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Decoding the complexities of human malaria through systems immunology

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Summary

The complexity of the *Plasmodium* parasite and its life cycle poses a challenge to our understanding of the host immune response against malaria. Studying human immune responses during natural and experimental *Plasmodium* infections can enhance our understanding of malaria-protective immunity and inform the design of disease-modifying adjunctive therapies and next-generation malaria vaccines. Systems immunology can complement conventional approaches to facilitate our understanding of the complex immune response to the highly dynamic malaria parasite. In this review, recent studies that used systems-based approaches to evaluate human immune responses during natural and experimental *P. falciparum* and *P. vivax* infections as well as during immunization with candidate malaria vaccines are summarized and related to each other. The potential for next-generation technologies to address the current limitations of systems-based studies of human malaria are discussed.

Keywords

malaria immunity; malaria immunology; host response; systems biology; systems immunology; systems vaccinology; transcriptomics; gene expression profiling; microarray analysis; *Plasmodium falciparum*; *Plasmodium vivax*

> "The processes of disease are so complex that it is excessively difficult to search out the laws which control them, and, although we have seen a complete revolution in our ideas, what has been accomplished by the new school of medicine is only an earnest of what the future has in store."
>
> Sir William Osler

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CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare.

INTRODUCTION

Clinical malaria encompasses a spectrum of syndromes that initially manifest as the intermittent fevers and rigors typical of “uncomplicated” malaria, which if not promptly treated, can rapidly progress to severe disease, most notably the devastating neurological complications of cerebral malaria. Although much progress has been made towards reducing malaria morbidity and mortality over the last century, over 200 million people still suffered from the disease in 2017, leading to ~435,000 deaths[1]. Among the human infective *Plasmodium* species, *Plasmodium falciparum* is the most prevalent malaria parasite in Africa and much of Asia and is responsible for cerebral malaria and the overwhelming majority of malaria deaths; however, *Plasmodium vivax* also causes significant morbidity, being widely distributed across the Asia Pacific and the Americas. Control of the female anopheline mosquito, the vector of human malaria, and prompt access to malaria diagnosis and effective anti-malaria treatment have played key roles in reducing the global malaria burden and eliminating malaria in several countries[2]. However, further progress towards malaria eradication will require additional tools, such as vaccines that induce durable protection[3]. It is also critical to develop effective adjunctive therapies to improve survival during severe malaria before eradication is achieved[4]. The development of these tools would be aided by a more complete understanding of the processes that govern the balance between protective immunity and immunopathogenesis during malaria infection. Thus, studying human immune responses during natural and experimental *Plasmodium* infections can enhance our understanding of malaria-protective immunity and inform the design of disease-modifying adjunctive therapies and next-generation malaria vaccines.

However, the complexity of the *Plasmodium* parasite and its life cycle poses a challenge to our understanding of the host immune response against this organism. *Plasmodium* is an intracellular pathogen that directly invades two cell types in humans: liver cells (hepatocytes) and red blood cells (erythrocytes). After entering the human host as sporozoites via the bite of an infected *Anopheles* mosquito, *Plasmodium* parasites initiate the liver stage of infection by invading hepatocytes where they develop and replicate for ~7 days without causing symptoms. The parasites then rupture from hepatocytes into the bloodstream as merozoites and rapidly invade erythrocytes. Within erythrocytes, the parasite develops and replicates over 48 hours before rupturing out as daughter merozoites, each capable of invading a new erythrocyte. Blood-stage infection that begins as low-level, asymptomatic parasitemia can rapidly progress to mild or severe malaria or persist as a chronic, asymptomatic infection depending on the balance between protective and pathologic host immune responses, and possibly parasite factors that remain poorly understood. Not only does the parasite exist in multiple forms within the human host, but in the case of *P. falciparum*, also expresses over 5,000 protein-coding genes[5], many of which are highly polymorphic, presumably due to immune selective pressure[6,7]. Thus, a strategy to comprehensively understand the host immune response to natural malaria infection would have to account for the diverse clinical phenotypes in the context of multiple forms of the parasite, each expressing a potentially unique set of polymorphic antigens.

Systems biology can complement conventional approaches to broaden our understanding of the complex immune response to the highly dynamic malaria parasite. Although systems

biology has been defined a myriad of ways, it is generally described as an interdisciplinary approach that aims to unravel complex biological phenomena both conceptually and quantitatively via the integrated analysis of large-scale experimental data using computational and statistical methodologies, which may permit the accurate simulation of biological interactions and prediction of relevant outcomes[8]. In contrast to reductionist approaches, which attempt to dissect individual components of a system to address defined hypotheses, systems biology evaluates components within the context of the whole system or organism to address more general hypotheses while potentially generating more specific hypotheses. As a sub-specialization within systems biology, systems immunology attempts to integrate the cellular and molecular components of the immune system by applying computational methods to high-dimension data acquired from well-designed observational or experimental studies to better comprehend their interactions and function, especially in the context of immune perturbations such as infection or vaccination. Although *Plasmodium* parasites can directly affect several organs and tissues, including the liver, spleen, bone marrow and vascular endothelium, ongoing infections replicate within erythrocytes that predominantly circulate in the peripheral blood where they interact with host immune cells and molecules[9], making the interrogation of immunological responses possible even in pediatric studies conducted in malaria-endemic settings. Thus, computational approaches employed in malaria systems immunology studies to date (Table 1) have generally been applied to data generated by -omics analyses of peripheral blood components: *ex vivo* whole blood, cryopreserved peripheral blood mononuclear cells (PBMCs), PBMCs re-stimulated with parasite antigen *in vitro*, and cryopreserved serum or plasma.

Here, we review recent studies that used systems immunology approaches to evaluate human immune responses during *P. falciparum* and *P. vivax* infections and during immunization with candidate malaria vaccines. We will first discuss controlled human malaria infections (CHMI) in which individuals are infected with sporozoites either by the bite of mosquitoes or by direct venous inoculation, resulting in blood-stage infections approximately one week later. We next highlight recent systems vaccinology studies that assessed molecular signatures of immunogenicity and protection before and after immunization and challenge. Lastly, we discuss systems immunology studies of naturally transmitted *Plasmodium* infections that cover the spectrum of disease severity, from asymptomatic parasitemia to uncomplicated and severe malaria syndromes, including cerebral malaria and severe malaria anemia. The majority of these studies applied transcriptomic analysis of peripheral blood cells to assess differences in malaria-related clinical phenotypes or associated outcomes. A few notable studies further captured the complexity of the immune response by integrating transcriptomic analysis with data from genomic, parasitological, cellular, serological and/or functional assessments to gain further insight into malaria pathogenesis and immunity. We examine and summarize these recent studies in relation to each other to identify unifying concepts that might aid in our understanding of malaria, while also illuminating gaps in knowledge deserving of further investigation. Studies highlighted in this review are tabulated along with additional studies that applied transcriptomics, metabolomics, proteomics, or *Plasmodium*-specific antibody profiling by protein microarray to different clinical phenotypes associated with malaria (Table 2). Systems-based studies focusing primarily on

mouse and non-human primate models of malaria are beyond the scope of this review, but their importance as model systems have recently been discussed by others[34,35].

Blood transcriptomic assessments of the early host response to controlled human malaria infections

Experimental malaria infections began in the early 1900s when Austrian physician Julius Wagner-Jauregg inoculated patients with malaria-infected blood to induce fever as a treatment for neurosyphilis, which was at that time a terminal illness[90]. Although penicillin would eventually supplant "malariotherapy" as a more effective and safer therapy for syphilis[91], experimental malaria infection of humans has remained an invaluable tool for evaluating the *in vivo* efficacy of anti-malarial drugs and vaccines[92] and–with the adoption of standardized safety procedures–is now referred to as controlled human malaria infection (CHMI)[93]. CHMI allows for high resolution temporal assessments of parasite replication in blood, providing an optimal framework for systems immunology studies of the initial host immune response to blood-stage infection. As discussed later, CHMI after immunization with candidate malaria vaccines has become the standard approach for evaluating vaccine efficacy against homologous and heterologous parasite challenge prior to conducting field trials in endemic areas[92].

