Immune gene expression changes more during a malaria transmission season than between consecutive seasons.

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Abstract

Plasmodium parasites caused over 600,000 deaths in 2022. In Mali, *P. falciparum* is responsible for the majority of malaria cases and deaths and is transmitted seasonally. Anti-malarial immunity develops slowly over repeated exposures to *P. falciparum* but some aspects

of this immunity (e.g., antibody titers) wane during the non-transmission, dry season. Here, we sequenced RNA from 33 pediatric blood samples collected during *P. falciparum* infections at the beginning or end of a transmission season and characterized the host and parasite gene expression profiles of paired, consecutive infections. Our analyses showed that human gene expression changes more over the course of one transmission season than it does between seasons, with signatures consistent with the partial development of adaptive immunity during one transmission season, contrasting with the stability in gene expression during the dry season. By contrast, *P. falciparum* gene expression did not seem to vary significantly and remained stable both across and between seasons. Overall, our results provide novel insights into the dynamics of anti-malarial immunity development over short timeframes.

Introduction

In 2022, malaria caused over 600,000 deaths worldwide[1]. This mortality is primarily caused by *Plasmodium falciparum* infections in children under five years old[2], who lack protective immunity. Repeated exposure to malaria leads, first to development of immunity to severe malaria (typically occurring in early childhood) and, in later childhood, to immunity against clinical symptoms altogether[3, 4]. Prior studies have demonstrated development of both a cellular[5] and humoral[6] response to malaria upon repeated exposures. Memory CD4+ T cells specific for *Plasmodium* blood-stage antigens and skewed towards several T cell phenotypes (e.g., Th1, Tfh, Treg) have been observed[7, 8], but their role in protective antimalarial immunity remains controversial. In mouse models, Th1 cytokine-biased memory cells appear to protect against malaria[9], but further work is needed to characterize human T cell memory-mediated protection. Memory B cell populations specific for blood-stage antigens have also been shown

to develop with age and exposure[10] in a transmission-dependent pattern[11], leading to secretion of *Plasmodium*-specific antibodies[12-15] that contribute to controlling the parasitemia.

However, the development of the adaptive immune memory response may be complicated by inefficient priming of T cells by antigen presenting cells[4], dampening of the immune response by regulatory T cells[16], dysregulation of B and T cells[4, 17] or development of atypical memory B cell phenotypes[18-20]. In addition, anti-malarial immunity wanes during periods of low exposure[21-24], but the time scale of this waning and its underlying mechanisms remain unclear[25].

While individual acquisition and loss of anti-malarial immunity has been studied longitudinally over years, the kinetics of development and loss of anti-malarial immunity over both long- and short-time frames are still incompletely understood. Additionally, the parasite response to changing immune pressure in an infected human during these short periods remains elusive. Previous work has characterized the immune gene expression changes[26] associated with high and low numbers of repeated clinical malaria episodes across an eight year period[27], while changes in the expression of *P. falciparum* variant surface antigen, PfEMP1, have been linked to changes in immune status[28, 29]. Since the *P. falciparum* blood-stages are responsible for all clinical symptoms of malaria and since we have access to peripheral blood to examine the human immune response at this stage, studying host and parasite gene expression from infected blood can provide information on how peripheral malaria immunity develops over one transmission season, whether this immunity wanes during the dry season, and how the parasite responds to these changes.

In Bandiagara, Mali, malaria transmission is intensely seasonal, with a high transmission wet season from June to December and a low transmission dry season from January to May. Each child 0-14 years of age experiences on average 2.2 clinical malaria episodes during the high transmission season, compared to 0.275 during the low transmission season[30]. This high seasonality makes Bandiagara an ideal location to study the dynamics of antimalarial immunity

development and loss over short time frames. Here, we use dual RNA-sequencing analyses of whole blood samples collected during symptomatic *P. falciparum* infections that occurred i) at the beginning and end of one transmission season and ii) at the end and beginning of two consecutive transmission seasons, to study the dynamics of the anti-malarial immune response over a short time scale.

