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Title

scRNA-Seq reveals elevated interferon responses and TNF-α signaling via NFkB in monocytes in children with clinical malaria

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Abstract

Malaria causes significant morbidity and mortality worldwide, disproportionately impacting parts of Africa. Disease phenotypes associated with malarial infection can vary widely, from subclinical to life-threatening. To date, prevention efforts, particularly those related to vaccine development, have been hindered by an incomplete understanding of which factors impact host immune responses resulting in these divergent outcomes. We applied single-cell RNA-sequencing to compare the immunological phenotypes of peripheral blood mononuclear cells (PBMCs) isolated from children with clinical and subclinical malarial infections in an area of high malaria transmission in northern Ghana. On average, clinical pediatric malaria infections were characterized by a higher fractional abundance of monocytes and an upregulation of innate immune responses, including those to type I and type II interferons and tumor necrosis factor-alpha (TNF-α) signaling via NFκB. Further, in the clinical malaria group, we identified more putative interactions between antigen-presenting cells and proliferating CD4 T cells and naïve CD8 T cells driven by MHC-I and MHC-II signaling pathways, respectively. Together, these findings highlight transcriptional differences between immune cell subsets associated with disease phenotypes that may help guide the development of improved malaria vaccines and new therapeutic interventions for individuals residing in endemic areas.

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Background

In 2021, the malaria parasite was responsible for an estimated 417 million cases and 619,000 deaths, with the majority occurring in the WHO African region (WHO, 2022). However, the development of an effective vaccine to address this global health threat remains challenging due to an incomplete understanding of the parasite's biology and which host factors influence clinical responses to infection.

Individual instances of malaria infection can be subclinical, where individuals experience mild symptoms and do not seek treatment, or clinical, where symptoms become profound resulting in clinical presentation and medical intervention. Instructive factors include environmental exposures, transmission intensity, host genetics, parasite genetics, host-pathogen interactions, and host immune responses (Ademolue et al., 2017; Babiker et al., 2013; Bousema et al., 2014; Nyarko & Claessens, 2021). Illustratively, upregulation of interferon responses and p53 gene expression can attenuate inflammation and protect children from fever (Tran et al., 2019); and, when comparing children with asymptomatic and severe malaria, the genes most upregulated in severe cases are related to immunoglobin production and interferon signaling (Boldt et al., 2019). As reviewed previously, studies have postulated that these interferons can orchestrate immune regulatory networks to dampen inflammatory responses and restrict humoral immunity, thus playing a critical role as a wedge that determines protection versus permissiveness to malaria infection (Mooney et al., 2017; Sebina & Haque, 2018).

Similarly, it has been shown that the number and phenotype of cells responding to infection can vary with exposure to *Plasmodium* (de Jong et al., 2021). For example, Africans, who tend to have higher levels of exposure, have been shown to exhibit metabolic and platelet activation during malaria infection as compared to typically infection-naïve Europeans (de Jong et al., 2021). Similarly, children who experience high numbers of malaria episodes show upregulation of interferon-inducible genes and immunoregulatory cytokines, suggesting an immune modification to prevent immunopathology and severe outcomes during new infections (Bediako et al., 2019). Beyond differences in exposure and infection history, the strain responsible for each infection can also alter immune response dynamics and disease pathogenesis (Crompton et al., 2014; Ioannidis et al., 2014).

Since so many factors can influence host response dynamics to infection (e.g., exposure, the timing of infection), some studies have implemented tightly regulated models of malarial infection, such as controlled human malaria infections (CHMI). CHMI studies have identified several pathways, including toll-like receptor signaling (Kazmin et al., 2017), platelet activation (de Jong et al., 2021), interferon signaling (de Jong et al., 2021; Loughland et al., 2020; Milne et al., 2021), and B-cell receptor signaling, that are involved in immunological modulation of *Plasmodium falciparum* infections (Tran et al., 2019). Although CHMI have identified several specific pathways involved in malaria, the differences and similarities in gene expression pathways in both CHMI and natural exposure reflect distinctive and unresolved immunopathological mechanisms elicited during *P. falciparum* infection. Collectively, these studies demonstrate the importance of obtaining a more comprehensive understanding of the host and pathogen factors that influence immune responses to inform the development of new therapeutic approaches and improved vaccines.

To date, most genomic analyses of immunological responses to malarial infection have been performed in bulk on blood, brain, liver, or spleen tissues (Lee et al., 2018). The majority of

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these studies have been conducted in children and show that clinical malaria or symptomatic infections, as mentioned above, are characterized by upregulated expression of genes involved in interferon signaling, antigen presentation, neutrophil-associated signatures, and B cell modules relative to healthy controls (Lee et al., 2018). Adults, meanwhile, present slightly varying responses: symptomatic Malian adults, compared to naïve individuals, had upregulated B cell receptor signaling but more modest upregulation of interferon responses, while symptomatic Cameroonian adults showed marked induction of genes related to interleukins and apoptosis compared to presymptomatic individuals (Ockenhouse et al., 2006; Tran et al., 2016). These inconsistencies may be related to patient history/exposure or the confounding effects of differences in how the various cell types respond, directly or indirectly, to infection, and the role of intercellular communication in these processes, as well as how overall cellular composition and communication influence clinical course. The emergence of single-cell transcriptomics provides a unique opportunity to examine the sources of this variability (Hie et al., 2020) by profiling abundance and transcriptomic variation across immune cell populations in individuals with high malaria exposure but divergent clinical phenotypes. Moreover, by examining the expression of ligands, receptors, and genes involved in intercellular signaling, we can identify the critical mediators of immune responses and the pathogenesis of malaria for subsequent validation (Jin et al., 2021).

Here, we present a comparative analysis of peripheral blood mononuclear cells (PBMCs) phenotypes in children with subclinical or clinical *P. falciparum* infections in an area with endemic malaria transmission. Our data describe in unprecedented detail, cell subsets and signaling pathways associated with disease severity to provide new insights into the immune response mechanisms that influence the course of *P. falciparum* infections.

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Results

Profiling pediatric malaria immune-cell populations using single-cell analysis

PBMCs from eleven children (aged 4-8 years) with subclinical (N=5) or clinical (N=6) *P. falciparum* infections in a high malaria transmission area (Navrongo, Ghana) were analyzed using single-cell RNA-sequencing (scRNA-Seq) to ascertain differences in expression patterns of immune response genes that might influence disease pathogenesis (***Figure 1a***). In this study, we defined the subclinical group as "malaria-positive individuals during community screening", while the clinical group was defined as "malaria-positive cases during health facility screening". The subclinical and clinical cohorts were selected because they were not significantly different in previously identified potential confounders, such as geographical location, age group, gender, clinical symptoms, housing conditions, and occupation (***Supplementary Table 1***). Each sample was profiled using the Seq-Well platform, a portable, simple massively parallel scRNA-Seq method (Gierahn et al., 2017). The resulting data were filtered to remove cells based on the fractional abundance of mitochondrial genes (<30%) and transcripts expressing in <20 cells. After variable gene selection, dimensionality reduction, clustering, cluster removal, and reclustering (***Methods***), we retained 18,303 transcripts and identified 10 distinct cell subsets in 18,176 cells, across the two groups of children (***Figure 1a, b; Supplementary Figure 1a***).

