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Systems immunology of human malaria

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Abstract

Plasmodium falciparum malaria remains a global public health threat. Optimism that a highly effective malaria vaccine can be developed stems in part from the observation that humans can acquire immunity to malaria through experimental and natural *P. falciparum* infection. Recent advances in systems immunology could accelerate efforts to unravel the mechanisms of acquired immunity to malaria. Here we review the tools of systems immunology, their current limitations in the context of human malaria research, and the human 'models' of malaria immunity to which these tools can be applied.

Keywords

systems immunology; malaria; *Plasmodium falciparum*

Malaria: a global disease in need of global immunological analyses

Plasmodium falciparum causes more than 200 million cases of malaria each year (http://www.who.int/malaria/world_malaria_report_2011/en/), with greater than one million deaths by one recent estimate [1]. Widespread implementation of artemisinin-combination therapy and long-lasting insecticide bed nets has been linked temporally to declines in the incidence of malaria in certain areas of Africa [2], but such gains are constantly threatened by the emergence of drug-resistant *P. falciparum* parasites and insecticide-resistant mosquito vectors [3, 4]. Thus, the development of an effective malaria vaccine is widely viewed as a key step toward malaria control and elimination, yet the leading malaria vaccine candidate, RTS,S, which targets the circumsporozoite protein, appears to confer only partial, short-

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lived protection against malaria [5]. Optimism that a highly effective malaria vaccine can be developed stems in part from the observation that humans can acquire immunity to malaria through natural and experimental *P. falciparum* infection [6, 7]. Efforts to unravel the mechanisms of acquired immunity to malaria have generally relied on reductionist approaches in which one or a few immunological parameters are related to malaria risk. This approach has provided key insights into isolated facets of the host response to *P. falciparum* [6] but is unlikely to resolve the molecular and cellular interactions of the innate and adaptive immune response that ultimately generate the complex 'system' phenotype of immunity to malaria. Systems immunology, a still-evolving subfield of systems biology [8], seeks to understand and predict these complex interactions through integration and computational modeling of data generated from high-throughput molecular and cellular assays such as genome-wide RNA expression [9], multiplexed cytokine analysis [10], polychromatic flow cytometry [11], antibody profiling by protein array [12] and metabolomics [13]. The potential of this approach for advancing our understanding of the human immune response to pathogens and vaccines is evidenced by the recent formation of National Institute of Allergy and Infectious Diseases-sponsored, pathogen-specific Systems Biology Centers which focus on influenza, emerging respiratory viruses, tuberculosis and bacterial enteropathogens [14, 15]. Indeed, systems analyses have been used to identify molecular signatures that predict the immunogenicity of already licensed vaccines [16-18]. For example, the yellow fever vaccine was found to induce a gene expression profile detectable in human blood that predicted the subsequent magnitude of the vaccine-specific CD8+ T cell and neutralizing antibody response [17]. More recently, early molecular signatures induced by the inactivated influenza vaccine were identified that predicted vaccine-specific antibody titers [18]. Similar approaches have been used to discriminate active tuberculosis from other infectious and inflammatory diseases [19, 20] and to provide insights into the immunopathogenesis of autoimmune diseases [21]. Systems immunology, which has yet to be fully exploited in the context of human malaria research [14], offers a roadmap for addressing many outstanding questions related to malaria immunity (see 'Outstanding questions' box). Here, we review the tools of systems immunology, their current limitations in the context of human malaria research and the human 'models' of malaria immunity to which these tools can be applied. The success of systems immunology studies depends on the underlying experimental design; the quality of the clinical data and biospecimens obtained; and the collaborative efforts of clinicians, epidemiologists, immunologists, bioinformaticians and computational biologists. Although important in their own right, systems approaches to animal models of malaria [22] and *Plasmodium* biology [23, 24] are beyond the scope of this review.

The systems immunology toolbox

Human pathogens are detected by cells of the innate immune system (dendritic cells, macrophages, neutrophils, natural killer cells, basophils, eosinophils and mast cells) through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which recognize highly conserved pathogen-associated molecular patterns (PAMPs) [25]. PRRs also recognize nucleic acids and proteins (damage-associated molecular patterns or DAMPs) released from host cells damaged by pathogen invasion [26]. Through these initial host-pathogen interactions, the innate immune system provides immediate defense against pathogens and also helps direct the response of adaptive immune cells (B and T cells) which recognize and bind foreign antigens through a diverse repertoire of clonally expressed cell surface receptors [27]. The summation of receptor-ligand interactions drives innate and adaptive immune cells to transcribe genes into non-coding RNA and mRNA; mRNA is then translated into intracellular proteins (e.g. signaling molecules and antigen-processing machinery), membrane-bound proteins (e.g. co-stimulatory and adhesion molecules) and secreted proteins (e.g. cytokines, chemokines and

antibodies), which together shape the quality of the immune response to a given pathogen. Recently developed assays allow for high-throughput quantification of the molecular and cellular components of the immune system, and new analytical tools permit the interaction of these components to be analyzed, visualized and modeled into comprehensible representations of immune system behavior (Boxes 1 and 2).