Results

Dual RNA-sequencing to characterize human and P. falciparum gene expression

We extracted and sequenced RNA from whole blood samples collected during 33 symptomatic *P. falciparum* infections from 11 Malian children, aged 1-10 years (Table 1). All samples were collected during a patient-initiated visit due to self-identified malaria symptoms (e.g., fever, headache) and for which *Plasmodium* parasitemia was confirmed by light microscopy[30] (**Table 1**). The mean parasitemia was 64,338 parasites per µL of blood (range 225 – 198,325).

We included in this analysis blood samples from children that had 1) two *P. falciparum* symptomatic infections in the same transmission season, one at the beginning of the wet season and one at the end (n=8 pairs) (Early vs. Late comparison, in blue on **Figure 1**), and/or 2) two *P. falciparum* symptomatic infections in consecutive years, one at the end of the transmission season of year 1 and one at the beginning of the transmission season of year 2 (n=11 pairs) (Late vs. Early comparison, in red on **Figure 1**) (**Table 1**).

To confirm that *P. falciparum* caused all infections, we first mapped all reads to the genomes of *P. falciparum, P. vivax, P. ovale and P. malariae,* simultaneously, and found that more than 98% of *Plasmodium* reads mapped to the *P. falciparum* genome in each sample (**Supplemental**

97 **Table 1**). We then mapped all reads to the human and *P. falciparum* genomes, simultaneously.
98 We obtained 32-139 million reads mapping to human (49% to 99%) and 0.3 to 50 million reads
99 mapping to *P. falciparum* (0.3% to 50%), allowing robust characterization of host and parasite
100 transcriptomes **(Supplemental Table 1**).

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Table 1: Sample characteristics of the selected participants.

Early vs. Late analysis group									
Participant ID	Sex	Ethnicity	Age* (years)	Collection Date (dd-mm-yyyy)	Collection Season	Parasitemia (parasites per µL)	Temp. (°C)	Hgb conc. (g/dL)	Complexity of infection
A	F	Dogon	7	20-Aug-2010	Early wet	36,375	39.5	10.6	Monoclonal
				02-Nov-2010	Late wet	225	39.6	10.7	Monoclonal
B	M	Dogon	7	18-Sept-2009	Early wet	56,600	37.5	11.3	Polyclonal
				17-Sept-2010	Early wet	83,075	36.1	10.6	Polyclonal
				17-Nov-2009	Late wet	97,125	37.5	10.3	Monoclonal
				21-Nov-2010	Late wet	12,450	37.9	9,0	Polyclonal
C	F	Dogon	2	29-Sept-2010	Early wet	179,550	39.8	7.7	Monoclonal
				27-Nov-2010	Late wet	65,100	38.9	11.6	Monoclonal
D	M	Dogon	5	13-Aug-2012	Early wet	181,800	38.2	11.2	Polyclonal
				09-Nov-2012	Late wet	39,975	38.4	11.6	Polyclonal
E	M	Dogon	5	29-Sept-2010	Early wet	118,500	37.7	11.1	Monoclonal
				25-Dec-2010	Late wet	120,400	38.4	10.5	Polyclonal
F	M	Dogon	3	03-Sept-2010	Early wet	37,206	38.4	9.7	Monoclonal
				20-Nov-2010	Late wet	2,100	38.6	6.6	Monoclonal
G	M	Peuhl	5	17-Sept-2010	Early wet	91,350	38.8	12.1	Polyclonal
				12-Dec-2010	Late wet	48,325	39.3	11.2	Polyclonal