We manually annotated these 10 clusters using known RNA marker genes to identify B cells, CD4 T cells, CD8 T cells, natural killer (NK) cells, monocytes (Mono), and dendritic cells (DC) (***Supplementary Figure 1b & c***). To identify and enumerate cell subsets in our dataset at higher resolution, we opted to map our query dataset to an annotated multimodal reference dataset of PBMCs. First, we confirmed that all the cell subsets identified using manual annotation were present in the resultant UMAP (***Supplementary Figure 1d***). As expected, our reference mapped dataset recapitulated all PBMC subsets, including B, CD4 T, CD8 T, NK, Mono, and DC (these subsets are used throughout the work; ***Figures 1b and c***). We identified several subclusters, such as intermediate, memory, and naïve B cells; naïve, proliferating, effector memory and central memory CD8 and CD4 T cells; proliferating CD56+ NK cells; CD14+ and CD16+ Mono; plasmacytoid (pDC) and conventional (cDC) dendritic cells, and other cell subsets (***Figures 1d and e***). Since the reference dataset only annotated two monocyte clusters (CD14+ and CD16+), we hypothesized that there might be additional transcriptional heterogeneity describing actively responding monocyte subpopulations. Therefore, further sub-clustering was done which resolved the Mono into 3 large subpopulations (Mono 1, Mono 2, Mono 3) and 1 small cluster (Mono 4) (***Figures 1f, g and h***) based on previously reported markers (Villani et al., 2017). Taken together, these data distinguish nearly all distinct cell subsets that were present in PBMCs of children in both the subclinical and clinical groups.

Differences in relative cellular composition between the subclinical and clinical groups

Next, we asked whether there were significant differences in the relative proportions of cell types between the clinical and subclinical groups. We found that relative cell proportions of the major cell subsets (B, CD4 T, CD8 T, NK, Mono, and DC) varied between individuals (***Figures 2a & b, Supplementary Table 2***). Individuals with clinical malaria exhibited

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elevated levels of circulating Mono while individuals with subclinical malaria exhibited higher proportions of circulating B cells (Dirichlet-multinomial regression, *P*<0.01; ***Figure 2a, Supplementary Table 2***). Further analysis of the B cell subsets showed that the abundance of naïve and intermediate B cells was significantly reduced in the clinical malaria group compared to the subclinical malaria group (Dirichlet-multinomial regression, *P*<0.05; ***Figure 2b, Supplementary Table 2***). We also confirmed a significant expansion of both CD14+ and CD16+ monocyte subsets in clinical malaria compared to the subclinical malaria group (Dirichlet-multinomial regression, *P*<0.01; ***Figure 2b, Supplementary Table 2***). Although there is evident variation in cellular proportions of T lymphocytes among all the individuals (***Figures 2a & b*****),** we did not observe any significant difference in proportions of either CD4 or CD8 T cells between the clinical and subclinical groups (Dirichlet-multinomial regression, *P*>0.05; ***Figure 2b, Supplementary Table 2***). However, the proportions of naïve and central memory CD4 T cells were significantly higher in the clinical group compared to the subclinical group (Dirichlet-multinomial regression, *P*<0.01); ***Figure 2b, Supplementary Table 2***). NK cell frequency was also higher in clinical malaria suggesting that they may play a role during active disease (Dirichlet-multinomial regression, *P*>0.05; ***Figure 2b, Supplementary Table 2***). Among NK cells, the proliferating and CD56+ subsets were higher in clinical compared to subclinical malaria, but these differences were not statistically significant (Dirichlet-multinomial regression, *P*>0.05; ***Figure 2b, Supplementary Table 2***). Overall, the minor cell subsets of T cells and other cell types with low frequencies did not show differences in proportions between the groups but the main cell subsets had significant differences in proportions between clinical and subclinical malaria children.

Comparative analysis of inflammatory responses in children with subclinical and clinical infections

Having identified shifts in the composition of circulating immune cells between children with subclinical and clinical infections, we next asked whether gene expression differed within each immune cell subset between the two groups. Comparing clinical to subclinical malaria, we observed the largest transcriptional changes (measured by pairwise DE across cell types with adjusted P value < 0.05 and log fold change > 2) within B cells and Mono (***Figures 2c, Supplementary Table 3***)***.*** Apart from other B cell function genes, there was a general trend towards upregulation of inflammatory genes in B and T cells in children with clinical relative to those with subclinical infections, including *S100A8, CXCL8,* and *S100A9* (***Figure 2c***). Significant transcriptional changes were also observed in Mono, with genes such as *IFTM3, FCER1G,* and *CCL4* being upregulated in the clinical group compared to the subclinical group (***Figure 2c***). Clinical malaria was also associated with the upregulation of Major Histocompatibility Complex I (MHC-I) genes such as *HLA-A* and *HLA-C* which are involved in antigen presentation in Mono (***Figure 2c***). In CD4 and CD8 T cells, there was increased expression of some inflammatory factors such as *CXCL8* and *NFKBIA* in children with clinical infection relative to the subclinical group, suggesting direct sensing of parasite products during clinical malaria (***Figure 2c***). Using gene set enrichment analyses (GSEA), we found that clinical malaria involved robust induction of several innate immune response pathways such as TNF-α signaling via NF-κB, TGF-β signaling, IL6-JAK-STAT pathway, complement, IL-2-STAT5 signaling, inflammatory response, interferon-α response (IFN-α), and interferon-γ response (IFN-γ) (***Figure 3a, Supplementary Table 4***). We observed that although each cell type was enriched in one or more of these pathways, there was a unique molecular signature of the genes involved in each. Upregulation of IFN-γ and IFN-α response pathways in Mono were characterized by increased expression of genes such as *IFITM2, IFITM3, IL10RA,* and

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TNFAIP3, while in NK cells they were typified by genes such as *NFKBIA, CD69*, and *ISG20* (***Figure 3b & c, Supplementary Table 4***). Mono and natural killer cells upregulated TNF-α signaling via the NF-κB pathway with the induction of genes related to this pathway such as *IL1B* and *TNFAIP3* for Mono, and *IL7R, CD44,* and *NFKBIA* for NK cells (***Figure 3b & c, Supplementary Table 4***). Inflammatory responses in Mono were characterized by *IL10RA, IL1B*, and *CXCL8* while in NK cells they were driven by *CD69, IL7R, CXCL8,* and *NFKBIA* among others (***Figure 3b & c, Supplementary Table 4***). Thus, the enrichment of unique genes for each cell subset for similar pathways suggests a specific but concerted contribution of each cell subset toward the innate immune response during clinical malaria.

Relative enrichment of ISGs gene modules in monocytes of children with clinical relative to those with subclinical infections

Since IFN genes were significantly upregulated in Mono in children with clinical malaria relative to the subclinical group, we next sought to determine if entire gene modules were enriched. Interferon stimulated genes (ISGs) modules scores were significantly higher in B cells, DC, CD4 T cells, and Mono in children with clinical malaria compared to those with subclinical malaria (***Wilcoxon, adjusted P < 0.01 for all comparisons, Figure 4 a, b, d, e***)*;* however, there were no significant differences in ISG module scores in CD8 T cells and NK cells. Further examination of intra- and inter-individual variation in these module scores revealed substantial intra-individual variation in cells from the same participant and between cells of the same type from different participants (***Supplementary Figure 1e***)***.*** Of note, cytokine scoring for one subclinical participant was notably different from the others in the subclinical group (***Supplementary Figure 1e***): this participant (Subclinical 4) had reported having fever, headache, and chills previously just like some of the other subclinical participants and hence may have been progressing to clinical malaria (***Figure 1a***). Overall, our data show that Mono play a significant role in clinical malaria infections compared to subclinical infections through induction of the ISGs gene modules.