In one of the earliest transcriptomic studies of human malaria, Ockenhouse et al. employed Affymetrix GeneChip microarrays to assess gene-expression profiles of peripheral blood mononuclear cells (PBMCs) collected from 22 malaria-naïve North American adults experimentally infected with *P. falciparum*. Samples were collected on the day of first smear-detectable parasitemia, when subjects were either pre-symptomatic (n=16) or exhibited low-grade fevers consistent with blood-stage malaria (n=6), and compared to pre-infection baseline samples in paired analyses.[69] Irrespective of symptomatology, infection induced upregulation of genes encoding the pattern recognition receptors Toll-like receptor 1 (TLR1), TLR2, TLR4; the glycoprotein CD36; and the lipopolysaccharide co-receptor CD14, but downregulation of *TLR3* and *TLR5*. During early infection when parasitemia was at the limit of microscopic detection, pre-symptomatic subjects demonstrated upregulation of genes encoding IFN-γ and members of the IFN-γ signaling pathway as well as the inflammatory cytokine IL-1β and Caspase-1, which cleaves and activates IL-1 β to initiate pro-inflammatory responses. Increases in IFN-γ gene expression correlated with expression of CCL2, CXCL9, CXCL10 at both the gene and cytokine levels, providing evidence that transcriptomics was sensitive for detecting immune signatures even during clinically silent infections. Network analysis of genes overexpressed during *P. falciparum* blood-stage infection regardless of symptomatology revealed, perhaps not surprisingly, that the inflammatory cytokines TNF, IFN-γ, and IL-1β were the central nodes suspected of coordinating the immune response during early malaria. Similar activation of pro-inflammatory cytokines was also observed in subsequent CHMI studies that employed whole-blood RNA sequencing (RNA-seq) in malaria-naïve Dutch adults (n=5) at first detectable *P. falciparum* parasitemia[81] and in both malaria-naïve (n=7) and semi-immune (n=9) Colombian adults at first detectable *P. vivax* parasitemia[36].

Rothen et al. investigated whole-blood transcriptome changes following CHMI with *P. falciparum* in 10 Tanzanian adult male volunteers with prior malaria exposure using RNA-Seq[79]. Rather than evaluate gene expression changes at first detectable parasitemia, comparisons were made between expression at days 5, 9, and 28 post-CHMI with each other and the pre-infection baseline. Unexpectedly, differential gene expression was observed at day 5 post-CHMI during what was presumably the sub-clinical liver-stage, but not at day 9, which was on average ~2 days before microscopy detectable blood-stage parasitemia. Enrichment analysis of differentially expressed genes (DEGs) on Day 5 using blood transcription modules (BTMs) revealed overexpression of genes within modules related to the CORO1A-DEF6 network, platelet activation, regulation of localization, signaling events as well as processes involving translation and transcription. Grouping subjects by duration of prepatent period identified genes differentially expressed between early and average/late appearance of parasitemia, with over-representation of genes within modules linked to regulation of transcription, cell cycle, phosphatidylinositol signaling and erythrocyte development. One of the caveats with conducting CHMI studies in individuals with prior malaria exposure is high variability in the pre-patent period owing to differing levels of pre-existing blood-stage immunity, which introduces greater variation in the kinetics of host gene expression and reduces statistical power in detecting differential gene expression. More frequent sampling to capture the time of maximal differential gene expression can overcome this to some extent and would allow for systems analyses to determine which features of pre-existing malaria immunity influence the host response to CHMI.

Systems analyses of malaria vaccine candidates

Systems vaccinology is a sub-discipline of systems biology that attempts to unravel the complex interactions between components of the immune system after vaccination in order to better understand and predict vaccine responses. From a practical standpoint, systems vaccinology aims to determine molecular and cellular signatures induced by vaccination and detectable in peripheral blood that are predictive of vaccine immunogenicity and vaccine-induced protection. Such an approach has been applied to approved formulations of influenza[94–96], yellow fever[97,98], and pneumococcal[96] vaccines in order to identify early molecular or cellular signatures induced by immunization that are predictive of subsequent pathogen-specific adaptive immune responses. In the case of infections for which immunologic correlates of protection are known and well defined (e.g. hepatitis A, hepatitis B, tetanus), the identification of signatures predictive of immunogenicity can be used to more rapidly evaluate the efficacy of next-generation vaccines. On the other hand, infections for which reliable immunologic correlates of protection are not yet known, such as malaria, integrated assessments of vaccine-induced molecular signatures with immunogenicity and vaccine efficacy data may lead to the identification of novel correlates of protection. Analyses of baseline (pre-vaccination) signatures can also reveal predictors of post-vaccination immune responses[95], which can be used to identify individuals who may not respond optimally to vaccination. This last application is highly relevant to malaria vaccines, which appear to induce less robust antibody and cellular responses in malaria-exposed volunteers from endemic regions relative to naïve volunteers from non-endemic regions[99–101].

It is important to note that protection conferred by malaria vaccines can be defined in terms of protection from parasitemia (sterile protection) or from febrile malaria (clinical protection), with vaccine efficacy assessed by proportional analysis or time to event analysis (either parasitemia or clinical malaria). Thus, a systems analysis of malaria vaccine efficacy would assess molecular signatures predictive of sterile or clinical protection using classifiers dichotomized by protection status or by time to event, with the latter being most useful for partially effective vaccines that may not have provided complete protection during the study period but nevertheless delayed either parasitemia or clinical malaria. Partially effective vaccines provide an opportunity to determine differences between protected and non-protected vaccinees using systems-based approaches.

The first systems vaccinology studies of a malaria vaccine focused on RTS,S—the most advanced malaria vaccine to date, having been licensed for use outside the European Union by the European Medicines Agency in 2015[102]. The RTS,S vaccine consists of a recombinant fusion protein (RTS) containing the circumsporozoite (CSP) NANP repeat region (R), multiple CSP T-cell epitopes (T), and the hepatitis B surface antigen (HBsAg; S) assembled with native HbsAg (S) into virus-like particles with the CSP component exposed on the surface[103]. The licensed version of RTS,S is formulated with the adjuvant AS01[102]. RTS,S/AS01 provided 23–41% protective efficacy against all malaria episodes in sub-Saharan Africa children 5 to 17 months of age, with declining efficacy over the follow-up period [104].

Vahey and colleagues examined PBMC transcriptomes by Affymetrix GeneChip microarrays in a subset of 39 volunteers enrolled in a phase 2b trial comparing RTS,S adjuvanted with either AS01 or AS02 to determine the pre-CHMI challenge signatures predictive of vaccine immunogenicity and efficacy[86,105]. Among these vaccinees, 13 (33%) were protected from *P. falciparum* parasitemia. Gene expression profiles were evaluated at the pre-vaccination baseline; the day of the third vaccination (just prior to injection); at 24 hours, 72 hours, and two weeks after the third vaccination; and 5 days after challenge by CHMI via *P. falciparum*-infected mosquito bites. Significant changes in gene expression were observed 24 and 72 hours after the third vaccination relative to the day of the third vaccination, but no differences were observed between protected and non-protected groups. Gene set enrichment analysis (GSEA), a computational method that determines whether a pre-defined set of genes are significantly enriched in a concordant manner between two groups[22], revealed that the immunoproteasome degradation pathway was upregulated in protected relative to non-protected volunteers 2 weeks after the third vaccination, suggesting differential antigen presentation and processing of major histocompatibility complex peptides in RTS,S-induced malaria protection.