Late vs. Early analysis group									
Participant ID	Sex	Ethnicity	Age* (years)	Collection Date (dd-mm-yyyy)	Collection Season	Parasitemia (parasites per µL)	Temp. (°C)	Hgb conc. (g/dL)	Complexity of infection
A	F	Dogon	6	20-Aug-2010	Early wet	36,375	39.5	10.6	Monoclonal
				12-Dec-2009	Late wet	900	39.8	9.9	Polyclonal
B	M	Dogon	6	17-Nov-2009	Late wet	97,125	37.5	10.3	Monoclonal
				17-Sept-2010	Early wet	83,075	36.1	10.6	Polyclonal
C	F	Dogon	1	19-Dec-2009	Late wet	10,800	35.9	9.0	Monoclonal
				29-Sept-2010	Early wet	179,550	39.8	7.7	Polyclonal
				27-Nov-2010	Late wet	65,100	38.9	11.6	Monoclonal
				12-Aug-2011	Early wet	71,900	38.1	11.7	Polyclonal
E	M	Dogon	5	25-Dec-2010	Late wet	120,400	38.4	10.5	Polyclonal
				26-Jul-2011	Early wet	198,325	39.1	12.2	Polyclonal
G	M	Peuhl	4	18-Dec-2009	Late wet	1,125	38.3	9.7	Monoclonal
				17-Sept-2010	Early wet	91,350	38.8	12.1	Polyclonal
H	M	Dogon	10	26-Nov-2010	Late wet	47,475	36.2	10.9	Monoclonal
				11-Sept-2011	Early wet	30,475	37.5	10.8	Polyclonal
I	M	Dogon	2	21-Dec-2010	Late wet	186,350	38.3	10.8	Polyclonal
				26-Sept-2011	Early wet	43,800	38.9	10.3	Monoclonal
J	M	Dogon	2	1-Nov-2009	Late wet	43,800	39.4	8.5	Polyclonal
				20-Aug-2010	Early wet	37,550	37.6	6.4	Monoclonal
K	M	Dogon	2	23-Dec-2010	Late wet	15,100	40	9.8	Polyclonal
				30-Sept-2011	Early wet	62,100	39.7	11.9	Monoclonal
*age at first sampled infection.									

Late season symptomatic infections are characterized by a stronger adaptive immune response.

We first compared the human gene expression profiles generated from symptomatic infections, from the same child, at the beginning and at the end of one transmission season to investigate potential differences in immune response (n=8 pairs, **Table 1**). Of 9,181 expressed human genes, 130 genes were significantly differentially expressed (FDR<0.1) between symptomatic infections occurring early versus late in the season, after adjusting for parasitemia (**Supplemental Figure 1A, Supplemental Table 2**). Interestingly, genes with functions indicative of an adaptive immune response, such as T cell activation (e.g., CCL5[31], ADA[32]) and T and NK cell granules (e.g., GNLY[33], FGFBP2[34]), were significantly increased in expression during late season infections. By contrast, genes with functions indicative of an innate immune response, such as pro-inflammatory cytokines (e.g., IL-18[35]), interferon-stimulated genes (e.g., GBP1[36], GBP4[36], GBP5[36], PARP14[37]) and regulators of the innate immune system (e.g., CLIC4[38], LRRK2[39]), were significantly decreased in expression during late season infections. Overall, this result is consistent with partial development of adaptive immunity to parasites over repeated exposures throughout the season.

To determine whether these differences in gene expression resulted from changes in white blood cell proportion or true differences in gene regulation, we estimated the relative proportion of each immune cell type using gene expression deconvolution[40] and adjusted our differential expression analyses for the proportion each cell type (**Supplemental Table 2**). After adjusting for cell composition, only one gene, myosin light chain 9 (MYL9), remained differentially expressed between early and late season infections, with a higher expression in late season infections (**Supplemental Figure 1B, Supplemental Table 2**). As a myosin molecule, MYL9 has diverse roles in different cell types, and can interact with the T cell activation marker CD69 to induce inflammation during infections[41]. MYL9 has been reported by one study to be

expressed during treatment and recovery from malaria[42], and could potentially be involved in promoting an adaptive immune response to infection.

We then examined which immune cell types differed in relative proportion between early and late season symptomatic infections in the same individual. On average, we found that late season infections were characterized by a higher proportion of adaptive immune cells, whereas early season infections were characterized by a higher proportion of innate immune cells. Specifically, late season infections had proportionally more naïve B cells, CD8 T cells, and resting NK cells than early season infections **(**$p < 0.03$, **Figure 2A**). In contrast, early season infections had proportionally more activated NK cells, neutrophils, resting mast cells, plasma cells, and activated dendritic cells ($p < 0.05$, **Figure 2B**). These observations are consistent with a greater role of an innate response in early season infections, while the adaptive immune response dominates late in the transmission season, suggesting that there is a significant acquisition of anti-malarial immunity, even on this short time scale.

Changes in human gene expression are minimal across the dry season.