Role of MHC-I and MHC-II signaling pathways in cell-to-cell interactions

Next, we used our single-cell data to infer putative axes of cell-to-cell communication using signaling ligands, cofactors, and receptors**.** First, we discerned cell-to-cell interactions in the clinical malaria group and found that the number of interactions (ligand-receptor) originating from primary innate immune cells such as DC and Mono were greater than those originating from non-antigen presenting cells (***Supplementary Table 5***). However, our data show very few inferred cellular communication networks in subclinical infection (***Supplementary Table 6***). This analysis suggests a role for Mono as antigen-presenting cells in orchestrating pro-inflammatory responses by interacting with proliferating CD4 T cells, intermediate B cells, effector memory T cells, and naïve CD8 T cells in clinical infection (***Figure 5a***). Conventional DC also produced factors that interact with proliferating CD4 T cells and effector memory CD4 T cells, suggesting a concerted effort by antigen-presenting cells to activate the immune response in children with clinical malaria (***Figure 5a***). Communication probabilities indicated that MHC-I and MHC-II play a role in these interactions among other pathways. The most significant receptor-ligand pairs for HLA-A, HLA-B, HLA-C, HLA-E, HLA-G, and HLA-F ligands for MHC class I include CD8A, CD8B, LILRB2, and LILRB1 (***Figure 5b***). The leading intercellular ligand-receptor pairs with CD4 T cells as signal receivers were distinct HLA genes, with the highest relative contribution being driven by HLA-DRA and HLA-DRB1

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(***Figure 5c***). The other minor signaling pathways that were important in clinical malaria include MIF, RESISTIN, ANNEXIN, GALECTIN, ADGRE5, APP, CD22, CD45, SELPLG, CD99, CLEC, and TNF signaling networks. For the TNF signaling pathway, the $CD56^+$ NK cells showed to be interacting with Mono and also with proliferating CD4 T cells, effector memory CD4 and CD8 T cells, and cDC (***Figure 5d***). This cell communication network was mediated by *TNF* in the sender cells and *TNFRSF1B* in the receiver subsets (***Figure 5e***), and this corroborates the DE results (***Figure 5g***). We examined the expression levels of *TNFRSF1B* across all the cell subsets and found that indeed it was expressed in all the receiver cells (***Figure 5f***). Only the pDC and CD16 Mono showed cell-to-cell interactions with naïve and intermediate B cells and might be playing a role in B cell activation and development in children with subclinical infections through MHC class II molecules (***Figure 5g & h***). Therefore, exposure of innate immune cells to parasite ligands may potentially activate intracellular signaling cascades through cell-to-cell interactions to induce rapid expression of a variety of innate immune genes.

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Discussion

Here, we compared the dynamics of immune response between clinical and subclinical pediatric malaria infection using scRNA-Seq and identified a potential role for interferon responses and TNF-α signaling via NFκB in Mono during the clinical manifestation of pediatric malaria infection. We also found differences in the fractional abundances of PBMC cell subsets, with clinical malaria infections characterized by a proportional increase in Mono while subclinical malaria infections had a higher proportion of circulating B cells. We show cellular level variation in the expression of innate immune modules within and between individuals as well as between clinical phenotypes. Further, we identified a role for Mono and other innate immune cells through MHC-I and MHC-II molecules in driving cell-to-cell interactions with CD8 and CD4 T cells respectively. Together, our work recontextualizes the function of the innate immune cells in malaria, demonstrates how variable their responses can be, and links specific responses to clinical presentation.

Differential gene expression comparing clinical and subclinical infection phenotypes across cell types revealed a significant upregulation of genes associated with innate immunity in different cell types. We show that *CCL3* and *CCL4* (also known as macrophage inflammatory protein - MIP-1α and MIP-1β) were upregulated in Mono of clinical malaria children, suggesting their possible role in modulating clinical disease (Awandare et al., 2006). *CXCL8* was also highly upregulated in B cells, CD4, and CD8 T cells. Other studies have shown that circulating levels of CXCL8 and CCL4 can correlate with parasite density, and when found in the cerebrospinal fluid they can predict cerebral malaria mortality (Abrams et al., 2003; Armah et al., 2007; Ayimba et al., 2011; Ioannidis et al., 2014). Furthermore, the adaptive immune cell subsets (B cells and T cells) in clinical malaria expressed two alarmins (*S100A8* and *S100A9*) that are known to form calprotein heterodimer, an endogenous TLR4 ligand; this could suggest a possible role to silence hyperinflammation (Nahrendorf et al., 2021). In the clinical malaria group, we also show significant expression of *FCER1G* in B cells, Mono, and DC, which is induced by interferon-gamma and encodes for a gamma chain of the FC receptor and it is suggested to play an important role in controlling parasitemia and fever (Tran et al., 2019). Collectively, our data imply that both adaptive and innate immune cells cooperatively play a role during the pathogenesis of clinical malaria when compared to subclinical malaria children.

We showed that several immune-related pathways are activated by *Plasmodium* infection including the TNF-α signaling via NFκB pathway, interferon-gamma/alpha responses, IL2-STAT5 signaling, and inflammatory response pathway in clinical malaria individuals. Since the parasite life involves repeated cycles of red cell invasion and lysis, the release of pyrogenic cytokines that drive these pathways such as interleukins, interferons, and TNF in Mono and NK cells, can signify pathophysiological events occurring in clinical malaria (Gazzinelli et al., 2014; Ioannidis et al., 2014). These observations could also mean that children with clinical malaria were sampled quite early during the onset of the disease progression trajectory (Crompton et al., 2014). Our data are consistent with those previously described by integrating whole blood transcriptomics, flow cytometry, and plasma cytokine analysis (Tran et al., 2019), and our results further identify the cell subsets in which these pathways were more enriched. We show that each of the cell subsets has a unique signature of genes enriched in these immunogenic pathways with minimal sharing. Similar innate immune response pathways have also been shown in individuals with malaria in a few studies such as whole blood transcriptomics of the Fulani of West Africa (Quin et al., 2017),

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children repeatedly exposed to malaria (Bediako et al., 2019; Tran et al., 2019), controlled malaria infection studies (Loughland et al., 2020), and even mice models (Nahrendorf et al., 2021). We have now confirmed some of these observations and demonstrated that in the pediatric clinical state, robust upregulation of certain genes in specific cell subsets is associated with systemic inflammatory responses. Innate immune cells, such as Mono, DC, and NK cells, appear to be most reactive during clinical malaria infections, probably due to continuous exposure in a high transmission area as suggested by other studies (Boldt et al., 2019; Gray et al., 2007; Nahrendorf et al., 2021).

By collating gene modules of interferon-stimulated genes (ISGs), we show that there is a difference in the expression between the clinical and subclinical malaria groups across different cell subsets. ISGs are normally produced as a function of interferon responses (IFNs) (Mooney et al., 2017), which we observe to be enriched in clinical malaria children. IFNs are produced primarily by DC to activate other cells to produce ISGs (Spaulding et al., 2016), and we observed that B cells, T cells, Mono, and DC have higher ISG module scores in clinical malaria compared to subclinical malaria children. Notably, our data show that each cell or cell subset responds differently upon IFN activation with varying transcriptional responses of an ISG module between individuals. This variability was also observed for cytokine modules, NF-κB target modules, and HLA modules. Similarly, a previous CHMI study observed striking inter-individual variation in immune cell composition and immune responses, demonstrating that an individual can have a unique immune fingerprint (de Jong et al., 2021). Thus, the variations in immune responses that we observed could be attributed to the complexity of the *P. falciparum* life cycle with several developmental erythrocytic stages, duration of infections, and intensity of infection in each individual (Crompton et al., 2014). These findings on inter-individual variability in immune responses could provide insights when considering the design and evaluation of interventions that target host immunity in the control of malaria.