Rinchai and colleagues re-analyzed the microarray dataset generated by Vahey et al.[86], applying a modular repertoire approach in which transcriptomic differences are assessed for sets of genes (“modules”) rather than as individual genes. These modules have been identified to be coordinately expressed (“co-clustered”) across multiple human disease states, including HIV, tuberculosis, sepsis, systemic lupus erythematosus, systemic arthritis, and also following liver transplantation[30,106]. Module response was measured as the percentage of responsive genes (i.e. DEGs relative to just prior to the third vaccination)

within a given module. Protected volunteers had an increased interferon module response on day 1 post-vaccination compared to non-protected volunteers, while, intriguingly, non-protected volunteers had a sustained decrease in the interferon module response at days 3 and 14 after the third vaccination. Based on the decrease in abundance of interferon signatures in response to the third RTS,S vaccination among the non-protected individuals, the authors hypothesized that protective interferon responses may be antagonized by antigen-specific IgE induced by vaccination via mechanisms similar to that observed in human plasmacytoid dendritic cells[107]. Follow-up studies using serum from malaria-exposed Mozambican children enrolled in a phase 2b trial of RTS,S showed that RTS,S vaccination specifically induced CSP-specific IgE. However, post-vaccination levels were significantly lower in the non-protected group when compared to the protected group[86], a finding that did not support the authors' original hypothesis.

More recent transcriptomic analyses of the same RTS,S vaccination cohort (n=39) were performed by van den Berg and colleagues who used a different approach that allows for the assessment of gene expression changes over time and the incorporation of data trends in models to predict protection status[88]. Again, subjects were defined as 'protected' if they remained parasite negative for 4 weeks after challenge (n=13). However, among infected subjects, those who developed parasitemia were classified as 'delayed parasitemia' if their pre-patent period was longer than the longest pre-patent period in unvaccinated infectivity control subjects (n=11), while the remaining infected subjects were classified as 'not protected' (n=15). Gene expression was measured by Affymetrix GeneChip microarray at the time of the first and third vaccination as well 1, 3, and 14 days after the third vaccination. Serum IFN-γ levels measured before and 1 day after each dose showed consistent but non-significant increases in IFN-γ among protected subjects relative to non-protected subjects post-vaccination. Based on this evidence, the role of IFNγ in RTS,S-mediated protection was investigated. Genes within the IFNγ-signaling pathway from the proprietary Ingenuity pathways database[23] demonstrated increased expression 14 days after the third vaccination in protected subjects relative to not protected subjects. Multiway partial least squares discriminant analysis (N-PLS-DA) incorporated subject, gene, and time point variables to correlate variability in expression data with the binary outcome of protection vs. non-protection (excluding delayed parasitemia). Using the entire data set as features, N-PLS-DA identified 110 genes, 42 of which had immune-related functions, within predictive models that showed 78% prediction accuracy by double cross-validation. Limiting features to only genes within the IFNγ-signaling pathway still allowed for the identification of 44 genes within predictive models that showed 77% prediction accuracy. Taken together, the blood transcriptomic studies of this vaccination cohort suggest that the vaccine-induced interferon response plays an important role in RTS,S-mediated protection against CHMI challenge.

Kazmin and colleagues used systems biology approaches to evaluate immune responses in volunteers from two different RTS,S vaccine trials. In the first, 21 volunteers received three consecutive immunizations with RTS,S/AS01 (RRR regimen). In the second, 25 volunteers received two immunizations with RTS,S/AS01 followed by immunization with adenovirus 35 expressing CSP (ARR regimen)[60]. Both regimens resulted in ~50% protective efficacy after challenge by CHMI via *P. falciparum*-infected mosquito bite three weeks after the final immunization. Gene expression of PBMCs was determined by Affymetrix GeneChip

microarray at baseline; at each vaccination dose; on multiple days after each vaccination; and immediately before and after challenge. Vaccination with either regimen induced strong transcriptional responses in PBMCs at Day 1 or Day 6 after vaccination relative to the pre-vaccination baseline. Enrichment analysis with BTMs as gene sets to determine functionally relevant pathways induced by each regimen showed enrichment of BTMs related to cell cycle and B cells 6 days after each dose with persistence of the cell cycle BTM 14 days after the first vaccine dose and repression of the NK-cell-related BTM following vaccination. To assess molecular signatures of immunogenicity, BTM expression levels were then correlated to CSP-specific IgG antibody levels measured in serum collected on the day of CHMI challenge (day 77). The RRR regimen, which has been shown to induce robust anti-CSP antibody responses, induced expression of plasmablast, innate, and type I interferon (IFN)-related signatures within 6 days post-vaccination that positively correlated with these late CSP-specific IgG levels. In contrast, BTMs related to NK cells showed strong negative correlations to antibody levels, which may be interpreted as either negative regulation of B-cell responses by NK cells[108], or migration of NK cells from the periphery to the lymphoid organs where they may act on infected erythrocytes via mechanisms such as antibody-dependent cellular cytoxicity.[109] The ARR regimen did not induce a robust antibody response but enhanced frequencies of polyfunctional CSP-specific CD4+ T cells at Day 14 that were associated with protection, suggesting a mechanism of protection distinct from the RRR regimen. In the ARR arm, multiple modules related to innate immune activation, including antigen presentation, activated dendritic cells and monocytes, and inflammatory responses, were strongly associated with polyfunctional CSP-specific CD4+ T-cell response at day 14.

With regard to protection, the RRR regimen induced BTMs related to plasma cells, B cells, and cell cycle at day 1 after each vaccination that were positively associated with protection. In contrast, the ARR regimen induced innate immunity BTMs that positively correlated with protection. Intriguingly, both the RRR and ARR vaccination regimens induced NK-cell modules shortly after each vaccine dose that showed strong negative associations with protection. Lastly, the authors used discriminant analysis via mixed integer programming (DAMIP)[110] to generate predictive signatures from the RRR vaccination group and validated these signatures using the dataset from the aforementioned study by Vahey et al.[86] The successful predictive signatures were dominated by transcripts found in Day 56 (day of third vaccine dose) signatures, with a majority of these signatures containing the NK cell marker *KIR2DS1*. The authors show in their study the ability of three representative successful gene signatures to segregate protected and non-protected subjects in both the RRR group and the study by Vahey et al.

We recently performed whole-blood transcriptome profiling using RNA-seq to assess the composition and kinetics of direct *ex vivo* immune responses in 10 Dutch volunteers enrolled in a *P. falciparum* CPS vaccination trial in which 50% were sterilely protected after homologous CHMI challenge during the 21-day monitoring period[82]. To determine the time points that might provide the highest signal for blood transcriptome profiling during CPS, we assessed 15 time points before and after each of three CPS immunizations given 28 days apart and before and after challenge performed 19 weeks after the final immunization. Although no differences were observed between protected and non-protected groups, genes

within BTMs related to T cells, NK cells, and mitochondrial processes were overexpressed in protected volunteers 28 days after the first immunization (one day before second immunization) relative to study baseline. The upregulation of NK-cell genes in protected subjects here contrasts with the negative association between NK modules and protection seen in the RTS,S vaccine trial described above, perhaps owing to differences in the mechanisms of protection. Differences observed in the protected group immediately prior to the second immunization corresponded with predicted activation of pathways involved in EIF2 signaling, oxidative phosphorylation and enhancement of T-cell activation pathways. Notably, correlation of summarized expression of BTMs at post-vaccination time points revealed more interactions between BTMs in protected subjects than in non-protected subjects, with the greatest number of interactions involving adaptive immunity modules observed in protected subjects relative to non-protected subjects several weeks after the second and third vaccinations. This suggests greater complexity of interactions and cross talk between adaptive and innate cells in protected subjects after CPS immunization.