To begin to understand whether the gene expression associated with developing immunity changes during the dry season (i.e., between transmission seasons), we compared gene expression profiles of 11 pairs of samples from the same children collected during one symptomatic infection at the end of one transmission season and during one symptomatic infection at the beginning of the next transmission season (**Figure 1**, **Table 1**, **Supplemental Table 2**). In contrast to gene expression changes observed between infections occurring in the beginning and end of the same season, and despite the larger sample size (11 vs. 8), we only identified one gene (MARCO, a macrophage receptor) whose expression was significantly

different in this comparison (**Supplemental Figure 2**). This suggests that immunity remains relatively stable during the dry season and that the immune gene expression in response to parasites in the next season is very similar to that of the end of the previous season, in the absence of interval exposure to infected mosquitoes.

Despite the lack of detectable gene expression differences, we analyzed how proportions of immune cells may have changed between transmission seasons (**Supplemental Table 2**). We found that infections occurring late in one transmission season had significantly more naïve B cells than infections occurring early in the subsequent transmission season (**Figure 3**). This supports our above findings that late season symptomatic infections are characterized by a more adaptive immune cell signature, but the overall immune response is stable across the dry season. These data suggest that there is no detectable waning of immunity over the dry season, at least as measured by gene expression among immune cells detectable in the peripheral blood.

P. falciparum gene expression varies minimally over the course of a transmission season or between transmission seasons.

Since circulating *P. falciparum* parasites are exposed to the immune system in the blood, we might expect that, as the immune response changes over a transmission season, parasites would vary their gene expression to adapt to changing immune pressures. We first compared the expression of 2,574 *P. falciparum* genes from the 8 pairs of samples selected from the beginning and end of one transmission season (**Table 1**). Interestingly, compared to more than 100 differentially expressed human genes between these samples, we identified only nine parasite genes whose expression differed after adjustment for parasitemia (**Supplemental**

Table 3, Supplemental Figure 3). This suggests that, despite changes in host immunity, parasite transcriptional programs in peripheral circulation remain very similar, but may not reflect that of liver-resident parasites in situ. Of these nine differentially expressed genes, only three had annotated functions and two of the genes, PfPTP3 and PfPTP7, are involved in trafficking the variant surface antigen PfEMP1 to the RBC surface[43, 44] (the third one, PfFRM2, is involved in daughter merozoite formation [45]). This observation is interesting since *P. falciparum* has been shown, *in vitro*, to vary PfEMP1 expression in response to environmental changes[29], which likely includes host immune status. Additionally, one study of Kenyan children identified particular PfEMP1 subtypes associated with immune status to severe malaria[28]. An important limitation of the current study is that we did not analyze expression of the different PfEMP1 genes (due to the high sequence homology between PfEMP1 genes and high variability among parasites, it is difficult to map and analyze rigorously PfEMP1s from short read RNA-seq data). It will be important to follow up on this observation and characterize in future studies whether particular PfEMP1 subtypes are expressed at different times during the transmission season or at different levels of host immunity.

Because of potential changes in host immune pressure over the course of the dry season, we also compared parasite gene expression from 11 pairs of samples selected from the end of one transmission season and the beginning of the next transmission season. Of the 2,483 parasite genes expressed, we did not detect any differentially expressed genes in this analysis (**Supplemental Table 3, Supplemental Figure 4**). This could support our above results that the host immune response is stable, in the absence of ongoing *P. falciparum* exposure, between transmission seasons and *P. falciparum* parasites are exposed to similar environments during clinical infections. (We also did not observe any differences in the developmental stage composition between early and late samples from the same season, nor between late samples from one season and early samples from the next (**Supplemental Table 3**).

Discussion

Overall, our data suggest that adaptive immunity to *P. falciparum* partially develops over the course of one transmission season, with evidence from gene expression of activated T and NK cells in late season infections, while we did not detect any waning during the non-transmission (dry) season. Interestingly, despite this change in host immune pressure across the transmission season, we did not detect any substantial changes in the *P. falciparum* gene expression.