Our scRNA-Seq data enabled us to quantitatively infer and analyze cell-to-cell communication networks across all the innate and adaptive immune cells (Jin et al., 2021). This analysis enabled us to uncover coordinated interactions between innate and adaptive immune cells through various ligands. The cell-to-cell interactions in clinical malaria samples were driven by MHC class I and II signaling pathways, whereby antigen-presenting cells were shown to have more interactions with proliferating CD4 and naive CD8 T cells. The importance of HLA genes has long been demonstrated by Hill and colleagues who associated HLA-Bw53 antigen and DRB1*1302–DQB1*0501 haplotype to independently protect against severe malaria in West Africa (Hill et al., 1991). Thus, our observations on cell-cell interaction involving HLA molecules and T cells support the importance of these molecules during malaria infections, although we observed varying degrees of interactions in clinical malaria compared to subclinical groups. We also showed that within clinical malaria infections, there are contrasting interactions between various HLA I and HLA II molecules with CD8 or CD4 T cell receptors respectively this could relate to their tight regulation and antigen-presenting ability (Guermonprez et al., 2002; Rock et al., 2016). Activation of CD4 and CD8 T cells has been correlated with protective immunity to malaria, and they can differentiate into several functionally distinct subsets in the presence of various cytokines (Kurup et al., 2019). It was not surprising that we identified different fractional abundances of CD4 and CD8 T cell subsets in clinical compared to subclinical children, but we demonstrate that ultimately this results in varying degrees of interactions with Mono or DC. Future work should seek to identify the mechanisms that result in these variations and their impact in

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orchestrating phagocytic and humoral responses as this critical knowledge gap will be important in developing T cell-based malaria vaccines.

Overall, by using scRNA-seq on PBMCs obtained from malaria-exposed individuals in a high transmission area, this work sheds light on the interplay between peripheral immune cells during malaria infection, uncovering the genes and immune pathways that might play a significant role during clinical cases. Data presented here demonstrate that subclinical infections are characterized by the absence of the inflammatory response signatures observed in the clinical malaria group, suggesting that a muted innate immune response or disease tolerance plays a role in children exposed to high malaria transmission (Nideffer et al., 2023). The findings are relevant for guiding the development of malaria vaccines, as well as immunotherapeutics for alleviating clinical malaria disease and preventing progression to severe disease.

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Methods

Study Design and Sample Size

Children with subclinical or clinical *P. falciparum* infections were recruited in the Kassena-Nankana Municipality of the Upper East Region of Ghana. Children with subclinical infections were recruited at the community level, while those with clinical malaria were recruited at the Navrongo War Memorial Hospital. Malaria RDT (CareStart^{TM}, malaria Pf(HRP2), Access Bio, NY, USA) was used to screen children presenting with suspected malaria symptoms. Individuals with positive malaria tests who provided written informed consent were recruited into the study. Five milliliters of whole blood was collected and used for thick and thin blood smears for parasite identification and quantification. Five children with subclinical infections and six children with clinical infections were selected for cell transcriptomic analysis.

Seq-Well scRNA-Seq Workflow

Seq-Well scRNA-Seq S^3 workflow was performed according to the published methods (Gierahn et al., 2017; Hughes et al., 2020). In brief, $5x10^5$ PBMCs from each patient were dispensed into a single array containing barcoded mRNA capture beads (Supplementary Figure 1). The arrays were sealed with a Polycarbonate Track Etch (PCTE) membrane (pore size of 0.01μM), allowing cells to remain separated through the lysis and hybridization steps. mRNA transcripts were hybridized and recovered for reverse transcription using the Maxima H Minus Reverse Transcriptase in the first strand synthesis step. Exonuclease (I) was used to remove excess primers and mRNA was captured via poly-T priming of the poly-A mRNA. The captured mRNA underwent first-strand synthesis to generate single-stranded cDNA while bound to the beads. Enzymes with terminal transferase were used to create 3' overhangs and three cytosines. The overhangs are used in template switching, whereby a SMART sequence is appended to the overhang on both ends of the cDNA molecule during the first strand synthesis. Some templates fail to switch, resulting in loss of the mRNA; hence they are chemically denatured using 0.1M NaOH with random octamer with the SMART sequence in 5' orientation, and a second strand is synthesized. Whole transcriptome amplification of the cDNA was performed using the KAPA HiFi PCR master mix (Kapa Biosystems). Libraries were pooled and purified using AgenCourt AMPure XP Beads. The quality of the library was assessed using Agilent Tape Station with D5000 High Sensitivity tapes and reagents. Samples were barcoded as described in the Nextera XT DNA (Illumina, USA) segmentation method. Tagmentation was important because, after cDNA amplification and clean-up, there are usually very long cDNA molecules that need to be fragmented to be sequenced by Illumina. The Nextera XT DNA tagmentation method is effective and allows for the addition of adaptors and multiplex indexes at both ends of each fragment (Hughes et al., 2020). Finally, the amplified library was purified using SPRI beads, pooled, and sequenced using the NextSeq500 kit (Illumina, USA). Paired-end sequencing was performed with a read structure of 20 bp read one, 50 bp read two, and 8 bp index one as recommended for Seq-Well. The targeted sequencing depth was 100 million reads for all samples.

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Processing Reads

The raw data were converted to demultiplexed FastQ files using bcl2fastq (Version 5, Terra Workspace) using the Nextera XT indices and then aligned to the hg19 human genome using STAR aligner (Version 2.7.9) within the Broad Institute DropSeq workflow (Version 11, Terra Workspace). The data was cleaned using Cell Bender (V 0.2.0) with default settings, to remove ambient RNA (Fleming et al., 2019). The raw expression matrices and sample information were loaded into the open-source statistical software R (R version 4.2.1). An array with 45,691 gene features for 22,819 cells described data collected across 11 samples. The data were filtered to include only features expressed in more than 20 cells, and the resultant matrix described 18,303 gene features across 22,819 cells. A Seurat (Version 4.0) object was created, and the metadata was added to it to identify the participants (Hao et al., 2021). Cell cycle scoring was performed and computation of the percentage of mitochondria genes before integration. The object from each participant was transformed individually within the object using SCTransform followed by the selection of integration features, finding the anchors, and finally combined integration. Principal component analysis was performed to reduce the dimensionality of the data in order to identify clusters of cells with similar transcriptomic profiles. Clusters and cluster resolution were determined using FindNeighbors and a customized FindClusters function that showed that the best resolution was 0.523, with an average silhouette score of: 0.2 and 11 clusters. One cluster showed no cluster-specific genes and was removed as multiplets, leaving 18,176 cells. The remaining clusters were reclustered and re-embedded, resulting in 10 clusters with a resolution of 0.292, and an average silhouette score of: 0.301. The average number of transcripts and expressed genes were evaluated per cluster using half violin and boxplots. The clusters were projected to a two-dimensional space using the Uniform Manifold Approximation and Projection (UMAP) (McInnes et al., 2018) algorithm in Seurat.

Reference-Based Mapping

Immune cell subsets were identified using common cell markers to identify the Mono, T cells, B cells, NK cells, DC, and other immune cell populations. Uniform Manifold Approximation and Projection for Dimensional Reduction (UMAP) was used to embed the cell populations and color code based on the expression of surface markers. The clustered PBMC dataset in this study (query) was mapped to a reference CITE-Seq dataset of 162,000 PBMCS measured with 228 antibodies (Hao et al., 2021). The query data were projected into the same dimensional space as the reference dataset, thus separating the cells into the cell types present in the reference dataset. The method first projected the reference data transformation onto the query data, followed by the application of KNN-based identification of mutual nearest neighbors (anchors) between the reference and query. On an L2-normalized dimensional space, the reference data transferred continuous data onto the query data to annotate the scRNA data based on a weighted vote classifier. For visualization, reference-based UMAP embedding was used, considering that all the immune cell populations are well represented.

