Evaluation of Poly-G and natural TLR ligands for their ability to reverse Treg cell function and their effects on antitumor immunity. (A) Naive CD4+ T cells were mixed with Treg 102 and naturally occurring CD4+CD25+ T cells in the presence of different TLR ligands. (B) The reversibility of TLR8 siRNA–transduced (GFP+) and untransduced (GFP-) Treg cells in response to Poly-G10, ssRNA40, and CpG-A oligonucleotides. Uninfected parental and control siRNA-transduced Treg 102 cells and Poly-T10 served as controls. (C) Poly-G10–induced reversal of Treg cell function enhances antitumor immunity in vivo. Rag1-deficient mice were injected with human 586mel tumor cells on day 0 and then treated with autologous tumor-specific CD8+ T cells on day 3. Treg cells were pre-activated with OKT3 and washed before adoptive transfer. Tumor volumes were measured and presented as means ± SD (n = 6 mice per group). (D) Experimental procedures, tumor cells, and T cells were as in (C), except that Rag2-γC-deficient mice were used. P values in (B) and (C) were determined by the Wilcoxon rank-sum test.

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Materials and Methods Figs. S1 to S6

References and Notes

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Fig. 1. Sialic acid–dependent and –independent invasion and global transcription in *P. falciparum*. (A) Erythrocytes were treated with neuraminidase to remove surface sialic acid residues. The percentage invasion into neuraminidase–treated erythrocytes is shown relative to invasion into untreated erythrocytes. W2mefΔ175 has the EBA175 gene disrupted (6). W2mef/c2 and c4 were done (fig. S1A) and selected for growth on neuraminidase-treated erythrocytes to obtain W2m/c2/N and W2m/c4/N. After growth on untreated erythrocytes over several months, revertants were derived (W2m/c2/NR1 and W2m/c4/NR1), which were indistinguishable from W2mef. Revertants were reselected on neuraminidase-treated erythrocytes to derive W2m/c2/N2 and W2m/c4/N2. Reversion (W2m/c2/NR2 and W2m/c4/NR2) and reselection (W2m/c2/N3 and W2m/c4/N3) could be reproduced successively in both clones of W2mef. (B) Oligonucleotide array analysis comparing transcriptional profiles in W2mef (sialic acid–dependent) with W2mefΔ175 and W2mef/c4/N (both sialic acid–independent). The genes that differ in transcription most relative to sialic acid–dependent W2mef are annotated: EBA165, erythrocyte binding antigen 165; PfRh4, reticulocyte binding homolog 4; and EBA175, which was essentially undetectable in W2mefΔ175. See also fig. S2, A and B. (C) Real-time PCR. Gene expression in W2mefΔ175, W2mef/c4/N, and W2mef/c4/NR is expressed relative to W2mef for genes of the *ebl* and *PfRh* families. *P* < 0.05; **P < 0.01. A transcript for the EBA175 gene was undetectable in W2mefΔ175. See also fig. S2C.

To address this issue, two clonal lines of W2mef, W2mef/c2 and W2mef/c4 (fig. S1A), were selected on neuraminidase-treated erythrocytes to select parasites switched to sialic acid–independent invasion to derive W2m/c2/N and W2m/c4/N, both of which showed invasion comparable to that of W2mef. W2mef, W2m/c2 and W2m/c4 (fig. S1A), were derived from two independent transfections. Molecular sizes are shown on the left (in kb). The absence of the 2.7-kb wild-type band and the presence of a 2.1-kb band indicate that the *PfRh4* locus is disrupted in all clones. (C) Schizonts were probed with α-Rh4.1 (fig. S3B) (top) or α-Hsp70 (bottom) antibodies. W2mefΔ175 and W2mef/N (sialic acid–dependent) express *PfRh4*, which was absent in W2mef, W2mef/Rh4/c5, W2mef/Rh4/c1, and W2m/c2/NR (sialic acid–dependent) parasites. Molecular sizes are indicated on the left (in kb). The parasite lines 7GB, HB3, and 3D7 also express *PfRh4* and invade by sialic acid–independent pathways. (D) Expression of *PfRh4* over the asexual life cycle is shown for W2mefΔ175, and time points are indicated in hours.