Prior work has described the development of adaptive immunity to *P. falciparum* over long time frames[4, 6, 46-48] and subsequent waning during periods without consistent parasite exposure[21, 22, 49]. We observed an increase in naïve B cells, NK cells and CD8 T cells in late season infections, and an adaptive immune gene expression signature, suggesting that some adaptive immunity incrementally develops even within one transmission season. While the memory B cell population has been shown to slowly develop over multiple infections[10], our observations suggest that the B cell response to *P. falciparum* may begin to develop even over a few exposures (but see some limitations below). NK cells can also produce a memory-like response[50] and mediate efficient killing of infected RBCs in an adaptive-like response[51, 52] in cooperation with *P. falciparum*-specific antibodies developed as part of the humoral response[13, 15, 53, 54]. CD8 T cells are implicated in immunity to the liver stages of *P. falciparum*[55] and their enrichment in late season infections is consistent with developing immunity to this stage throughout the transmission season.

Surprisingly, we did not detect enrichment for memory B and T cells, specifically, which would have suggested anti-malarial immune memory development during one transmission season. This could partially be due to the limited resolution of our gene expression deconvolution technique in distinguishing precisely between memory and naïve lymphocyte populations, the

226 atypical, exhausted phenotype of memory lymphocytes that develop after malaria infection and
227 have unique gene expression profiles[17, 18, 20, 56, 57], or a true defect in memory cell generation
228 over few infections. Future work with more high-resolution techniques such as flow cytometry
229 will be necessary to confirm our results and disentangle these possibilities. Taken together, our
230 findings could suggest that despite development of an appropriate adaptive response after
231 malaria exposures (i.e., accumulation of B and T cells late in a season), impairment of the
232 memory response to *P. falciparum* potentially occurs even over a few symptomatic infections
233 during one season. Prior work has also suggested that the precise number of infections
234 (symptomatic or asymptomatic) experienced during a transmission season also strongly
235 influences the development of immunity during that season[58, 59]. Future basic immunology work
236 is warranted to validate these findings and further disentangle the relationship between
237 development of immunity and number of infections per season.

238 Interestingly, despite previous evidence of waning antibody titers over the dry season[21], we did
239 not detect any appreciable differences in adaptive immune-related gene expression over the
240 course of one non-transmission season. The lack of differences in gene expression and immune
241 cell composition between late season and subsequent early season infections suggests that the
242 response to infecting parasites remains relatively stable between the end of one season and the
243 beginning of the next, in Mali. While the lack of detectable gene expression differences is not
244 proof of the lack of immunologic differences, it is worth noting here that i) the sample size for
245 this analysis was slightly larger than for the analysis of the changes during one season and ii)
246 that the samples spanning one dry season were collected 7-10 months apart (compared to 2-3
247 months apart for the samples in the same transmission season, where ongoing exposure to *P.*
248 *falciparum* is occurring). Interestingly, a previous study has reported that parasites can persist
249 as sub-clinical infections through the dry season in Mali, with reportedly little effect on the host

immune response[60] and it is possible that these asymptomatic infections could help to maintain immunity through the dry season.

One important limitation of our current study is its small sample size, which limits overall study power. Individual differences in baseline gene expression, prior malaria exposure and immunity and number of infections experienced during the malaria season, likely impact gene expression and may confound our analyses but are difficult to control for due to the sample size. Indeed, prior work has identified age and number of exposures as important determinants in the development of immunity[49, 58, 61]. The Peuhl ethnicity has also been associated with genetic protection from malaria[62] and differences in immune gene expression between Dogon and Peuhl individuals could influence our findings. Here, we used paired analyses, comparing samples from the same individual collected during different time points throughout the season, to limit the influence of these individual variations but future work with larger cohorts, which can better control for these potential confounders, will be essential to confirm and strengthen findings presented here.

Additionally, because our samples were collected when a child presented to clinic with symptoms, all infections included in our analyses were identified and treated at different lengths of time after initial symptom presentation. Specifically, since the adaptive immune response takes several weeks to develop, the interval between infection and diagnosis may influence the gene expression profile. Additionally, treatment of infections could influence the development of a productive adaptive immune response, especially if parasite exposure is very short-lived. We also only included individuals in this study who presented with symptomatic disease, which could introduce an important sampling bias by studying only those individuals who did not yet develop anti-disease immunity to malaria.