Analyzing differences in samples

Cluster/sample composition was calculated to determine the proportion of cells per cluster and per cell type. Cell subsets that were significantly different between subclinical and

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clinical infection groups were identified by computing Dirichlet Regression using the DirichReg function in DirichletReg Package in R (Simmons, 2022). Differentially expressed (DE) genes were computed using the FindMarkers function on Seurat (Version 4.0), which we used to determine differentially expressed genes in the clinical and subclinical groups using MAST with significance at ($P<0.05$) and log fold change of > 0.2. Subclinical 4 was not included in the DE analysis due to high levels of cytokine module scores. DE genes were visualized using volcano-like plots and heatmaps to compare all the cell types between subclinical and clinical and malaria cases. The fgsea (R-package) was used to analyze the pre-ranked gene set enrichment analysis (GSEA). Module scores for HLA genes, ISG, NFκB target genes, and cytokines were analyzed using the AddModuleScore function in the Seurat R package. Statistical difference in module scores between the subclinical and clinical groups for each cell subset was computed using Wilcoxon sign-rank test with Bonferroni correction. Boxplots were used to visualize the module scores for each cell, denoting the median and interquartile range.

Cell-to-cell Interaction using CellChat

CellChat (Version 1.1.1) was used to quantitatively infer and analyze cell-to-cell communication networks (Jin et al., 2021). Statistically significant intercellular communication between cell groups was identified using permutation tests, and interactions with a significance level of less than 0.05 were considered significant (Jin et al., 2021). Heatmaps were used to visualize each signaling pathway and their cell-cell communications, highlighting the number of interactions, the sources (ligands) of the interactions, and the receivers (receptors) of the interactions. The relative contribution of each ligand-receptor pair to the overall signaling was shown in bar plots. The relative contribution provides a measure of a particular ligand-receptor interaction in a particular cell-cell signaling network. This measure demonstrates the importance or significance of the interaction in mediating cell communication between the cell types and potential functional relationships. It is calculated by comparing the expression levels of different cell receptor and ligand genes between the cell types while accounting for all the possible interaction pairs within a signaling network.

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Author's contributions

G.A.A, TDO, LA, YB, and AKS contributed to design and conceptualization; CMM, LA, DA, YA, and NKN contributed to sample collection and processing. VM, RD, and CMM contributed to performing Seq-Well experiments; CMM performed the data analysis and drafted the manuscript. VM, VA, SB, NKN, TDO, LA, and AKS contributed to data analysis and drafting of the manuscript. All authors read and approved the manuscript.

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Availability of the data

All data generated or analyzed during this study are included in this published article and its supplementary information files. The scRNA-Seq data are available at https://cellatlas-cxg.mvls.gla.ac.uk/PBMC.Pediatric.Malaria.Ghana.

Ethics approval and consent to participate

Ethical clearance was obtained from the Noguchi Memorial Institute of Medical Research, University of Ghana **(IRB 0000908),** and the Ghana Health Service **(GHS-ERC 008/02/19).** All participants were provided with written informed consent before inclusion in the study.

Consent for publication

Not applicable.

Competing interests

A.K.S. reports compensation for consulting and/or SAB membership from Honeycomb Biotechnologies, Cellarity, Ochre Bio, FL86, Relation Therapeutics, Senda Biosciences, IntrECate biotherapeutics, and Dahlia Biosciences unrelated to this work.

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Figures

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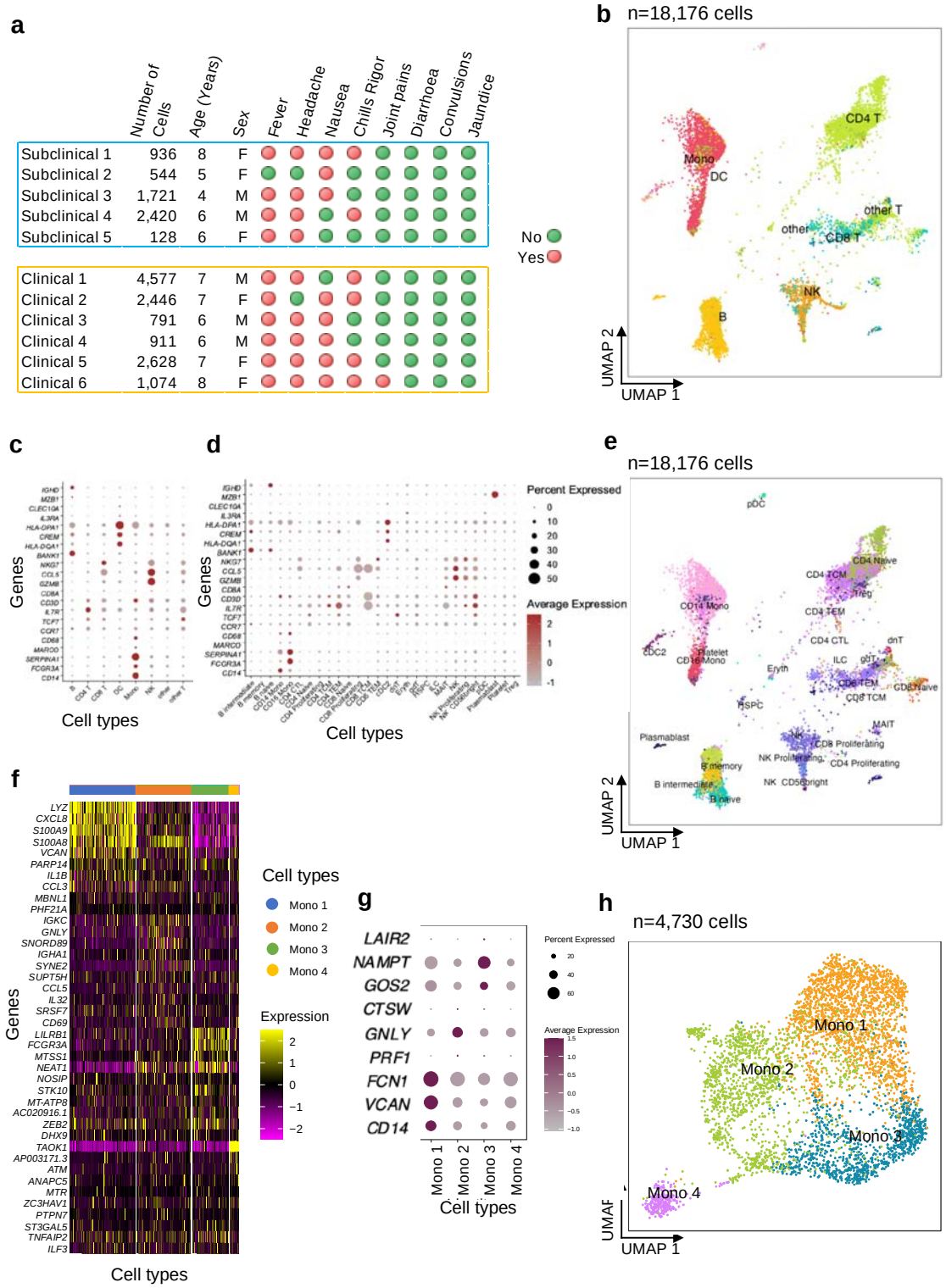

Figure 1 | Analysis of scRNA-Seq data from subclinical and clinical malaria individuals. a) PBMCs collected from eleven individuals and the clinical characteristics of the participants, such as age, sex, and symptoms. The color indicates the yes and no responses of the study participants. **b)** Uniform manifold approximation and projection (UMAP) plot of 22,819 cells from eleven participants colored by identities of 10 cell clusters; mainly B cells, T cells, and Mono. **c)** Expression levels of cluster-defining marker genes

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organized by color intensity to show the average expression of the marker in that particular cell type and the proportion of cells with non-zero expression shown by the size of the dot. **d)** Markers used to annotate the subclusters to various cell subsets showing average expression and fraction of cells expressing the marker. **e)** Reference mapped dataset showing the predicted subclusters of B, CD4 T, CD8 T, NK, Mono, and DC cell subsets. Reference-defined cell subsets were generated from CITE-seq reference of 162,000 PBMCS measured using 228 antibodies (Hao et al., 2021). **f)** UMAP of re-clustered and re-embedded Mono showing four subclusters of the CD14 and CD16 Mono. **g)** Markers used to identify monocyte subclusters. **h)** Mono top 10 highly expressed genes in each subcluster.