Microarrays of *P. falciparum* (7) were used to reveal any transcriptional switch between W2mef versus W2mefΔ175 and W2mef/c4/N (Fig. 1B and fig. S2, A and B). *PfRh4* (PFD1150c) (8) and *EBA165* (PFD1155w) were the only genes to show reproducible and significant transcriptional differences, *PfRh4* and *EBA165* occur within the *P. falciparum* genome in a head-to-head orientation on chromosome 4, and it is possible they are coregulated (9). The protein encoded by *PfRh4* is homologous to other reticulocyte binding–like proteins disrupted (10). W2mefΔ175 has the EBA175 gene disrupted (6). W2mef/c2 and c4 were done (fig. S1A) and selected for growth on neuraminidase-treated erythrocytes to obtain W2m/c2/N and W2m/c4/N. After growth on untreated erythrocytes over several months, revertants were derived (W2m/c2/NR1 and W2m/c4/NR1), which were indistinguishable from W2mef. Revertants were reselected on neuraminidase-treated erythrocytes to derive W2m/c2/N2 and W2m/c4/N2. Reversion (W2m/c2/NR2 and W2m/c4/NR2) and reselection (W2m/c2/N3 and W2m/c4/N3) could be reproduced successively in both clones of W2mef. (B) Oligonucleotide array analysis comparing transcriptional profiles in W2mef (sialic acid–dependent) with W2mefΔ175 and W2mef/c4/N (both sialic acid–independent). The genes that differ in transcription most relative to sialic acid–dependent W2mef are annotated: EBA165, erythrocyte binding antigen 165; PfRh4, reticulocyte binding homolog 4; and EBA175, which was essentially undetectable in W2mefΔ175. See also fig. S2, A and B. (C) Real-time PCR. Gene expression in W2mefΔ175, W2mef/c4/N, and W2mef/c4/NR is expressed relative to W2mef for genes of the *ebl* and *PfRh* families. *P* < 0.05; **P < 0.01. A transcript for the EBA175 gene was undetectable in W2mefΔ175. See also fig. S2C.

Fig. 2. Disruption of *PfRh4* and expression of *PfRh4* protein in *P. falciparum*. (A) Schematic of the wild-type and disrupted *PfRh4* loci. The *hDHFR* gene was inserted into *PfRh4* by double crossover recombination. Southern analysis was performed using Hind III (H)–digested DNA with probe Pr3'. (B) Southern blot of the *PfRh4* locus in W2mef and clones of W2mefΔRh4 (fig. S1B). Clones 1 to 4 and clones 5 to 9 were derived from two independent transfections. Molecular sizes are shown on the left (in kb). The absence of the 2.7-kb wild-type band and the presence of a 2.1-kb band indicate that the *PfRh4* locus is disrupted in all clones. (C) Schizonts were probed with α-Rh4.1 (fig. S3B) (top) or α-Hsp70 (bottom) antibodies. W2mefΔ175 and W2mef/N (sialic acid–dependent) express *PfRh4*, which was absent in W2mef, W2mef/Rh4/c5, W2mef/Rh4/c1, and W2m/c2/NR (sialic acid–dependent) parasites. Molecular sizes are indicated on the left (in kb). The parasite lines 7GB, HB3, and 3D7 also express *PfRh4* and invade by sialic acid–independent pathways. (D) Expression of *PfRh4* over the asexual life cycle is shown for W2mefΔ175, and time points are indicated in hours.
proteins (PfRh), including PfRh1 (PFD0110w) and PfRh2b (MAL13P1.176) that have been implicated in *P. falciparum* invasion of erythrocytes (10–12), and this family is related to other invasion proteins in *Plasmodium yoelii* (13) and *Plasmodium vivax* (14). EBA165 is a member of the erythrocyte binding-like (ebi) family that includes EBA175; however, current data suggest that it is a transcribed pseudogene (15). Sequencing of *EH4165* in W2mefΔ175 and W2mef showed that frameshift mutations were present, suggesting that it was unlikely to encode a protein and was only activated by its proximity to PfRh4. Additionally, antibodies to putative EBA165 did not bind to the predicted protein in W2mefΔ175 (fig. S3A).

To validate transcriptional changes seen in microarrays, we used real-time polymerase chain reaction (RT-PCR) of sialic acid–dependent parasites W2mef and W2m/c4/N, and sialic acid–independent parasites W2mefΔ175 and W2m/c4/N (Fig. 1C and fig. S2C). Transcription of *PfRh4* and *EBA165* increased ~60- to 80-fold in the sialic acid–independent lines compared with the sialic acid–dependent lines, confirming the microarray results. In comparison, other members of the *ebi* and *PfRh* families showed relatively minor increases in transcription. These results suggest that activation of the *PfRh4* gene is required for switching from sialic acid–dependent to –independent invasion.

We constructed transgenic parasites in which the *PfRh4* gene was disrupted (W2mefΔRh4/c1-9) and used PfRh4-specific antibodies to determine expression patterns (Fig. 2 and fig. S3B). Attempts to disrupt *PfRh4* in the sialic acid–independent parasite lines 3D7 and 7G8 were unsuccessful; although this does not verify that *PfRh4* is essential in these sialic acid–independent parasites, it does suggest a functionally important role in the invasion of normal erythrocytes. Specific antibodies did not detect PfRh4 in sialic acid–dependent parasites W2mef and W2m/c4/N, nor in any schizonts and free merozoites with the exception of RAP-1, which is localized to the body of the rhoptries. Scale bar, 5 μm.