Conclusions

In this work, we described the transcriptional profiles of the host and parasite during uncomplicated *P. falciparum* infections occurring at the beginning and end of consecutive transmission seasons. We found that the human immune response changes more over the course of one transmission season than between transmission seasons, despite a lower power (n=8 vs n=11) and a shorter time frame (2-3 months vs 7-10 months). This observation suggests that the immune response to *P. falciparum* changes over a transmission season to adopt an adaptive immune signature later during the transmission season, while it remains relatively stable between transmission seasons. In contrast, we found that *P. falciparum* gene expression varies minimally over this short time scales. Overall, this study contributes new insights into anti-malarial immunity development over repeated exposures during the short time scale of one transmission season. These findings have important implications for understanding the development of protective immunity to malaria that could be exploited by future vaccine and prevention efforts.

Materials and Methods

Ethics approval and consent

Individual informed consent/assent was obtained from all children and their parents. The study protocol and consent/assent processes were approved by the institutional review boards of the Faculty of Medicine, Pharmacy and Dentistry of the University of Maryland, Baltimore and of the University of Sciences, Techniques and Technologies of Bamako, Mali (IRB numbers HCR-HP-00041382 and HP-00085882).

Samples

We selected 55 whole blood samples, collected directly in PAXgene blood RNA tubes, from children experiencing a symptomatic uncomplicated malaria episode caused by *Plasmodium falciparum* parasites at the beginning or end of the transmission season in Mali (i.e., June to December). The presence of parasites and the parasite species were initially determined by light microscopy using thick blood smears. All infections were successfully treated with antimalarial drugs according to the Mali National Malaria Control Programme standards.

Case Definition

Children were classified, by the field clinicians, as experiencing symptomatic uncomplicated malaria if they i) sought treatment from the study clinic, ii) experienced symptoms consistent with malaria (i.e., fever, headache, joint pain, abdominal pain, vomiting or diarrhea), and iii) *Plasmodium* parasites were detected, at any density, by thick blood smear, and if they lacked any signs of severe malaria (e.g., coma, seizures, severe anemia) [30].

Generation of RNA-seq data

We extracted RNA from whole blood using MagMax blood RNA kits (Themo Fisher). Total RNA was subjected to rRNA depletion and polyA selection (NEB) before preparation of stranded libraries using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB). cDNA libraries were sequenced on an Illumina NovaSeq 6000 to generate ~60-156 million paired-end reads of 75 bp per sample. To confirm that *P. falciparum* was responsible for each malaria episode, we first aligned all reads from each sample using hisat2 v2.1.0[63] to a fasta file containing the genomes of all *Plasmodium* species endemic in Mali downloaded from PlasmoDB [64] v55: *P. falciparum* 3D7*, P. vivax* PvP01*, P. malariae* UG01, and *P. ovale curtisi* GH01. After ruling out coinfections and misidentification of parasites, we aligned all reads using hisat2 to a fasta file containing the

P. falciparum 3D7 and human hg38 genomes i) using default parameters and ii) using (--max-intronlen 5000). Reads mapping uniquely to the hg38 genome were selected from the BAM files generated with the default parameters. Reads mapping uniquely to the *P. falciparum* genome were selected from the BAM files generated with a maximum intron length of 5,000 bp. PCR duplicates were removed from all files using custom scripts. We then calculated read counts per gene using gene annotations downloaded from PlasmoDB (*P. falciparum* genes) and NCBI (human genes) and the subread featureCounts v1.6.4[65].

Gene expression analysis

Read counts per gene were normalized into counts per million (CPM), separately for human and *P. falciparum* genes. Only human or *P. falciparum* genes that were expressed at least at 10 CPM in > 50% of the samples were retained for further analyses. Read counts were normalized via TMM for differential expression analyses. Statistical assessment of differential expression by the time during the season in which a sampled infection occurred was conducted, separately for the human and *P. falciparum* genes, in edgeR (v 3.32.1)[66] using a quasi-likelihood negative binomial generalized model. We used a paired design to make intra-individual comparisons of gene expression between early and late season infections to minimize interindividual effects such as differing levels of developed immunity due to age and exposure history. All models were corrected for the parasitemia of each infection. Adjusted models were corrected for the cell composition of each sample (see below). All gene expression analyses were corrected for multiple testing using FDR [67] (FDR = 0.1).