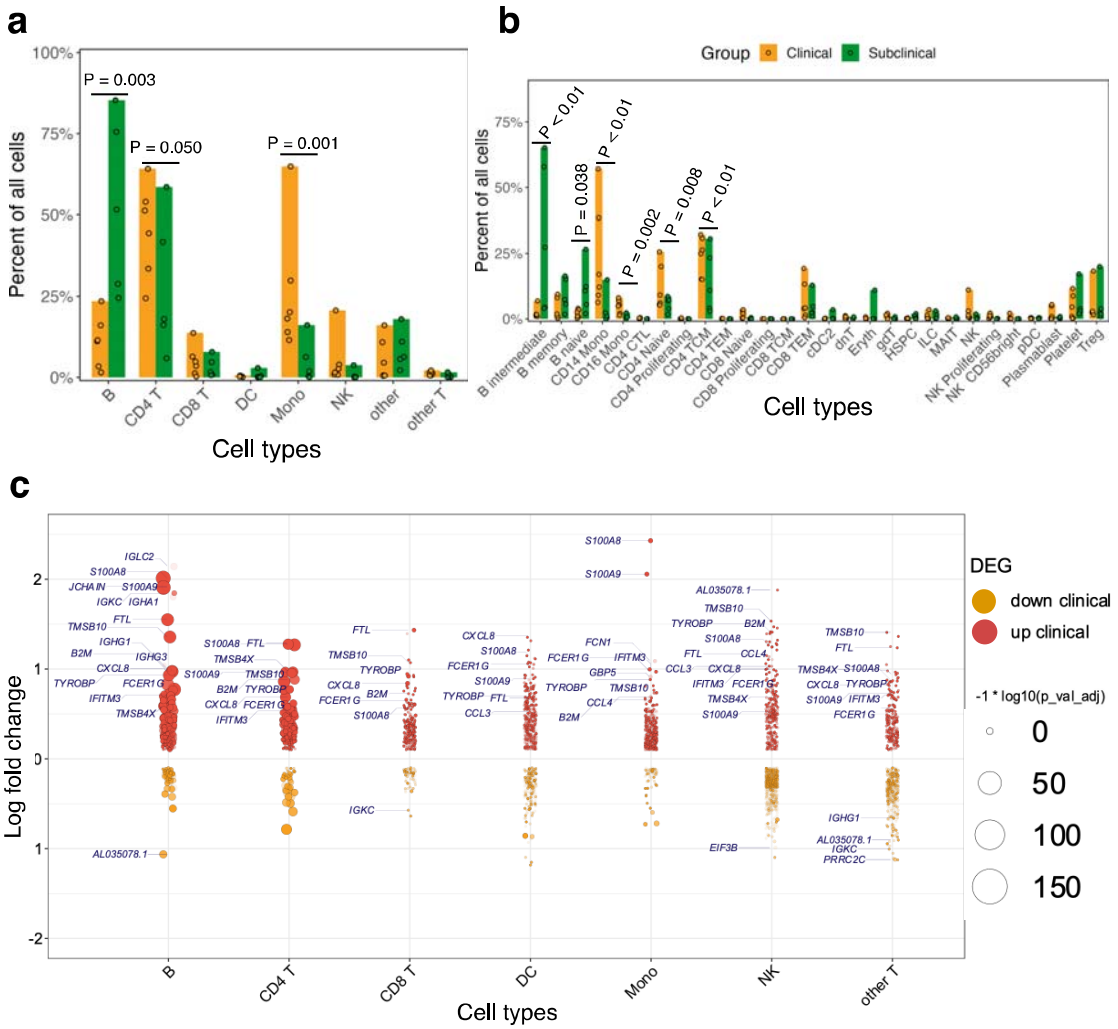

Figure 2 | Profiling of immune cells from clinical and subclinical malaria children. a). Relative cell proportions of the major cell subsets within subclinical and clinical malaria groups. Statistical tests were conducted using the Dirichlet Multinomial Regression in the DirichletReg package in R (Simmons, 2022). The dots represent individual proportions while the color scheme represents the clinical and subclinical groups. **b)** Relative proportions of minor cell subsets compared between subclinical and clinical malaria groups. Cell proportions per group and P-value are shown in **Supplementary Table 1. c)** Violin-like plots showing genes that are differentially expressed between subclinical and clinical infections.

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The x-axis shows the Log_2 fold change against the cell subsets (y-axis) – i.e., B cells, Mono, CD4 T cells, CD8 T cells, other T cells, dendritic cells (DC) and natural killer **(**NK) cells. The color scheme is based on the upregulated (up clinical) and downregulated (down clinical) genes in clinical samples and the size of the point represents the adjusted P value. The frequency shows the number of comparisons in which the gene is significantly expressed in the cell subset.