Systems biology studies of naturally transmitted *Plasmodium* infections

Systems immunology analyses conducted with malaria-naïve volunteers enrolled in vaccine trials and CHMI studies have the distinct advantage of knowing the precise timing of the immunological perturbation (either vaccination or infection) and, in most cases, biological specimens can be sampled with high resolution and under ideal conditions. As such, in-depth assessments of the complexity of the host response to vaccination and/or infection over time can be achieved. In contrast, systems analyses of naturally transmitted *Plasmodium* infections must take into account a multitude of additional variables that could affect the interpretation of the data. These variables, listed in Table 3, can be both immunological and non-immunological in nature and can profoundly shape the quality and magnitude of the host response to *Plasmodium* infection. We previously reviewed the earliest studies that employed blood transcriptomic approaches to assess host responses during natural malaria infection[111]. Here, we examine more recent studies, referencing earlier investigations in their context and highlighting studies that extended beyond blood transcriptomics to incorporate other immunological, genetic, parasitological, and environmental variables into the analysis. A more complete listing of these studies along with those that rely primarily on data generated from a single platform are found in Table 2.Idaghdour and colleagues examined whole-blood transcriptomes of Beninese children with uncomplicated *P. falciparum* malaria (n=94) and age-matched *P. falciparum* negative controls from the city of Cotonou (n=61)[57]. Gene expression profiling was performed using Illumina BeadChips. Parasite load was noted to exert a profound influence on the whole-blood transcriptome, with *P. falciparum* infection and parasitemia class accounting for the majority of the transcriptomic variation in the combined dataset when modeled as a function of other variables such as sickle hemoglobin (HbS) genotype, sex, total blood counts, and ancestry. Enrichment analysis using the C2, C3, and C5 collections of the MsigDB database showed that, relative to controls, acute malaria induced signatures of innate immunity (including upregulation of IFN-inducible genes, neutrophil-associated modules, and markers of Fcγ-receptor mediated phagocytosis) but repressed adaptive signatures (including down-regulation of T cells, B cells, and cytotoxic T cell signaling pathways). Correlations of parasite load and average transcript abundance within each of six modules derived from cell

type-specific expression profiles[94] revealed significant effects of parasitemia on cell-type specific transcription and/or cellular composition. The authors used a mouse model of malaria to confirm the effect of high parasitemia on host gene expression observed in humans, including the strong induction of modules related to IFN response, antigen processing and presentation, the proteasome; suppression of the B-cell module; and regulation of Fcγ-receptor mediated phagocytosis. Lastly, a genome wide association test compared malaria cases and controls to explore a possible genetic basis for differential gene expression. Using a model that accounted for single nucleotide polymorphisms (SNP), malaria status, SNP by malaria status interaction, gender, location, and blood cell counts, significant genotype-by-infection interactions were identified for *PRUNE2*, *SLC39A8*, *C3AR1*, *PADI3*, and *UNC119B*.

To investigate differences between cerebral malaria phenotypes, Feintuch and colleagues performed whole-blood transcriptional profiling by Affymetrix GeneChip microarray with samples obtained from 97 Malawian children from the Blantyre Malaria Research Project with cerebral malaria grouped by the presence (Ret+CM, n=64) or absence (Ret-CM, n=33) of malarial retinopathy, which is microvasculature pathology in the ocular fundus indicative of sequestration of parasitized erythrocytes in the cerebral vasculature[51]. Compared to Ret-CM, the Ret+CM phenotype had increased parasite biomass as measured by PfHRP2 levels; similar parasitemia; decreased hematocrit and platelets; and increased mortality. Gene set enrichment analysis using genes differentially expressed between CM phenotypes, with adjustment for peripheral parasitemia (not parasite biomass) and Gene Ontology (GO) pathways, identified an association between Ret+CM and the GO slim categories “cell adhesion”, which included genes encoding multimerin 1 (MMRN1), P-selectin (SELP), CD9, α-integrins and β-integrins; “cytokines”, which included genes encoding monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α); and the GO pathways related to degranulation of platelets. Plasma cytokines associated with inflammation such as TNF, MCP-1, and IL-10 were increased in Ret+CM relative to Ret-CM. Notably, extracellular matrix (ECM) pathways were also upregulated in Ret+CM children, with enrichment in genes encoding neutrophil-related proteins that included neutrophil collagenase (matrix metalloproteinase 8 [MMP8]), human neutrophil elastase (HNE), and secretory leukocyte peptidase inhibitor (SLPI). Plasma levels of neutrophil primary granule proteins HNE, myeloperoxidase (MPO), and proteinase 3 (PRTN3) were also significantly increased in Ret+CM relative to Ret-CM. This evidence suggestive of enhanced neutrophil activation during Ret+CM was also associated with neutrophil dysfunction, as neutrophils from Ret+CM children exhibited decreased chemotactic responses relative to neutrophils from Ret-CM children. The Ret-CM phenotype was positively associated with the GO slim categories “Immune response” which included TLR signaling pathways; “apoptosis”, and “antigen presentation”, as well as stress-response, proteasome complex, and heme biosynthetic process pathways. Interestingly, increased expression of *GATA1*, which encodes for the transcription factor involved in the growth and differentiation of erythrocytes and megakaryocytes, and genes encoding γ globin, which along with α globin form the subunits of fetal hemoglobin, were observed in Ret-CM children despite none of the participants having hemoglobinopathies such as sickle cell trait or α-thalassemia, suggesting relatively decreased erythropoiesis in Ret+CM children. Taken

together, the Feintuch study suggests that children with cerebral malaria and evidence of retinopathy on exam have activated and perhaps dysfunctional neutrophils.

In a study by Lee and colleagues, dual RNA-seq was used to examine whole-blood transcriptomes of 46 Gambian children with *P. falciparum* uncomplicated malaria (n=21) and severe malaria (n=25, severe malarial anemia excluded), which allowed for an integrated analysis of host and parasite gene expression[64]. Deconvolution of host and parasite gene signatures revealed heterogeneity in leukocyte composition and parasite intraerythrocytic development stage between clinical groups. Thus, differential gene expression analysis between clinical groups included adjustments for leukocyte composition and parasite stage to remove their potential confounding effects. Notably, among genes overexpressed in severe malaria relative to uncomplicated malaria were neutrophil granule-related genes including *MMP8*, *OLMF4*, *DEFA3*, and *ELANE*. Colony-stimulating factor 3 (CSF3/GCSF), Fas cell surface death receptor, and prostaglandin E receptor 2 signaling were predicted to be activated by Ingenuity upstream regulator analysis in severe malaria. Given that host gene expression has been shown to be strongly influenced by parasitemia[51,57], which differed between the two groups, the authors examined the relationship between host gene expression and parasite load. Differential host gene expression between the uncomplicated and severe malaria groups was minimally changed after adjustment for parasite density but was significantly reduced by ~99% after adjustment for PfHRP2, which is a biomarker for total parasite biomass that accounts for both circulating and sequestered parasites. Thus, the authors concluded that the host response in severe malaria is an appropriate response to excessive pathogen load.

In the same study, weight-gene co-expression network analysis (WGCNA) was applied to paired host and parasite gene expression data to identify co-expressed group of genes which were termed “modules” named after the “hub” gene with the greatest number of connections within the module[64]. Eleven modules included both host and parasite genes, with a module connected by *HSPH1* [heat shock protein family H (Hsp110) member 1] having the greatest balance of genes of both species, being highly enriched in genes related to human heat shock response and parasite RNA metabolism. Distinct modules associated with disease severity included an *MMP8*-connected module enriched in defense response genes and reflective of granulopoiesis, an *OAS1*-connected module enriched for Type 1 IFN response genes, and a *LYSMD3*-connected module centered around IFN-γ. Comparison of co-expression networks for uncomplicated and severe malaria identified the *MMP8*-connected module as a unique feature of severe malaria, with predicted regulation by CEPBA, CSF3/GCSF, and TNF. Plasma levels of the neutrophil-related proteins defensin A3, elastase, and MMP8 significantly correlated with gene expression, but with MMP8 correlating only in severe malaria subjects.