Analysis of gene expression

Knowledge of the human genome sequence has spawned technological advances that make it possible to quantify mRNA transcript levels for every known human gene in a single assay. Genome-wide expression profiling often serves as the first unbiased step toward characterizing the global functional state of biological systems. In the case of the human immune system, gene expression profiling often involves extraction of RNA from isolated (e.g. T cells or other immune cell subsets) or mixed (e.g. whole blood leukocytes or blood mononuclear leukocytes) immune cell populations, conversion of RNA transcripts to complementary DNA (cDNA) via reverse transcription, and quantification of transcripts through hybridization or sequencing methods. Commercial hybridization assays depend on the binding of cDNA 'targets' to complementary oligonucleotide 'probes' attached to microarrays or microscopic beads [28, 29]. The relative quantity of each transcript is determined by fluorescent labeling of cDNA prior to hybridization. Gene expression profiling by RNA sequence analysis (RNA-Seq) has emerged as an alternative to hybridization due to the efficiency and decreasing cost of next-generation sequencing technology [30]. In brief, RNA-Seq involves ligation of cDNA to sequence adapters, PCR amplification and sequencing of cDNA, and mapping the sequence reads back to a human reference genome. Recent methods also allow for direct sequencing of RNA without conversion to cDNA, although the read lengths have been relatively short [31]. Unlike hybridization-based assays, RNA-Seq can detect novel and variant RNA species that occur through gene fusions, splice variants and post-transcriptional modifications—the mechanisms by which 26 000+ human genes give rise to approximately 1 million distinct proteins. RNA-Seq also has lower background and a higher dynamic range, which increases the sensitivity for detecting rare transcripts [30]. However, the reproducibility of RNA-Seq is dependent on sequencing depth for common and rare transcripts; thus, a small number of highly expressed genes, such as globin mRNA in whole blood, can give misleading correlation coefficients [32]. This limitation can be overcome by depletion of abundant transcripts (e.g. globin mRNA) prior to RNA sequencing [33]. Regardless of the method used, the results of genome-wide expression profiling should be confirmed by quantitative RT-PCR analysis of a subset of differentially expressed genes of interest and, when feasible, by quantification of gene products. Data from genome-wide expression experiments can both generate and test hypotheses which can be further tested in more refined and iterative follow-up studies in humans, animal models and *in vitro*.

Analysis of gene products

Gene expression profiling has proved to be a powerful tool for assessing global effects of immune system perturbations. However, RNA transcripts are only part of a complex system that also includes proteins (e.g. cytokines, chemokines, antibodies and cell-surface molecules) and metabolites that ultimately shape the quality of the immune response. Ideally, all proteins induced during an immune response could be directly measured rather than relying on surrogate measures of RNA, but this is not yet possible with current proteomic technology [34, 35]. Instead, multiplexed protein assays are often used to validate the differential expression of selected subsets of genes at the protein level and to focus analyses on proteins of particular interest. Generally, these assays employ either fluorescently labeled microscopic beads or solid-state arrays bound with antibodies specific

for the proteins of interest [10]. These platforms can be adapted to detect virtually any polypeptide but are primarily used to quantify cytokines, chemokines and growth factors in serum or in supernatants of stimulated leukocytes. Customized assays can measure up to 190 analytes from a single sample [36]. These assays, which have been used in several studies of human *P. falciparum* infection [37, 38], are an important complement to genome-wide expression analysis.

The integration of metabolomic data with transcriptomic and proteomic data can further enhance our understanding of the human immune response to *P. falciparum* infection. Metabolomics is the study of small molecule metabolites (e.g. hormones, signaling molecules and intermediate metabolites) that arise from all cellular processes in a biological system. Within the context of malaria research, metabolomic studies have focused primarily on understanding *P. falciparum* parasite biology [13, 39] rather than the host response to *P. falciparum* infection. Metabolomic studies will likely provide important insights into the pathophysiology underlying the various clinical syndromes associated with *P. falciparum* infection, such as asymptomatic infection, febrile uncomplicated malaria, cerebral malaria, severe anemia and respiratory distress [40, 41], and have the potential to identify host biomarkers that relate to malaria risk. The feasibility of human metabolomic profiling in malaria-endemic areas was recently demonstrated by a study in Uganda in which schistosomiasis-infected and uninfected individuals were distinguished by metabolic profiling [42]. Currently, no single method exists to analyze the complete set of human metabolites. Mass spectrometry (MS) obviates the need for analyte-specific probes by identifying and quantifying ionized analytes based on mass-to-charge ratios [13]. When combined with metabolite separation techniques such as liquid chromatography, MS can provide exquisite sensitivity and resolution. Further resolution can be achieved with tandem MS, in which ionized analytes are fragmented before undergoing additional rounds of mass spectrometry, thereby providing structural information on amino acid sequence and post-translational modifications. The latter will be essential to systems immunology given the pivotal role that protein phosphorylation plays in regulating signaling pathways. Quantitative characterization of the phosphoproteome using tandem MS techniques, or less expensive assays that employ phosphoprotein arrays for analysis of the most common kinase signaling pathways, can provide insight into the dynamic changes of host cellular response to infectious stimuli, thereby facilitating construction of computational models with better predictive capability [43].

Antibodies are known to play a critical role in protecting against naturally acquired malaria [44], but the antigen specificity of both the protective antibodies and the CD4+ T helper cells that support the generation of protective antibodies remains unclear. Recent progress towards high-throughput analysis of B and T cell repertoires is discussed in Box 3.