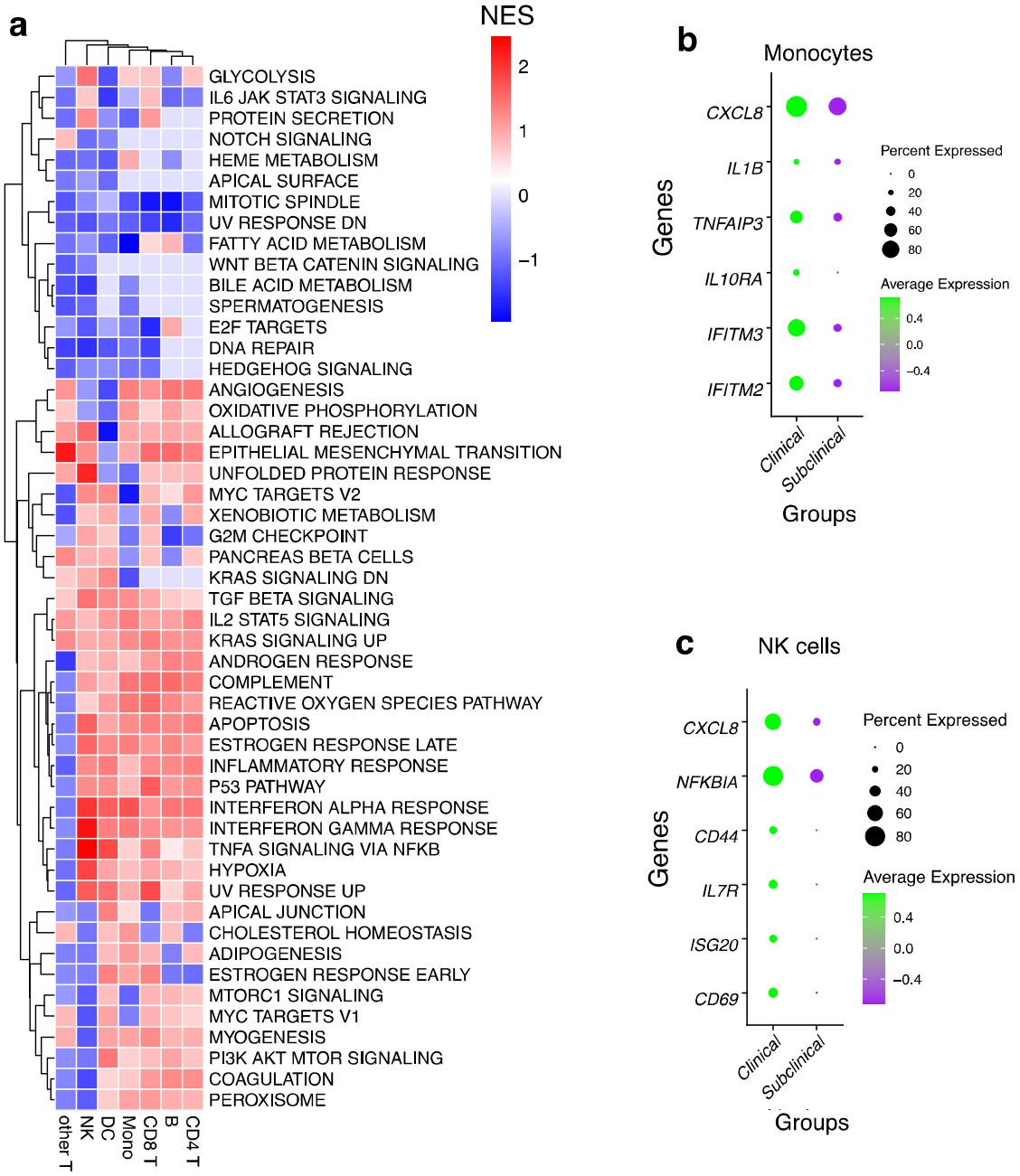

Figure 3 | a) Pathway analysis using an immunologic signature geneset enrichment analysis (GSEA) and the color scheme is based on the normalized enrichment score of genes DEG in clinical malaria. **b)** Dot plots showing some of the leading edge genes in IFN-γ and IFN-α

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response, TNF-α signaling via NFκB & inflammatory response pathways in Mono and, **c**) NK cells. Dot size represents the fraction of cell subsets expressing a given gene. The dot color indicates scaled average expression by gene column.

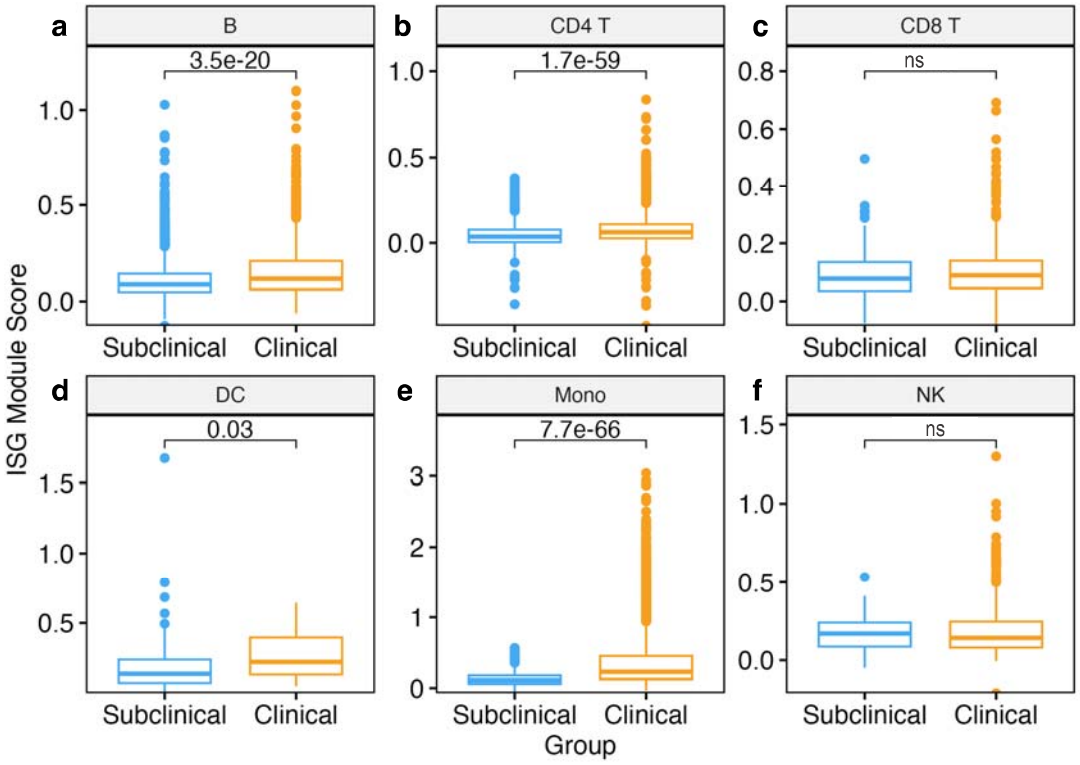

Figure 4 | Module score analysis of innate immune gene modules. a) Boxplot showing interferon-stimulated gene (ISG) module scores per cell subset compared between clinical and subclinical malaria individuals in B cells, **b)** CD4 T cells, **c)** CD8 T cells, **d)** DC e) Mono, **f)** NK cells. Module scores are computed using the AddModuleScore function in the Seurat R package. Statistical significance between the clinical and subclinical of each cell subset was computed using Wilcoxon sign-rank test with Bonferroni correction. Non significant differences are indicated by ns.

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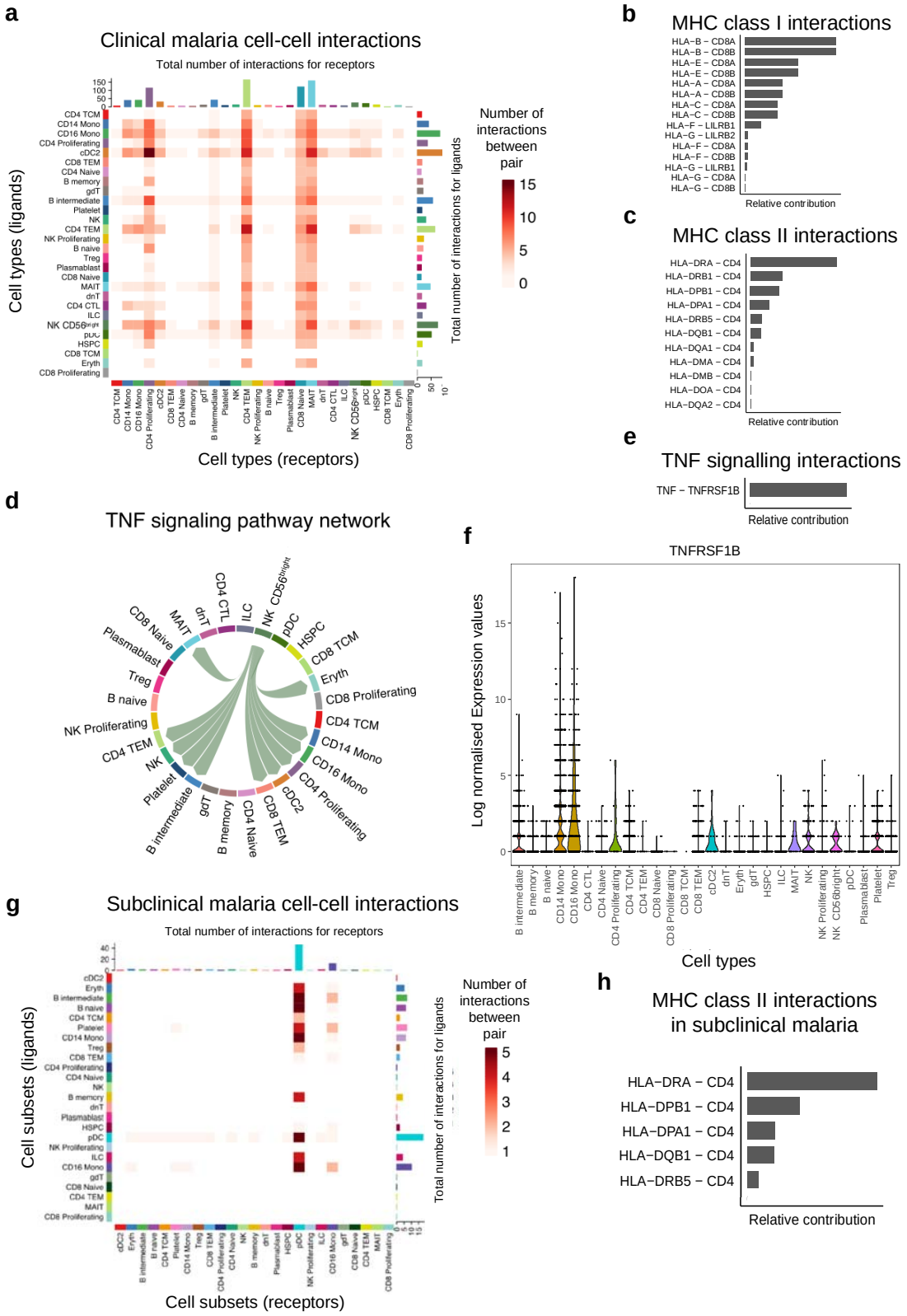