To gain insight into the molecular and cellular processes that differentiate cerebral malaria and severe malarial anemia, we (Tran and colleagues) used Illumina Expression Beadchips to compare genome-wide transcriptional profiles of whole blood obtained from Ugandan children with retinopathy-positive cerebral malaria (n = 17) or severe malarial anemia (n = 17) enrolled in a larger study[67]. Gene expression data was also compared to healthy, community children negative for *P. falciparum* infection (n = 12) and correlated with

hematological indices and relevant plasma biomarkers. Unsupervised clustering analysis showed that global differences in gene expression were influenced primarily by clinical phenotype, parasite biomass as measured by PfHRP2, sickle cell homozygosity (HbSS), platelet count, and hemoglobin levels. Subsequent statistical analyses included adjustments for PfHRP2 levels and HbSS status but not platelet counts or hemoglobin given the role of the latter two variables in defining the respective cerebral malaria and severe malarial anemia phenotypes[134,135]. When each was compared to healthy controls, cerebral malaria appeared to induce greater transcriptional activity relative to severe malarial anemia. Differential enrichment of functional pathways between clinical phenotypes was assessed using the CERNO test (a variant of Fisher's combined probability test) with BTMs or KEGG biological pathways as gene sets.

Relative to controls, both cerebral malaria and severe malarial anemia groups showed upregulation of modules related to dendritic cell activation, monocyte, and inflammatory/ Toll-like receptor/chemokines modules, but downregulated of modules related to natural killer and T cells. A direct comparison of cerebral malaria and severe malarial anemia revealed significant downregulation of erythroid-related modules; inhibition of heme biosynthesis pathways; predicted inhibition of GATA1 and the erythropoiesis-stimulating hormone erythropoietin (EPO); and decreased expression of a reticulocyte-specific gene signature in cerebral malaria. These findings were consistent with the prior study by Feintuch et al. (reviewed above) which found decreased *GATA1* expression in retinopathy-positive cerebral malaria relative to retinopathy-negative cerebral malaria[51]. Hematological indices of reticulocytosis were decreased during cerebral malaria relative to severe malarial anemia despite evidence of an appropriate increase in plasma levels of erythropoietin in children in the study and the entire parent cohort (n=196), strongly suggesting ineffective erythropoiesis during cerebral malaria. Reticulocyte-related gene expression negatively correlated with expression of the module related to viral sensing and interferon-regulatory factor 2 (IRF2) targets networks (M111.1) as well as plasma concentration of the chemokine interferon gamma-induced protein 10 (CXCL10), which is an inflammatory chemokine known to inhibit human hematopoietic progenitors[136].

To investigate the impact of cumulative malaria exposure on the immune system, Bediako and colleagues examined whole-blood transcriptomes using RNA-seq along with cellular and cytokine profiles in Kenyan children aged 7 to 10.5 years who either had a high (≥8) or low (<5) number of prior malaria episodes (n = 21 in each group), as well as age-matched malaria-naïve children from a cohort in a nearby community (n = 27)[37]. Malaria episodes were defined as body temperature above 37.5 °C with ≥ 2500 parasites/μl of blood. All children were free of malaria for the previous 110 days and were negative for *Plasmodium* by PCR at the time of sampling. None of the children carried the HbS allele. Differential gene expression analysis could not distinguish between the low-episode and naïve groups but did reveal significant DEGs between the high and low- groups involving innate immunity T-cell differentiation, and regulation of adaptive immune cells. Modular repertoire analysis of the RNA-seq data revealed upregulation of interferon-related modules comprising genes involved in inflammatory responses and type I and type II interferon signaling. Analysis of plasma cytokines and chemokines revealed significantly higher plasma IL-10 levels and a trend toward increasing inflammatory cytokines (TNF, IL-6) in

the high vs. naïve group. Flow cytometric analysis showed that prior malaria exposure was associated with increased numbers of γδ T cells, $CD11c^+$ B cells, $CD4^-CD8^-$ T cells, and dendritic cells in both of the malaria- experienced groups compared to the naïve group. In addition, relative to the low-episode group, the high-episode group demonstrated significant expansion of γδ T cells, with this being the only subset evaluated that differed between the two malaria-experienced groups. Cellular deconvolution of whole-blood transcriptomes to infer cell-specific gene expression profiles showed transcriptional alterations in $CD8^+$ T cells, neutrophils, and B cells in high-episode children. Specifically, enrichment analysis of significantly altered genes in $CD8^+$ T cells showed upregulated enrichment of genes related to lymphocyte activation such as *LAT*, *LCK*, and *CD40* in the high-episode children. Within the B-cell profile, there was increased expression of *IGHE*, which encodes for IgE, in the high-episode children. The main conclusion of this study was that high prior malaria exposure is associated with immune modifications characterized by upregulation of interferon-inducible genes and enhanced activation of neutrophils, B cells, and $CD8^+$ T cells.

In order to better understand the cellular mechanisms of naturally acquired protection to uncomplicated, febrile malaria, we recently performed a systems biology analysis of children enrolled in a prospective cohort study of malaria immunity conducted in Kalifabougou, Mali, where malaria transmission is intense and seasonal[83]. During the first year of the cohort study, participants were followed weekly by active clinical surveillance and by symptom-triggered passive surveillance through self-referral. Retrospective PCR analysis of dried blood spots collected during clinic visits allowed us to determine the first time point at which an individual became positive for *P. falciparum* parasitemia. Combining this PCR data with clinical visit data allowed us to determine the time to progression to febrile malaria after initial parasitemia. Among a subset of the cohort consisting of children aged 6 to 11 years who started the malaria season negative for *Plasmodium* by PCR, three clinical phenotypes were identified: children who became parasitemic with *P. falciparum* and never progressed to fever during the remainder of the season (Immune, n =20); children who became parasitemic with *P. falciparum* but later progressed to fever within 2–14 days (Delayed Fever, n = 34); and children who were already febrile at their first parasitemic visit (Early Fever, n = 26). Consistent with the protective role of malaria-specific antibodies in naturally acquired immunity, antibody profiling by protein array showed increased magnitude and breadth of *P. falciparum*-specific IgG in Immune children relative to the susceptible Early Fever group. Whole-blood transcriptional profiling by RNA-seq was performed at baseline and at the first PCR-detectable parasitemia of the malaria season to determine if pre-infection transcriptional signatures and the transcriptional response to incident infection differed among the clinical phenotypes. The Immune children demonstrated a distinct molecular signature of B-cell enrichment and interferon activation that resembled that found in children with high malaria exposure in the Bediako study[37]. Flow cytometric analysis of PBMCs collected at the pre-infection baseline revealed an increased proportion of B cells in the Immune children along with increased expression of the IL-4 inducible, low-affinity IgE receptor CD23 on both atypical and activated memory B cells. However, it was unclear which cells were the source of IL-4 for the increased CD23 as there were no differences in Th2 transcription factors GATA3 or STAT6 in $CD4^+$ T cells between groups. Notably, relative to the Early Fever group, both the Immune and Delayed

Fever children showed increased whole-blood expression of *TP53* as well as targets of p53, suggesting that activation of p53 pathways in peripheral blood was associated with protection from initial fever during incident blood-stage infection. Flow cytometry showed that expression of p53 in monocytes was increased in children protected from initial fever relative to children in the Early Fever group. Prior or recent malaria exposure could possibly explain these differences, given that p53 in monocytes increased during acute malaria relative to baseline and was higher in malaria-exposed Malian adults relative to malaria-naïve North American adults. Pharmacological activation of p53 using the MDM2/HDM2 inhibitor nutlin-3 reduced the production of TNF in malaria-naïve monocytes stimulated with *P. falciparum* lysate *in vitro* relative to vehicle-treated controls. Using a mouse malaria model, we showed that "super p53" mice, which have one extra copy of the gene encoding p53, had a blunted pro-inflammatory response relative to wild-type mice soon after infection with *P. yoelii* 17XNL. However, the kinetics of parasitemia were similar in both super p53 and wild-type mice and the blunting of inflammation was transient, occurring only during early blood-stage infection. Taken together, the data suggests that p53 activation plays a role in the initial control of malaria-induced inflammation without impacting parasitemia.