Analysis of cell phenotype and function

Flow cytometry is widely used for phenotyping and sorting cells of the immune system and for multifunctional analysis of innate or antigen-specific cellular responses following *in vivo* infection/vaccination or *ex vivo* stimulation (reviewed in [11]). Generally, flow cytometry involves staining cells with a panel of fluorophore-conjugated antibodies directed against cell surface and intracellular proteins and detecting cells that have been suspended in a liquid stream as they pass by multiple lasers and fluorescence detectors. Identification of specific cell subsets producing the gene products of interest is of particular importance, as cell subsets represent discrete units that can be used to monitor and manipulate the system in subsequent experiments done *in vivo* or *in silico.* A major limitation of fluorescence-based flow cytometry is the spectral overlap of fluorescent dyes, which limits the number of colors that can be reliably distinguished [11]. One approach to overcoming this limitation employs

lanthanide-tagged antibodies to label cellular proteins [45], which can be detected by time-of-flight MS. Because there is no spectral overlap, the number of parameters that can be measured simultaneously in a single cell is only limited by the number of available heavy metal isotopes with low biological background [45]. A recent study employed this technology to measure 34 parameters in hematopoietic bone marrow cells simultaneously, with labeling of 13 cell surface markers and 18 intracellular markers (including phosphorylated proteins), and demonstrated that stimulation with physiologically relevant molecules induced cell subset-specific signaling phenotypes that localized to pathway and cell-specific boundaries [46]. Such systems-level insights gained from single-cell analyses can eventually be integrated with '-omics' approaches to provide a more comprehensive description of the immune system.

Tissue issues: the relevance, limitations and feasibility of peripheral blood analysis

Analyses of the human immune response to *P. falciparum* infection are generally restricted to the molecules and cells circulating in peripheral blood, but emerging methods may enable studies of other tissues relevant to *P. falciparum* infection such as skin, liver, spleen and placenta (Box 4). Cytokines, metabolites and antibodies are released systemically so changes in the levels of these molecules in response to infection can be readily detected in blood. Likewise, changes in the frequency, phenotype and function of circulating leukocytes can be ascertained by flow cytometry and gene-expression analysis, since leukocytes constantly traffic between blood and secondary lymphoid tissues as they patrol for foreign antigens. Although analysis of peripheral blood is clearly germane to blood-borne pathogens such as *P. falciparum,* changes in cellular immune responses observed in peripheral blood may not reflect the changes occurring in secondary lymphoid organs and reticular connective tissue. Moreover, leukocyte circulation patterns are not fixed, but vary with changes in expression levels of chemotactic and adhesion molecules; thus, changes in RNA transcript levels in peripheral blood can reflect either migration of leukocytes in and out of peripheral blood or *de novo* gene expression [47]. Several strategies have been developed in an attempt to distinguish these two possibilities. For example, blood leukocytes stimulated *ex vivo* with the antigen or pathogen of interest can determine if gene expression is induced in a fixed population of cells [16, 17]. Another approach involves isolating cell subsets of interest (e.g. B cells, T cells, monocytes, dendritic cells) and analyzing their gene expression profiles separately [18], but this can be prohibitively expensive and increases the probability of irrelevant gene expression due to *ex vivo* manipulation of cells. A related but less costly approach is to quantify cell subsets by flow cytometry to determine if differential gene expression between comparison groups can be explained by differences in cell composition. Lastly, several groups have devised statistical methods to 'deconvolute' cell-type-specific gene expression from mixed cell populations [18, 48-50]. It is not clear that a consensus approach to this limitation is emerging, and often multiple methods are applied.

High-throughput RNA, protein and metabolite assays require small volumes of blood and minimal up-front sample processing, making it feasible to obtain high-quality biospecimens for systems immunology studies even in remote malaria-endemic areas. For example, small volumes of whole blood collected from children under field conditions in Africa were found to yield sufficient quantities of high-quality RNA for genome-wide expression profiling (Tuan Tran *et al.*, unpublished) [51]. The collection of high-quality RNA in the field is facilitated by commercially available reagents that block RNA degradation in whole blood for up to five days at room temperature. Measurements of cytokines and metabolites, which are less stable than antibodies at room temperature, require < 100 microliters of serum. Degradation of cytokines and metabolites can be minimized by snap-freezing serum at the study site with portable liquid nitrogen tanks or 'dry shippers', key research tools that are

increasingly available in Africa (http://www.who.int/vaccine_research/documents/Lee_LN_Infrastructure_in_Africa_MalVac_00_.pdf). Isolation of leukocytes from blood for flow cytometry and gene-expression analysis is also feasible in malaria-endemic settings with nominal investments in basic lab training and equipment [52]. To ensure the success of systems immunology studies and to facilitate cross-study comparisons, it is critical to implement rigorous quality control measures to minimize and account for non-biological batch effects, which may be introduced during biospecimen collection and processing.

The human 'models' of *P. falciparum* infection to which systems immunology can be applied

Progress toward the development of highly effective vaccines for HIV, tuberculosis and malaria has been frustratingly slow [53], but the distinctive bright spot for malaria vaccine and immunology researchers is the availability of human experimental and natural 'models' of *P. falciparum* infection and malaria immunity [7, 41, 54-56]. The application of systems immunology to these models is likely to offer fundamental insights into how the human immune system responds to infectious diseases generally and may also accelerate malaria vaccine development by illuminating the molecular and cellular interactions of the innate and adaptive immune responses that confer protection against malaria.