Figure 5 | Primary innate immune cells dominate the cell-to-cell interactions with other cell subsets. a) Heatmap showing the number of interactions between the PBMCs cell subsets. The y-axis shows the signal senders and the x-axis shows the signal receivers. **b)**

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Relative contribution of ligand receptor pairs during clinical malaria infections in the MHC class I signaling pathway and **(c)** MHC class II signaling networks, respectively. A higher relative contribution indicates the magnitidue of contribution of the ligand-receptor and its significant role in the MHC I or II signalling networks. **d)** Cell communications through the TNF signaling pathway and the arrows indicate signal sender to receiver. **e)** Relative contribution of the TNF-TNFRSF1B ligand-receptor pair towards the TNF signaling pathway. **f)** Violin plots showing the expression levels of the TNFRSF1B in the Seurat object for the cell subclusters. **g)** Heatmap comparison showing the overall signaling between all cell subclusters and the number of interactions. **h)** Relative contribution of MHC class II signaling pathway during subclinical infections.

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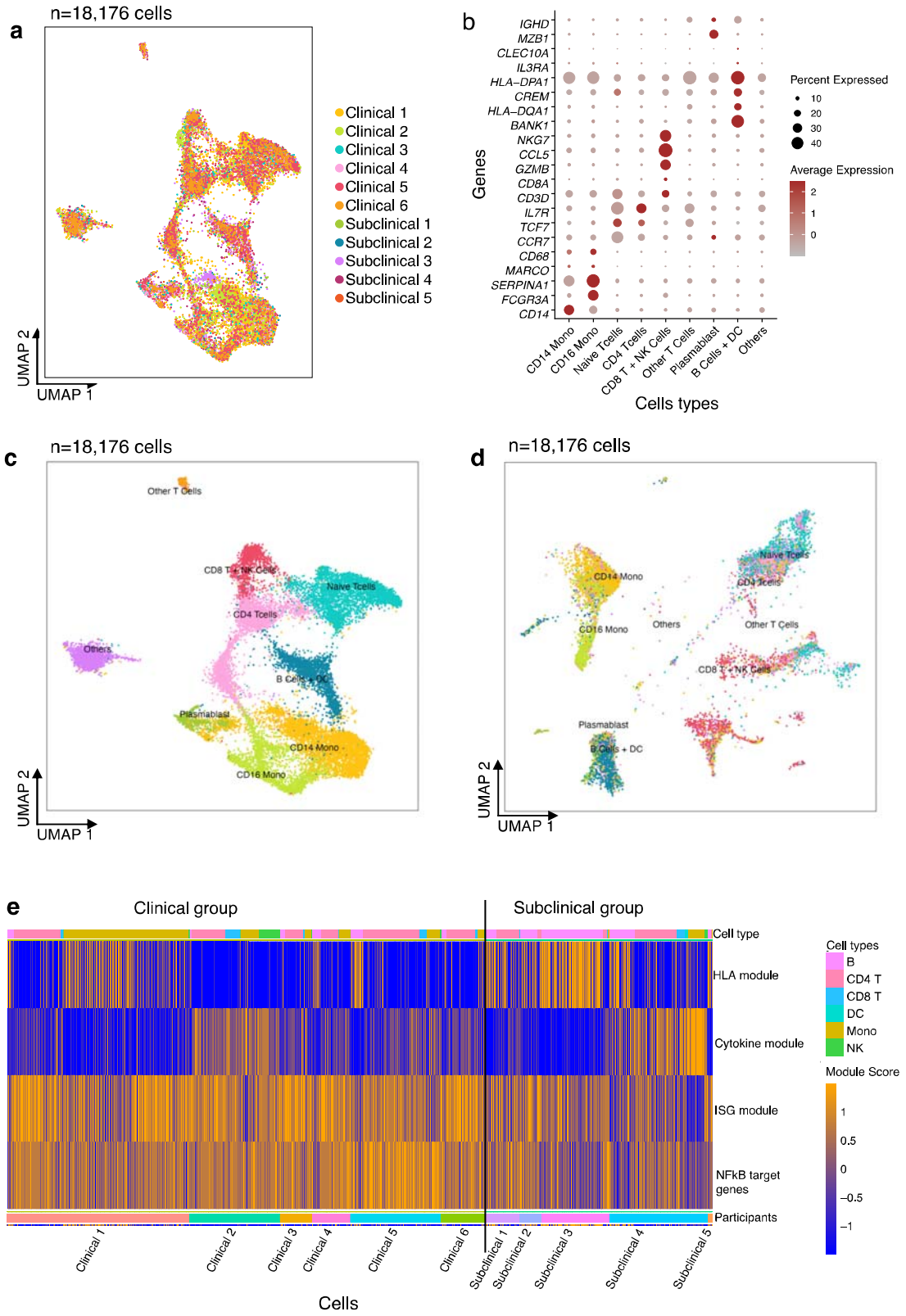

Supplementary Figure 1 | Cluster identification and annotation. a) UMAP of 22,819 cells from all participants, showing 10 clusters in the dataset (following iterative Louvian clustering). **b)** Dot plots showing genes used to manually annotate the clusters and show the

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fraction of cells expressing it and the non-zero expression. Dot size represents the fraction of cell types (rows) expressing a given gene (columns). The dot color indicates scaled average expression by gene column. **c**) UMAP colored by various manually annotated clusters based on the cell markers **d)** UMAP showing cell clusters identified from a reference-mapped dataset but labeled with the manually annotated cluster identities. **e)** Heatmap showing overall module score for each cell, and grouped based on each participant and all the cell types, and overall study groups. The color scheme represents a scale for module scores.

Supplementary Tables

Supplementary Table 1: Descriptive statistics of the study participants

Supplementay Table2: Dirichlet Regression analysis of proportions of various cell types compared between the two groups

Supplementary Table 3: Differentially expressed genes between clinical and subclinical individuals

Supplementary Table 4: Pathway Analysis of differentially expressed genes between clinical and subclinical groups

Supplementary Table 5: Leading Pathways in cell-cell interaction analysis for clinical malaria group

Supplementary Table 6: Leading Pathways in cell-cell interaction analysis for subclinical malaria group