(E) *PfRh4* is expressed more highly in CSL2 schizonts grown on neuraminidase-treated erythrocytes (CSL2/c4/N) than on untreated erythrocytes. Schizonts were probed with α-Rh4.1 (top) or α-Hsp70 (bottom). Molecular sizes are indicated on the left (in kD).
of the W2mefΔRh4 cloned lines (Fig. 2C). In contrast, PIRh4 was expressed in sialic acid–independent lines W2mefN and W2mefΔ175, as well as in 7G8, HB3, and 3D7. Western analysis was performed during the course of the 48-hour asexual blood stage life cycle of W2mefΔ175 parasites. PIRh4 was detected in mature schizonts (Fig. 2D), concomitant with apical organelle development and expression of other ligands involved in the invasion process (1, 16).

W2mΔRh4 parasites were grown on normal or neuraminidase-treated erythrocytes to determine if they could switch to sialic acid–independent invasion. W2mefΔEBA181 parasites (17) were generated in the same way as W2mefΔRh4, and as expected, this line was able to switch to sialic acid–independent invasion (fig. S4A). Although W2mΔRh4 parasites grew normally on untreated erythrocytes (Fig. 3), they were unable to switch to sialic acid–independent invasion even after prolonged culture on neuraminidase-treated erythrocytes (Fig. 3 and fig. S3C), suggesting that invasion was completely blocked at the first generation. Therefore, transcriptional activation of the PIRh4 gene and expression of the PIRh4 protein are required for switching of W2mef from sialic acid–dependent to –independent invasion.

We constructed two independent transgenic parasite lines that expressed PIRh4 as a chimeric protein with green fluorescent protein (GFP) to determine if subcellular localization of PIRh4 is consistent with a role in merozoite invasion; the results were identical (figs. S1C and S5). The W2mef-Rh4GFP parasites could switch invasion pathways and invaded neuraminidase-treated erythrocytes efficiently, indicating that activation and function of PIRh4 were preserved (Fig. 4C). The GFP-tagged PIRh4 protein showed the expected increase in molecular weight (Fig. 4A). Segmenting schizonts and merozoites of W2mef-Rh4GFP1N/2N displayed fluorescence apical to the nucleus (Fig. 4B, fig. S5D). PIRh4 colocalized well with PIRh2a/b in segmenting schizonts, and the overlap condensed into a single apical dot in free merozoites in which PIRh2a and b are present in the neck of the rhoptries (11, 12) (Fig. 4D). PIRh4 was more apical than RAPI, a protein located within the body of the rhoptries (18). Therefore, PIRh4 is located at the apical tip of free merozoites, consistent with a direct function in invasion of erythrocytes.

We tested several sialic acid–dependent strains for growth on neuraminidase-treated erythrocytes to determine if the ability to switch invasion pathways and use different receptors for invasion is a general property of P. falciparum. Cloned lines of CSL2 (fig. S1D) were sialic acid–dependent but adapted to sialic acid–independent invasion in a similar way to W2mef (Fig. 4C) in association with elevated expression of PIRh4 protein (Fig. 4E).

We have shown that activation of sialic acid–independent invasion is regulated by differential gene expression and silencing of PIRh4. Activation of PIRh4 occurs at a low level, and these variant parasites can be selected for growth on erythrocytes lacking sialic acid or by genetic ablation of the EBA175 gene. Silencing of the active PIRh4 gene occurs over time when parasites are returned to normal erythrocytes, showing that the switch in invasion pathways can occur in both directions in the presence of functional EBA175. The activation of PIRh4 in response to loss of EBA175 function suggests that the PIRh and ebl protein families show some overlap with respect to their function in invasion. The ability to switch receptor usage for invasion from sialic acid–dependent to –independent pathways represents a previously unknown strategy to evade host receptor polymorphisms and immune mechanisms and has important implications for the design of vaccines against malaria parasites.

References and Notes
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Computational Improvements
Reveal Great Bacterial Diversity
and High Metal Toxicity in Soil

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The complexity of soil bacterial communities has thus far confounded effective measurement. However, with improved analytical methods, we show that the abundance distribution and total diversity can be deciphered. Reanalysis of reassociation kinetics for bacterial community DNA from pristine and metal-polluted soils showed that a power law best described the abundance distributions. More than one million distinct genomes occurred in the pristine soil, exceeding previous estimates by two orders of magnitude. Metal pollution reduced diversity more than 99.9%, revealing the highly toxic effect of metal contamination, especially for rare taxa.

For any complex system, the number and relative abundance of parts is fundamental to a quantitative description of the system. Quantification provides a framework to compare equilibrium and dynamic properties and, for biological communities, to evaluate perturbations such as pollution, global climate change, and foreign species encroachment. To quantify plant and animal communities, ecologists survey the number and relative abundance of species (i.e., species-abundance distributions) (1, 2). However, effective measurement of bacterial species-abundance distributions has eluded...