The 'model' of natural *P. falciparum* infection in endemic areas

In areas of intense *P. falciparum* transmission, non-sterile clinical immunity to the blood stages of *P. falciparum* infection can be acquired after years of repeated infections [57-59]. Immunoglobulin (Ig) G is the only immune effector known unequivocally to contribute to naturally acquired malaria immunity in humans, as demonstrated by studies in which the passive transfer of purified IgG from malaria immune adults to children with acute malaria resolved fever and parasitemia [44]. However, the nature of the innate and adaptive immune response that promotes the acquisition of protective IgG, the antigen specificity of protective IgG, and mechanisms by which other components of the innate and adaptive immune systems contribute to protection from malaria in humans remains unclear [6]. Also unclear is the extent to which regulation of *P. falciparum*-induced inflammation contributes to protection from 'uncomplicated' febrile malaria and severe malaria and the mechanisms by which this might occur, as well as the mechanisms by which *P. falciparum* modulates the host immune response to evade clearance. 'Systems' approaches can potentially illuminate the complex molecular and cellular networks underlying these phenomena [60].

Recent studies have demonstrated the feasibility of conducting genome-wide expression analysis in malaria-endemic settings to investigate the immune responses of malaria susceptible individuals during natural *P. falciparum* infection [61-63]. A Stanford University cDNA lymphochip microarray was used to compare whole blood gene expression profiles of Kenyan children hospitalized with *P. falciparum* malaria (n=15), bacterial infections (n=2), lower respiratory tract infections (n=2), presumed viral infections (n=2) or bacterial/malaria co-infection (n=1) [61]. Convalescent blood samples obtained from subjects with malaria two weeks after hospital discharge (n=6) were also analyzed. The authors observed upregulation of gene expression profiles related to neutrophil and erythroid-related cell activity in children with acute malaria. Another study compared peripheral blood mononuclear cell (PBMC) gene expression profiles of Brazilian adults (n=21) during acute *P. falciparum* malaria and 3-4 weeks after anti-malarial treatment [63]. A custom Operon microarray chip (Harvard University, Cambridge, MA) was used for seven subjects and Illumina HumanWG-6 v2.0 Expression Beadchips (Illumina Inc., San Diego, CA) for the remaining subjects. The authors observed enhanced expression of genes encoding TLRs,

TLR-signaling proteins and elements of IFN signaling pathways during acute malaria relative to convalescent controls. A third study [62] employed Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA) to examine PBMC gene expression profiles of Cameroonian adults presenting with *P. falciparum* malaria (n=15) and *P. falciparum*-naïve US adults (n=22) 9-12 days after experimental *P. falciparum* infection when *P. falciparum* parasites were first detected by blood microscopy, and the majority of subjects were asymptomatic. Compared to *P. falciparum* uninfected US adults, both natural and experimental infections were associated with enhanced expression of genes encoding key components of the innate and adaptive immune response, including TLRs, TLR signaling proteins, pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-1β), IFN-γ signaling proteins (e.g. JAK2 kinases, STAT1, IRF-1), and proteins involved in antigen processing and presentation through MHC class I and II pathways. Interestingly, genes involved in immune regulation (e.g. IL-10), apoptosis and MAP kinase signal transduction pathways were upregulated by natural but not experimental *P. falciparum* infection, possibly reflecting differences in prior *P. falciparum* exposure, the duration and intensity of infection before treatment, and other factors such as co-infection. Although the above studies all utilized hybridization-based platforms, array platforms used were different from each other, which precludes direct, quantitative comparisons among the signatures observed in these three studies. Differences in subject age, time points, tissue type (i.e. whole blood versus PBMCs) and analytical methods further limit comparisons. However, the results generally point toward upregulation of inflammatory pathways through PRRs during acute *P. falciparum* malaria, as might be expected in subjects who are susceptible to febrile malaria.

Going forward, it will be crucial to improve upon these pioneering studies by applying the full set of systems immunological tools to adequately powered, longitudinal cohort studies in which *P. falciparum* infection and malaria episodes are reliably detected through active parasitological and clinical surveillance respectively, and in which high quality biospecimens are collected before, during and after asymptomatic and symptomatic *P. falciparum* infections (Figure 1). The predictable timing and intensity of *P. falciparum* transmission from year to year in many endemic areas makes such studies feasible [64]. Although resource and labor intensive, this approach increases the probability of correctly classifying study subjects as malaria immune or susceptible and, importantly, permits each subject to serve as his or her own uninfected control—a critical point since there appears to be significant inter-individual variation in baseline gene expression profiles among healthy individuals in malaria endemic areas (Tuan Tran *et. al*, unpublished) [65]. The substantial cost of systems assays such as gene expression and antibody profiling can be reduced by conducting retrospective, nested case-control studies within larger prospective cohort studies (Figure 1) such that systems analysis is only applied to well-characterized subsets of age-matched malaria immune and susceptible subjects—only as many as necessary to achieve the desired statistical power (reviewed in [66]). Further cost reductions could be achieved by implementing ‘mesoscale’ profiling methods in which data obtained from initial global systems analysis are used to derive a smaller gene or protein signature that could be quantified across larger studies [67]. To the extent possible in observational studies, it is important to account for known and potential modulators of *P. falciparum* exposure (e.g. bednet use), malaria risk (e.g. sickle-cell trait), and immune responses (e.g. age, gender, ethnicity, co-infections, nutritional status, co-morbidities, vaccination history, use of immunomodulatory drugs, etc.) [68]. A nested case-control study design can also be applied retrospectively to Phase IIb and Phase III trials of partially effective malaria vaccines in malaria endemic areas (Figure 1) [54, 66, 69]). This could help define the relationship between early, vaccine-induced molecular signatures (days 1-7 after vaccination) and the subsequent quality and magnitude of vaccine-specific cellular and antibody responses (days 14-28) and may help identify biomarkers or ‘signatures’ of vaccine efficacy [16-18, 70]. Ideally, predictive ‘signatures’ of naturally acquired or vaccine-induced immunity to malaria