Next-generation studies of systems immunology in human malaria

The majority of published studies that have investigated the immune response to human malaria using high-throughput or highly multiplexed platforms (e.g. RNA-seq, gene microarrays, protein arrays, multiplex bead arrays for cytokines and chemokines) rely on measurements of bulk blood cells or serum analytes that are in essence the summation of responses from a heterogenous collection of cell types. Such bulk measurements are most sensitive to changes in the most abundant cell subsets and may not detect changes in relatively rare cell types such antigen-specific B or T cells[137]. The studies reviewed above employed transcriptomics using RNA isolated from bulk cell samples such as whole blood or PBMCs to evaluate differences between malaria-related clinical phenotypes. The main dilemma in bulk transcriptomics is distinguishing whether differential transcript abundance between comparison groups is due to gene expression differences within cells or differences in cell composition in the blood sample. Resolving this dilemma may involve adjusting for cellular composition with contemporaneous complete blood counts or with estimates of cell subset composition derived from deconvolution techniques that take advantage of cell subset-specific signatures derived from sorted immune cells [17,18,138]. However, adjustments for cellular composition are not ideal given that it may overcorrect for gene expression changes or unnecessarily remove variations in cell composition that are actually specific to one comparison group. Highly multiplexed polychromatic flow cytometry of contemporaneously collected blood cells is one approach to measure differences in protein expression in a cell subset-specific manner[139]. As such, standard immunotyping of cells directly *ex vivo* or after cryopreservation can be complemented with *in vitro* stimulation with *Plasmodium* parasites to assess malaria-specific function. However, conventional polychromatic flow cytometry is mainly used for testing specific hypotheses generated by analysis of bulk transcriptomic data given limitations in the number of potential detectable targets to assay (both in terms of parameter limit and antibody availability) and the natural bias towards evaluating cell subsets with known functions.

The next generation of systems immunology studies will employ newer technologies to address the limitations inherent with bulk measurements. Single-cell (sc) technologies provide high-resolution assessments of the variation in the genomes, transcriptomes, and protein expression between individual cells and have recently been reviewed elsewhere in the context of immunological studies in general[137,140–142]. Single-cell immunoglobulin and T-cell receptor gene sequencing has been used to profile the respective repertoires of memory B cell and T cells during the host response to malaria[143–145]. Here, we specifically discuss the potential of single-cell technologies for systems-based interrogations of the immune response to human malaria.

Single-cell RNA-seq platforms became possible with the development of low-input RNA-seq protocols and allow for unbiased transcriptional profiling of even rare immune cell types. There are several general approaches for scRNA-seq. Individual cells can be 1) captured in barcoded gel beads (10X[146] and inDrop[147]) or brittle resin droplets (Drop-Seq[148]) or 2) physically separated in 96-well plates using fluorescence-activated cell sorting or in microfluidic chips. After cell lysis, RNA is reversed transcribed and amplified by PCR with subsequent RNA-seq libraries prepared as cellular barcoded pools or from individual cells. Sequencing can be performed from the 3’ end of the transcript to detect only gene expression or for full-length transcripts to determine splice variants, B-cell receptor and T-cell receptor repertoires in addition to gene expression.

In studies of acute malaria using cryopreserved PBMCs, confounding due to differences in immune cell composition among malaria syndromes[64,67,149] can be readily resolved by scRNA-seq provided that samples demonstrates adequate viability (generally >90%). Applying scRNA-seq to acute malaria blood samples collected *ex vivo* would be more physiologically relevant and allows for the interrogation of neutrophils and other granulocytes but could be challenging given the logistic hurdles of implementing such technologies in malaria-endemic regions and the need to remove erythrocytes and non-viable cells that would otherwise contribute to unwanted sequencing reads. Dual-RNA-seq has been applied to bulk whole blood from malaria patients to study the host-parasite “interactome”,[9,64] but is limited to cases with high parasite densities (>10,000 parasites/μl, unpublished data) and thus probably not reliable for investigating less complicated malaria or asymptomatic infections. Here, scRNA-seq can potentially allow transcriptional profiling of individual *Plasmodium*-infected erythrocytes and human leukocytes collected *ex vivo* from patients with low parasitemia, provided that these target components can be effectively separated from both uninfected erythrocytes and non-viable cells.

Single-cell technologies can also be used to evaluate the epigenetic landscape of immune cells in unprecedented detail. For instance, applying single-cell Assay for Transposase Accessible Chromatin (scATAC-seq)[150,151] with scRNA-seq on the same immune cell preparations can provide insight on the impact of malaria or prior malaria exposure on the coordinated accessibility and expression of genes involved in control of the host immune response and the degree of regulatory variation within cell subsets. Combined with single-cell bisulfite sequencing, which provides a map of the DNA methylome[152,153], a more complete understanding of cell-state dynamics and epigenetic heterogeneity within immune cells during different malaria syndromes or malaria-immune phenotypes can be ascertained.

Protocols have been developed to generate transcriptomes and methylomes from the same single cells[154,155] as well as simultaneous profiling of chromatin accessibility, DNA methylation and the transcriptome using single-cell nucleosome, methylation and transcription sequencing (scNMT-seq)[156]. Joint profiling of single-cell chromatin accessibility and transcriptomes can now be performed in each of thousands of cells using single-cell combinatorial indexing methods[157], with the scalable throughput being particularly useful for the analysis of peripheral blood samples from malaria patients that are often comprised of highly heterogeneous immune cell subsets.

Mass cytometry (as known as cytometry by time of flight or CyTOF) combined with single-cell fluidics is a relatively new, commercially available technique that can extend the number of quantifiable parameters beyond those available with conventional flow cytometry. Rather than using fluorochromes that are inherently limited by spectral overlap, mass cytometry relies on monoclonal antibodies tagged with rare-earth-metal isotopes that have minimal signal overlap, which currently allows for the simultaneous interrogation of nearly 50 immune markers including cell surface proteins, intracellular cytokines, and phosphorylated proteins[158,159]. Such expansion of parameters would provide deeper insight into the functional and activation states of immune cells in response to *Plasmodium* during natural exposure or after *in vitro* stimulation.

Lastly, more complete understanding variable immune responses in individuals living in malaria-endemic areas may require detailed assessments of co-infections and commensal microorganisms, both which can shape the host response to malaria and vaccination[160–162]. Future studies could integrate metagenomic analyses of microbiota from the skin and mucosal surfaces (e.g. the gut and urinary tract) with immunological signatures in the peripheral blood to assess the potential impact that specific microorganisms may have on acute malaria or immunization to malaria vaccines in an unbiased manner.

Summary

For over a decade ‘systems’ tools have been used to investigate the human immune response to malaria infection and vaccine candidates. These unbiased approaches have begun to provide insights into the nature of protective and pathologic immune responses to malaria, as well as potential correlates of immunogenicity and protective efficacy of vaccine candidates. Such advances may not have been readily achieved through hypothesis-driven, reductionist approaches. As more datasets become available from studies that vary by study population, clinical phenotype, and vaccine regimen, it is likely that common themes will continue to emerge that lead to a more comprehensive understanding of the complex and multi-faceted nature of immune response across various malaria-endemic settings. Many of these datasets have generated novel hypotheses of immunity to malaria that will require further testing in model systems to better elucidate the mechanisms of protection and immunopathogenesis. The limitations of current studies, most notably the inability of bulk approaches to adequately attribute differences in molecular abundance to increased cellular activity or changes in cell composition, could potentially be addressed with single-cell technologies. When coupled with other recently developed technologies such as mass cytometry, the resolution and interpretability of systems approaches will continue to increase.

Comprehensive systems studies conducted in malaria-endemic settings may need to account for co-infections and variations in commensal flora by linking metagenomic profiles to distinct molecular signatures associated with malaria response phenotypes to provide a more complete understanding of the human immune response during naturally transmitted malaria.