Gene expression deconvolution

CIBERSORTx [40] was used to estimate, in each sample, the proportion of i) human immune cell types and ii) *Plasmodium* developmental stages. To deconvolute human gene expression profiles, we used as a reference LM22 [68], a validated leukocyte gene signature matrix using 547 genes to differentiate 22 immune subtypes. A custom signature matrix derived from *P. berghei* scRNA-seq data was used for *P. falciparum* stage deconvolution, using orthologous genes between the two species [69]. Relative proportions of each human immune cell type and *P. falciparum* blood stage from each sample are available in **Supplemental Table 4.**

Statistical Analysis

All statistical analyses not mentioned above were conducted in R (version 4.0.3). Paired t-tests were used to compare cell proportions between groups.

Complexity of infection

We used samtools[70] mpileup to call the genotype at each sequenced position in all samples directly from the RNA-seq reads. We removed positions within *Plasmodium* multi-gene families due to inaccurate mapping of reads within these regions because of high sequence variability. We then calculated the reference allele frequency (RAF) at each position directly from the resulting files. To determine the complexity of each infection (i.e., monoclonal vs. polyclonal), we visualized graphically the distribution of RAF in each sample. Samples with a U-shaped curve, with the RAF for most positions being either 0 or 1, were considered monoclonal. Samples with RAF between 0 and 1, representing a substantial deviation from the U-shaped curve, were considered polyclonal[71].

Data and Code Availability

All sequence data generated in this study are deposited in the Sequence Read Archive under the BioProject XXX. Custom scripts are available at https://github.com/tebbenk/seasonality.

Figure Legends

Figure 1: Schematic of the sampling strategy. The black bars show the number of symptomatic malaria cases reported in the entire longitudinal cohort[30] across four years. The blue and red arrows illustrate the sampling strategy of paired infections (i.e., from the same child) that would be selected for, respectively, Early vs. Late comparisons (to examine the development of immunity over one transmission season) and Late vs. Early comparisons (to examine the loss of immunity over one dry season).

Figure 1: Change in the relative proportion of immune cell types between symptomatic infections occurring early and late in the transmission season. Each panel shows the proportion of one WBC subset estimated by gene expression deconvolution, with the thin black lines joining estimates from the same individual. **A) Cell types that are enriched in late season infections.** The panels correspond, from left to right, to naïve B cells, CD8 T cells, resting NK cells. **B) Cell types that are enriched in early season infections.** The panels correspond, from left to right and top to bottom, to activated NK cells, neutrophils, resting mast cells, plasma cells and activated dendritic cells. All comparisons utilize student paired T-test with significance defined as $p \leq 0.05$. Note the difference in y-axis scale due to differences in the proportion of each immune cell subtype.

Figure 2: Change in the relative proportion of naïve B cells between symptomatic infections occurring late and early in subsequent transmission seasons. The panel shows

the proportion of naïve B cells estimated by gene expression deconvolution, with the thin black lines joining estimates from the same individual, compared with a paired T-test.

Supplemental Figure 1: Differences in host gene expression between infections occurring early and late during one transmission season. Each point represents one gene plotted according to the fold-change and the p-value. Red points represent genes that are more highly expressed in late season infections. Blue points represent genes that are more highly expressed in early season infections. **A)** Before adjustment for cell composition **B)** After adjustment for cell composition.

Supplemental Figure 2: Differences in host gene expression between infections occurring late in one transmission season and early in the next, unadjusted for cell composition. Each point represents one gene plotted according to the fold-change and the p-value. Red points represent genes that are more highly expressed in late season infections. Blue points represent genes that are more highly expressed in early season infections.

Supplemental Figure 3: Differences in *P. falciparum* gene expression between infections occurring early and late during one transmission season, unadjusted for cell composition. Each point represents one gene plotted according to the fold-change and the p-value. Red points represent genes that are more highly expressed in late season infections. Blue points represent genes that are more highly expressed in early season infections.

Supplemental Figure 4: Differences in *P. falciparum* gene expression between infections occurring late during one transmission season and early in the next, unadjusted for cell

composition. Each point represents one gene plotted according to the fold-change and the p-value. Red points represent genes that are more highly expressed in late season infections. Blue points represent genes that are more highly expressed in early season infections.

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