identified by one group would be validated independently by groups in other malaria-endemic settings [18, 20]. Targeted genome sequencing at loci that are expressed differentially between susceptible and protected individuals may identify genomic variants that correlate with transcriptional and clinical responses to *P. falciparum* infection. Because gene-expression datasets from independent studies are amenable to statistical meta-analysis [71], cross-study comparisons could potentially move us toward predictive models of malaria immunity that transcend geographically and genetically disparate populations. The challenge would be to minimize study heterogeneity by harmonizing study designs, assay platforms and analytical methods—an effort that could be facilitated by forming a consortium of malaria immunology centers, analogous to the Malaria Genomics Epidemiology Network, which has focused on understanding how human and *Plasmodium* genetic variants shape the biology and epidemiology of malaria [72].

The 'model' of human experimental P. falciparum infection

Currently, five institutions in the United States and Europe conduct experimental *P. falciparum* infection studies of healthy adult volunteers [73], primarily to test the efficacy of pre-erythrocytic vaccine candidates in Phase IIa trials of malaria-naïve adults prior to Phase IIb trials in endemic areas [74]. Under strictly controlled conditions, volunteers are exposed to the bites of laboratory-reared *P. falciparum*-infected mosquitoes that inject sporozoites into the skin and bloodstream. Within minutes to hours, sporozoites travel to the liver and invade hepatocytes. During the clinically silent liver stage, each sporozoite gives rise to tens of thousands of asexual parasites called merozoites. After approximately 6 days, merozoites rupture out of the liver into the bloodstream and rapidly multiply during 48-hour cycles of erythrocyte invasion, replication, erythrocyte rupture and release of infectious merozoites. In *P. falciparum*-naïve volunteers, parasites become detectable in the blood by microscopy an average of 11 days after infection, at which point curative anti-malarial therapy is administered [74, 75]. *P. falciparum*-naïve volunteers often experience 2-3 days of 'mild' malaria symptoms such as fever, chills, headache, myalgias and fatigue [75]. Since the mid-1980s, over 1300 volunteers have been experimentally infected with *P. falciparum* [73, 74] and severe or life-threatening malaria has never been reported [75].

Remarkably, sterile immunity to experimental *P. falciparum* infection can be induced by exposing volunteers to the bites of irradiated, *P. falciparum*-infected mosquitoes [56]; through vaccination with RTS, S (approx. 30-50% efficacy) [76]; or by exposing volunteers to the bites of *P. falciparum*-infected mosquitoes monthly for three months while on chloroquine prophylaxis [77, 78]. Chloroquine only kills blood-stage parasites and thus allows full exposure to sporozoites and liver-stage parasites and only transient exposure to blood stage parasites.

The highly controlled and predictable nature of the human experimental *P. falciparum* infection model lends itself to high-resolution 'systems' analysis of the host immune response during the liver and early blood stage of the parasite life cycle. It will be of great interest to build upon the aforementioned work of Ockenhouse *et al.* [62] by analyzing blood samples and possibly skin biopsies [79, 80] (Box 4) collected at strategic time points before and after experimental infection of naïve volunteers. In this way, a detailed 'natural history' of the early innate immune response to sporozoite inoculation, liver stage infection and early blood stage infection, as well as the later adaptive immune response, can be delineated (Figure 1). Using a similar sampling strategy, this approach can also help define the mechanisms by which immunity to human experimental *P. falciparum* infection is induced by irradiated sporozoites, infection under chemoprophylaxis, or partially effective malaria vaccine candidates in Phase IIa trials (Figure 1). By comparing 'systems' profiles obtained from natural and experimental infection studies, we may gain important insights into the

mechanisms underlying the differential rate of immune acquisition for these two models as well as the immunological consequences of chronic versus acute *P. falciparum* exposure.

Concluding remarks

The application of systems immunology to human models of natural and experimental *P. falciparum* infection promises to provide fundamental insights into the immune response to complex pathogens and will likely advance our understanding of the mechanisms underlying immunity to malaria. Whether this knowledge ultimately aids malaria vaccine development remains to be seen. However, given the current lack of highly effective malaria vaccine candidates in the pipeline, new approaches are clearly needed, and systems immunology is arguably the most promising approach to have arisen in recent years.