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Table 1

Methodologies and tools commonly used in published systems studies of malaria immunity in humans

Objective	Methodology or Tool	Applicable to	Reference(s)
Determine differences between phenotypes or groups	Cufflinks/Cuffdiff	RNA-seq	Trapnell et al. 2012[10]
	DESeq2	RNA-seq	Love et al. 2014[11]
	edgeR	RNA-seq	Robinson et al. 2010[12]
	empirical Bayes moderated t test (limma)	microarrays	Ritchie et al. 2015[13]
	Power Law Global Error Model (PLGEM)	microarrays	Pavelka 2004[14]
	significance analysis of microarrays (SAM)	microarrays	Tusher 2001[15]
	voom-limma	RNA-seq	Ritchie et al 2015[13]
Deconvolution of transcriptomic data from heterogeneous tissue	Immune Response in Silico (IRIS)	transcriptomic datasets	Abbas et al. 2009[16]
	immunoStates	transcriptomic datasets	Vallania et al. 2018[17]
	leukocyte signature matrix (LM22; CIBERSORT)	transcriptomic datasets	Newman et al. 2015[18]
Functional and pathways analysis	Camera	molecular -omic datasets	Wu and Smyth 2012[19]
	DAVID functional annotation	molecular -omic datasets	Huang et al. 2007[20,21]
	Gene set enrichment analysis	molecular -omic datasets	Subramanian et al. 2005[22]
	Ingenuity (for pathways analysis; proprietary)	molecular -omic datasets	Kramer et al. 2014 [23]
	Coincident extreme ranks in numerical observations (CERNO; implemented via tmod)	molecular -omic datasets	Zyla et al. 2019[24]
Gene Correlation Network analysis	weighted gene co-expression network analysis (WGCNA)	molecular -omic datasets	Langfelder and Horvath 2008[25]
Integrative analysis with *Plasmodium* genome or proteome; protein arrays for antibody profiling	PlasmoDB *Plasmodium* genomics resource	Plasmodium -omic datasets	Aurrecoechea et al. 2009[26]
Gene sets and transcriptional modules	blood transcription modules	transcriptomic datasets	Li et al. 2014[27]
	ImmuneSigDB (C7 collection)	transcriptomic datasets	Godec et al. 2016[28]
	Kyoto Encyclopedia of Genes and Genomes (KEGG)	molecular -omic datasets	Kanehisa and Goto 2000[29]
	modular transcriptional repertoire	transcriptomic datasets	Chaussabel et al. 2014[30]
	Molecular signatures database (MSigDB)	molecular -omic datasets	Liberzon et al. 2011[31]
Platforms with accessible graphic user interfaces	DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp)	molecular -omic datasets	Huang et al. 2009[32]
	Galaxy (https://galaxyproject.org)	molecular -omic datasets	Afgan et al. 2016[33]
	Gene set enrichment analysis (http://software.broadinstitute.org/gsea/index.jsp)	molecular -omic datasets	Subramanian et al. 2005[22]
	Ingenuity (for pathways analysis; proprietary)	molecular -omic datasets	Kramer et al. 2014 [23]
	tmod (http://tmod.online/)	transcriptomic datasets	Zyla et al. 2019[24]

Table 2.

Human malaria studies that have employed systems-based approaches

Study	Year	*Plasmodium* species	Comparison groups	Study design	Immune status	Age	Primary platform
Arévalo-Herrera et al.[36]	2016	*Plasmodium vivax*	asymptomatic infection and uncomplicated malaria	CHMI	naïve or semi-immune	adults	protein microarrays
Bediako et al.[37]	2019	*Plasmodium falciparum*	high and low prior malaria exposure	prospective cohort	semi-immune	children	RNA-seq transcriptomics
Boldt et al.[38]	2019	*Plasmodium falciparum*	asymptomatic infection, uncomplicated malaria, severe malarial anemia, and cerebral malaria	cross-sectional	semi-immune	children	microarray transcriptomics
Chakraborty et al.[39]	2018	*Plasmodium falciparum* and *Plasmodum vivax*	severe and uncomplicated malaria	cross-sectional	semi-immune	adults	microarray transcriptomics
Chakravorty et al.[40]	2007	*Plasmodium falciparum*	*in vitro* co-culture of human umbilical vein endothelial cells with *P. falciparum* infected erythrocytes and uninfected erythrocytes	*in vitro* culture	naïve	not applicable	microarray transcriptomics
Chattopadhyay et al.[41]	2011	*Plasmodium falciparum*	*in vitro P. falciparum* sporozoite infected and non-infected HepG2-A16 liver cells	*in vitro* culture	not applicable	not applicable	microarray transcriptomics
Chen et al.[42]	2010	*Plasmodium vivax*	uncomplicated malaria	cross-sectional	semi-immune	adults	protein microarrays
Chen et al.[43]	2015	*Plasmodium vivax*	*P. vivax* exposed	cross-sectional	naïve or semi-immune	adults	protein microarrays
Colborn et al.[44]	2015	*Plasmodium falciparum*	uncomplicated malaria	cross-sectional	semi-immune	children	microarray transcriptomics
Cordy et al.[45]	2019	*Plasmodium coatneyi and Plasmodium falciparum*	uncomplicated malaria and chronic *Plasmodium* infection	cross-sectional	semi-immune	adults	plasma metabolomics in macaques with validation in humans
Crompton et al.[46]	2010	*Plasmodium falciparum*	malaria exposed	prospective cohort	semi-immune	children and adults	protein microarrays
Dent et al.[47]	2015	*Plasmodium falciparum*	malaria exposed	prospective cohort	semi-immune	children and adults	protein microarrays
Dobbs et al.[48]	2017	*Plasmodium falciparum*	uncomplicated malaria, convalescent malaria, and asymptomatic *P. falciparum* infection	longitudinal analysis	semi-immune	children	microarray transcriptomics
Doolan et al.[49]	2008	*Plasmodium falciparum*	volunteers immunized with radiation-attenuated P. falciparum sporozoites and naturally exposed donors	longitudinal analysis (vaccine study) and cross-sectional (natural malaria exposure)	previously malaria-naïve or semi-immune	children and adults	protein microarrays

Study	Year	*Plasmodium* species	Comparison groups	Study design	Immune status	Age	Primary platform
Dunache et al.[50]	2015	*Plasmodium falciparum*	antigen-stimulated PBMCs from volunteers in either RTS,S/AS02A and thrombospondin-related adhesive protein (TRAP) malaria vaccine trials	vaccination and CHMI	previously malaria-naïve	adults	microarray transcriptomics
Feintuch et al. [51]	2016	*Plasmodium falciparum*	retinopathy-positive and retinopathy-negative cerebral malaria	cross-sectional	semi-immune	children	microarray transcriptomics
Felgner et al.[52]	2013	*Plasmodium falciparum*	uncomplicated malaria	CHMI and natural infection	naïve or semi-immune	adults	protein microarrays
Franklin et al.[53]	2009	*Plasmodium falciparum*	uncomplicated malaria	cross-sectional	semi-immune	adults	microarray transcriptomics
Gardinassi et al.[54]	2018	*Plasmodium vivax*	pre-symptomatic and uncomplicated malaria	CHMI	naïve or semi-immune	adults	integrated metabolomics and transcriptomics
Grangeiro de Carvalho et al.[55]	2011	*Plasmodium falciparum*	*in vitro* co-culture of NK cells with *P. falciparum* infected erythrocytes and uninfected erythrocytes	*in vitro* co-culture	naïve	adults	microarray transcriptomics
Grangeiro de Carvalho et al.[56]	2011	*Plasmodium falciparum*	*in vitro* co-culture of NK92 cells cells with *P. falciparum* infected erythrocytes and uninfected erythrocytes	in vitro co-culture	naïve	adults	microarray transcriptomics
Idaghdour et al.[57]	2012	*Plasmodium falciparum*	uncomplicated malaria	case-control	semi-immune	children	Integrated transcriptomics and genomics
Kamuyu et al.[58]	2018	*Plasmodium falciparum*	malaria-exposed and malaria-naïve	cross-sectional	naïve or semi-immune	adults	protein microarrays
Kassa et al.[59]	2011	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	cross-sectional	semi-immune	adults	metabolomics
Kazmin et al.[60]	2017	*Plasmodium falciparum*	volunteers immunized with RTS,S (RRR) and RTS,S/AS01 following a primary immunization with adenovirus 35 (Ad35) vector expressing circumsporozoite protein (ARR)	vaccination and CHMI	naïve	adults	microarray transcriptomics
Keller et al.[61]	2009	*Plasmodium falciparum*	*in vitro* culture of PBMCs from malaria-exposed and malaria-naïve donors with and without *P. falciparum* hemozoin	*in vitro* co-culture	naïve or semi-immune	children and adults	microarray transcriptomics
Krupka et al.[62]	2012	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	longitudinal analysis	semi-immune	children	microarray transcriptomics
Kumar et al.[63]	2018	*Plasmodium falciparum*	cerebral malaria	case-control	semi-immune	children and adults	proteomics of frontal lobe samples