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Boxes

Outstanding questions: key unresolved questions in malaria immunology

1. What constitutes immunity to *P. falciparum* malaria? The *P. falciparum* parasite encodes ~5400 gene products and likely produces numerous biologically active materials. Which of these products activate cells of the innate immune system, and through which receptors and pathways does this occur? Which products are targets of specific B and T cell adaptive immunity? Are there *P. falciparum* products that interfere with the acquisition of immunity, and by what mechanisms does this occur?
2. Children in malaria endemic areas acquire immunity to 'mild' or 'uncomplicated' malaria only after years of exposure to numerous bites from *P. falciparum*-infected mosquitoes. Why does the acquisition of immunity to malaria take so long? How does the frequency or persistence of *P. falciparum* infection influence the acquisition of immunity? What is the duration of this immunity, and what components of protective responses are lost or maintained in the absence of ongoing *P. falciparum* exposure? How does the genotype and transcriptional state (e.g. variant surface antigen expression) of the infecting *P. falciparum* parasite influence the clinical course of infection and the acquisition of immunity?
3. In endemic areas, *P. falciparum* infection in children under the age of five can result in severe disease and death, accounting for nearly 25% of childhood mortality in Africa. The most deadly forms of severe disease, cerebral malaria and respiratory distress, have many of the features of uncontrolled inflammation or sepsis. Children apparently become resistant to severe disease after only a few infections. What immunity are they acquiring? How long-lived is this immunity? Is the longevity of immunity to severe malaria influenced by continued parasite exposure? Does the regulation of *P. falciparum*-induced inflammation contribute to protection from severe malaria, and, if so, by what mechanisms? To what extent are the same immune mechanisms at play in protection from mild malaria?
4. Malaria in pregnant women can have serious, life threatening consequences for both the mother and her baby. Placental malaria occurs, in part, because the placenta provides a new adult tissue to which the parasite can adhere and replicate. Most women become immune to placental malaria after one or two pregnancies. What is the nature of this immunity?
5. How is the clinical course of *P. falciparum* infection and the acquisition of immunity influenced by co-infections with other pathogens, super-infection with *P. falciparum* or the individual's own microbiota?
6. Malaria is transmitted through the saliva of a female *P. falciparum*-infected mosquito as she takes a blood meal. How does the mosquito's saliva affect the immune response in the skin and the clinical course of infection? What role do cutaneous immune networks play in regulating the immune response during the early and later phases of *P. falciparum* infection?
7. What differences exist between protective immune responses induced by natural *P. falciparum* infection, experimental *P. falciparum* infection (irradiated infected mosquitoes or infection under chemoprophylaxis) or partially effective subunit vaccines? Can early molecular signatures induced by experimental infection or candidate malaria vaccines predict the subsequent magnitude, quality and

longevity of effector and memory B and T cell responses? Which of these responses correlate with protection?

Box 1. An overview of integrated systems biology and malaria immunology

The complexity of malaria immunology makes it the ideal discipline for an integrated systems immunology approach. The availability of annotated host and parasite genomes and the development of high throughput technologies to globally measure and analyze biological parameters at the molecular and cellular levels have made this approach technically feasible. A systems approach makes no assumptions and thus does not pre-determine a set of parameters to study. With the aid of computational tools such as unsupervised clustering and pathways analysis, an unbiased approach in data gathering can reveal unanticipated relationships between different compartments of the immune system. Several hurdles to the successful implementation of systems immunology to the study of malaria must be addressed, including the practical issues of conducting intensive, adequately powered studies in endemic settings where resources and infrastructure may be limited. At the very least, the field research team must be equipped to address the medical needs of the participants, properly process biological samples for subsequent analysis and catalogue large amounts of clinical data. The successful transformation of clinical parameters and biological samples collected at field sites into large '-omics' datasets used for building predictive models of malaria immunity will require the concerted efforts of clinicians, epidemiologists, data managers, immunologists, bioinformaticians and computational biologists. Another concern is the need for developing and validating predictive computational models that can help integrate discovery science with hypothesis-driven science, a problem not unique to malaria immunology but magnified by the complex lifecycle of the parasite within the human host. Central to the goal of defining a molecular signature of immunity or susceptibility is modeling how host-parasite interactions initiate signaling pathways that ultimately lead to cytokine production, cellular proliferation/exhaustion and effector function. By incorporating studies of functional *P. falciparum* genomics (reviewed in [23]), a truly integrated approach can be achieved that can illuminate systems-level mechanisms of host-parasite interactions. The ideal model would identify critical receptor-ligand interactions and signaling pathways required for immunity, which would guide subsequent research using *in vitro* techniques and animal models to confirm the roles of the individual genes and gene products involved in those critical pathways.

Box 2. Overview of data management and analysis

The large and complex datasets generated by high-throughput systems biology assays often require more time and effort to process and analyze than to generate [81]. An upfront data management strategy that streamlines data capture, storage and organization can increase the efficiency of subsequent data processing and analysis. To be fully exploitable, the database must integrate the output from cellular and molecular assays, but must also include quality control data, clinical and sample information, and details of the study design. Many tools have been developed to improve the data management workflow (reviewed in [82, 83]), but they often require customization to meet the unique needs of a given project.