Study	Year	*Plasmodium* species	Comparison groups	Study design	Immune status	Age	Primary platform
Lee et al.[64]	2018	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	cross-sectional	semi-immune	children	whole-blood RNA-seq transcriptomics
Leopold et al.[65]	2019	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	nested case-control	semi-immune	adults	plasma metabolomics with *in vitro* validation
Lu et al.[66]	2014	*Plasmodium vivax*	uncomplicated malaria	cross-sectional	naïve or semi-immune	adults	protein microarrays
Nallandhighal et al.[67]	2019	*Plasmodium falciparum*	cerebral malaria, severe malarial anemia, malaria exposed community controls	nested case-control	semi-immune	children	microarray transcriptomics with plasma biomarkers
Nnedu et al.[68]	2011	*Plasmodium falciparum*	malaria exposed with HIV co-infection	cross-sectional	semi-immune	adults	protein microarrays
Ockenhouse et al.[69]	2006	*Plasmodium falciparum*	pre-symptomatic infection and uncomplicated malaria	CHMI and natural infection	naïve or semi-immune	adults	microarray transcriptomics
Orikiiriza et al.[70]	2017	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	cross-sectional	semi-immune	children	lipidomics
Portugal et al.[71]	2014	*Plasmodium falciparum*	*in vitro* co-culture of PBMCs with *P. falciparum* infected erythrocytes and uninfected erythrocytes	longitudinal analysis	semi-immune	children	microarray transcriptomics
Quin et al.[72]	2017	*Plasmodium falciparum*	malaria exposed	cross-sectional	semi-immune	adults	RNA-seq transcriptomics and genome-wide DNA methylation
Ray et al.[73]	2012	*Plasmodium falciparum* and *Plasmodium vivax*	uncomplicated malaria	cross-sectional	semi-immune	adults	proteomics
Ray et al.[74]	2012	*Plasmodium vivax*	uncomplicated malaria	cross-sectional	naïve or semi-immune	adults	proteomics
Ray et al.[75]	2015	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	cross-sectional	semi-immune	adults	proteomics
Reuterswärd et al.[76]	2018	*Plasmodium falciparum*	uncomplicated malaria and severe malaria	case-control	semi-immune	children	proteomics
Rojas-Peña et al.[77]	2015	*Plasmodium vivax*	uncomplicated malaria	case-control	naïve or semi-immune	adults	quantitative RT-PCR array and RNA-seq transcriptomics
Rojas-Peña et al.[78]	2018	*Plasmodium falciparum* and *Plasmodum vivax*	malaria exposed	longitudinal analysis	semi-immune	adults	RNA-seq transcriptomics
Rothen et al.[79]	2018	*Plasmodium falciparum*	pre-symptomatic infection and uncomplicated malaria	CHMI	semi-immune	adults	RNA-seq transcriptomics
Torres et al.[80]	2015	*Plasmodium falciparum*	uncomplicated malaria and asymptomatic infection	cross-sectional	naïve or semi-immune	adults	protein microarrays

Study	Year	*Plasmodium* species	Comparison groups	Study design	Immune status	Age	Primary platform
Tran et al.[81]	2016	*Plasmodium falciparum*	uncomplicated malaria and asymptomatic infection	CHMI and natural infection	naïve or semi-immune	adults	RNA-seq transcriptomics
Tran et al.[82]	2019	*Plasmodium falciparum*	volunteers immunized with sporozoites under chemoprophylaxis (CPS)	vaccination and CHMI	naïve	adults	RNA-seq transcriptomics
Tran et al.[83]	2019	*Plasmodium falciparum*	asymptomatic infection and uncomplicated malaria	nested case-control in a prospective study	semi-immune	children	RNA-seq transcriptomics
Tripathi et al.[84]	2009	*Plasmodium falciparum*	*in vitro* co-culture of human brain microvascular endothelial cells with *P. falciparum* infected erythrocytes and uninfected erythrocytes	*in vitro* co-culture	naïve	not applicable	microarray transcriptomics
Uplekar et al.[85]	2017	*Plasmodium falciparum* and *Plasmodum vivax*	asymptomatic infection, uncomplicated malaria	cross-sectional	semi-immune	adults	protein microarrays
Vahey et al.[86]	2010	*Plasmodium falciparum*	volunteers immunized with the RTS,S malaria vaccine	vaccination and CHMI	previously malaria-naïve	adults	microarray transcriptomics
Vallejo et al.[87]	2018	*Plasmodium vivax*	pre-symptomatic and uncomplicated malaria	CHMI	naïve or semi-immune	adults	RNA-seq transcriptomics
van den Berg et al.[88]	2017	*Plasmodium falciparum*	volunteers immunized with the RTS,S malaria vaccine	vaccination and CHMI	naïve	adults	microarray transcriptomics
Yamagishi et al.[89]	2014	*Plasmodium falciparum*	uncomplicated malaria	cross-sectional	semi-immune	children and adults	RNA-seq transcriptomics

Table 3.

Variables that can potentially confound systems studies of malaria immunity

Variable	Comments	Suggested References
age	In long-term residents of endemic areas, age is inextricably linked to malaria exposure. Additionally, there are age-related differences in immune cell composition and immunity that are independent of malaria exposure.	112,113
previous malaria exposure	Prior malaria episodes can generate immunity that is protective against clinical malaria but can also shape the quality of the immune response.	113,114
erythrocyte polymorphisms	Erythrocyte variants such as sickle hemoglobin (HbS) and thalassemias can adversely affect parasite growth and protect against malaria.	115,116
spatial factors	Microgeography including residence near mosquito-breeding sites, near health clinics, and within clusters of asymptomatic carriers can influence malaria exposure.	117,118
helminth co-infections	Co-infection with helminths can affect malaria risk and the quality of the immune response.	119,120
parasite biomass	Parasite biomass, which includes the amount of sequestered parasite, can influence the host response to *P. falciparum* malaria. This is typically measured using plasma histidine-rich protein 2 (HRP2) levels.	9,51
nutrition status	Although evidence on the effect of malnutrition of malaria risk remains inconclusive, primary childhood malnutrition impairs host defense mechanisms, potentially increasing the risk and severity of infection.	121,122
microbiota	Gut microbiota composition has been shown to affect risk of *P. falciparum* infection in humans and malaria severity in a mouse malaria model.	123–125
timing of blood sampling	Duration of parasitemia is generally not known at time of blood sampling in naturally infected individuals. Thus, there can be significant variability in immunological responses between subjects based on the natural history of acute malaria.	126–128
parasite genetics	The parasite employs antigenic diversity and variation as immune evasion mechanisms. For example, certain parasite variants, such as those expressing PfEMP-1 variants with EPCR-binding phenotype, have been associated with cerebral malaria.	129–131
host genetics	In addition to erythrocyte variants, other unknown host genetic determinants can potentially affect malaria severity.	132,133