In the analysis of genome-wide expression experiments, samples from individual donors can be grouped using unsupervised clustering based on similarity of patterns of gene expression without regard to clinical phenotype, thus providing an estimation of how well expression patterns *per se* fit with clinical status while possibly revealing unanticipated classifications. Supervised analysis can identify transcripts that are differentially expressed between two pre-defined groups (e.g. malaria immune versus susceptible) for class comparison. Differentially expressed transcripts from a 'training set' can be fitted into a mathematical model using class prediction algorithms such as *k*-nearest neighbor, support vector machines, artificial neural networks, decision tree-base methods, and linear and quadratic discriminant methods. The predictive accuracy of the resultant mathematical model is often tested using transcript data from an independent group with known clinical phenotypes (referred to as the 'validation set'). Annotation of the human genome allows genes to be grouped into functional categories such that large lists of differentially expressed genes can be more easily visualized, understood and communicated. For example, pathway analysis of differentially expressed genes provides systems-level insight into the functional significance of changes in gene expression. Several pathway analysis software packages exist that help visualize the expression data in the context of a pathway, class or interactions and employ statistical methods to determine if any of the pathways are significantly enriched between groups. Among the most widely used pathway analysis programs are Ingenuity, GeneSpring, Pathway architect, MAPPfinder and Onto-Express. Pathway and functional information used by these programs are derived from annotated databases of genes and gene products, which include the Gene Ontology project (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and GenMAPP. The functional components of the transcriptional host response to infection can also be determined and visualized using transcriptional modules [19]. Transcriptional modules were identified from transcriptional profiles generated from PBMCs of individuals with one of several disease states. The modules were constructed from sets of transcripts that were coordinately expressed using unsupervised clustering analysis, accounting for the tendency for certain sets of genes to co-cluster in a subset of diseases. Functional associations were assigned to each module by literature profiling. This modular analysis framework allows for visual identification of patterns in gene regulation and changes in cellular abundance and has been used to identify disease-specific, whole-blood modular signatures from patients with inflammatory conditions such as tuberculosis [20].

Box 3. High-throughput analysis of B and T cell repertoires

Passive transfer studies in humans proved that IgG plays a critical role in acquired immunity to malaria [6, 44], but the antigen specificity of protective antibodies remains unclear [84]. Two technical bottlenecks have hindered studies seeking to correlate malaria immunity with specific antibody responses—the ELISA-based platform, which is limited to evaluating one antigen at a time, and traditional cloning and protein expression and purification methods that have made < 0.5% of the 5000+ predicted *P. falciparum* proteins [85] available for analysis [86]. The latter has been made particularly difficult by the A/T richness of the *P. falciparum* genome [85], which can result in early translation termination and heterogeneous polypeptide products during recombinant protein expression. The availability of *P. falciparum* genome sequence data and high-throughput cloning and *in vitro* cell free transcription/translation systems has led to the development of high-density protein microarrays of known and hypothetical *P. falciparum* proteins expressed at all stages of the parasite life-cycle [87-91]. Thus, in a single assay with < 50 microliters of plasma or serum, the specificity of antibodies against a broad array of *P. falciparum* antigens can be compared in malaria-immune and susceptible individuals. It will be of interest to examine the relationship between host genome-wide expression profiles during the early stages of *P. falciparum* infection and the subsequent breadth, magnitude, isotype and IgG subclass composition of *P. falciparum*-specific antibody responses. Also, by integrating antibody profile data with *P. falciparum* proteomic databases (www.plasmodb.org), the proteomic features of highly immunogenic proteins may be identified. Further insights into antibody repertoires that protect against malaria may come through tracking the mutational evolution and use of Ig variable regions in response to natural and experimental *P. falciparum* infection [92]. An effective antibody-based malaria vaccine must stimulate CD4+ T helper cells to orchestrate the generation of memory B cells and long-lived plasma cells in germinal centers. The search for antigen-specific T cells that correlate with protection from malaria is daunting given the size of the *P. falciparum* proteome and the low frequency of any given antigen-specific T cell. However, despite significant technical challenges, progress is being made toward the development of various high-throughput, proteome-wide methods to profile CD4+ and CD8+ T cell responses to *P. falciparum* (reviewed in [91], [93]) and other human pathogens [94, 95].

Box 4. Thinking outside the blood

Recent innovations may allow for 'systems' analyses of the human immune response to *P. falciparum* to be extended beyond the peripheral blood compartment to the skin, liver and spleen. For example, a sufficient quantity of high-quality RNA can be isolated from minimally invasive skin biopsies (~1.5 mm diameter) for genome-wide expression profiling [96]. Analysis of dermal tissue obtained shortly after experimental human *P. falciparum* infection may offer insights into the potential role that cutaneous immune networks play in regulating the host immune response during the early phases of *P. falciparum* infection [80, 97]. The nature of the host response during the liver stage of *P. falciparum* infection that is permissive to repeated natural infections and potentially protective under certain conditions of human experimental infection (i.e. irradiated *P. falciparum*-infected mosquitoes, repeated *P. falciparum* infections under chloroquine prophylaxis) remains unclear and is obviously difficult to study in humans. Remarkable advances in tissue engineering have led to the development of human ectopic artificial livers (HEALs) which can be efficiently established in immunocompetent mice with normal liver function and which have the potential to be integrated with studies of humanized immunity [98]. *Ex vivo* perfusion of human spleens isolated from patients already undergoing left splenopancreatectomy for benign pancreas tumors has offered an otherwise inaccessible window into the interactions between parasitized erythrocytes and whole splenic tissue [99]. Gene expression profiling of *P. falciparum*-infected spleen tissue may illuminate early innate responses to blood stage infection that are otherwise not detectable in peripheral blood. Although not the focus of this review, pregnancy-associated malaria remains a major health threat in malaria-endemic areas [100, 101]. Placental tissue and cord blood are amenable to systems analysis, and gene expression analysis has been used to investigate the host response to placental malaria [102]. Further understanding of immunity to placental malaria would benefit from studies that assess gene expression and cellular composition in placental tissue and cord blood obtained from primigravidae and multigravidae women in endemic areas. Finally, the interaction between the gut microbiome and the host immune system has generated considerable interest. The content of the gut microbiota has been shown to influence both local [103, 104] and systemic [105, 106] immune responses. As an example of the latter, gut microbiota have been shown to be a source of peptidoglycan that systemically primes the innate immune system, enhancing the killing of pathogens by bone marrow-derived neutrophils [106]. It has also been shown that the introduction of pathogens can alter the makeup of microbial communities within the host [107]. With respect to malaria, experimental infection of mice with *P. berghei* has been associated with alterations in metabolite profiles that may be indicative of disturbances within the gut microbiome [108]. Given the potential for cross-talk between the microbiome and the immune response to *P. falciparum* infection, detailed genomic and transcriptomic analyses of the gut microbiome before and after *P. falciparum* infection and in malaria-immune versus susceptible individuals may reveal novel relationships between microbiota and malaria.

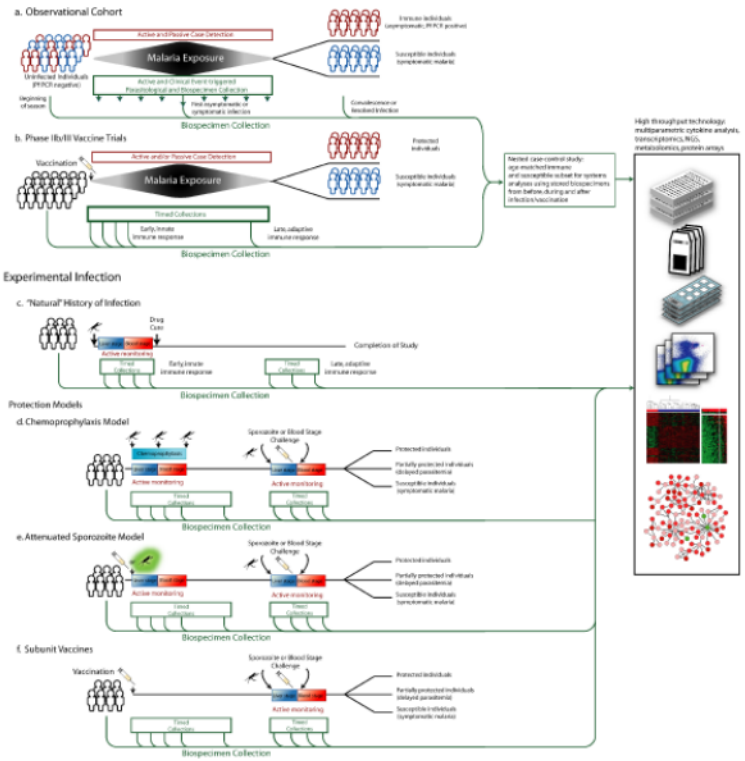

Figure 1. Human 'models' of *P. falciparum* infection

Systems immunology can be applied to natural **(a-b)** and experimental **(c-f)** 'models' of *P. falciparum* infection. **(a)** For example, in areas of seasonal *P. falciparum* transmission, uninfected individuals enrolled in observational cohort studies before the malaria season can be classified prospectively during the ensuing malaria season as either malaria immune (asymptomatic *P. falciparum* infection) or susceptible (symptomatic *P. falciparum* infection). At the end of the malaria season, a nested case-control analysis can then be applied in which immune responses of age-matched immune and susceptible individuals are compared using stored biospecimens collected before, during and after *P. falciparum* infection. For any given study, the design (e.g. timing and frequency of biospecimen collection) will vary with the dynamics of *P. falciparum* transmission at the study site, the scientific questions of interest and the available resources. **(b)** A nested case-control study design can also be applied to Phase IIb and III malaria vaccine trials in malaria-endemic areas to identify relationships between vaccine-induced innate immune responses and the subsequent quality of vaccine-specific cellular and antibody responses and to potentially identify biomarkers or 'signatures' of vaccine efficacy (reviewed in [66]). **(c-f)** The controlled and predictable nature of human experimental *P. falciparum* infection permits high-resolution systems analyses of the host immune response at precise time points during the liver and early blood stages of infection. **(c)** Analysis of biospecimens collected before, during and after *P. falciparum* infection in naïve and previously exposed individuals can yield key insights into the 'natural history' of the human response to the early stages of *P. falciparum* infection. **(d-e)** Protection from malaria can be experimentally induced through repeated *P. falciparum* infections while on chemoprophylaxis [7] **(d)** and through exposure to irradiated sporozoites **(e)**. Analyses of biospecimens collected before, during and after the 'immunization' and 'challenge' phases of these models may yield important insights into the mechanisms by which these models induce protection. **(f)** Systems analysis of Phase I and IIa malaria vaccine trials in which *P. falciparum*-naïve individuals are vaccinated and then challenged with infective mosquito bites may reveal early vaccine-induced molecular

signatures of immunogenicity and efficacy that inform subsequent vaccine development and evaluation. Abbreviation: Pf, *P. falciparum*.