Adaptive T cells regulate disease tolerance in human malaria

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impact statement | malaria attenuates T cell activation to increase host fitness

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Abstract

Immunity to severe malaria is acquired quickly, operates independently of pathogen load and represents a highly effective form of disease tolerance. The mechanism that underpins tolerance in human malaria remains unknown. We developed a re-challenge model of falciparum malaria in which healthy naive adult volunteers were infected three times over a 12-month period to track the development of disease tolerance in real-time. We found that parasitaemia triggered a hardwired emergency myeloid response that led to systemic inflammation, pyrexia and hallmark symptoms of clinical malaria across the first three infections of life. In contrast, CD4+ T cell activation was quickly modified to reduce the number and diversity of effector cells upon re-challenge. Crucially, this did not silence critical helper T cell functions but instead prevented the generation of cytotoxic effectors associated with autoinflammatory disease. Tolerised hosts were thus able to prevent collateral tissue damage and injury. Host control of T cell activation can therefore be established after a single infection and in the absence of anti-parasite immunity. And furthermore, this rapid host adaptation can protect vital organs to minimise the harm caused by systemic inflammation and sequestration.
Introduction

The epidemiology of human malaria clearly shows that immunity develops in two distinct phases. First, individuals acquire protection against severe life-threatening disease and in areas of high transmission this occurs very quickly (often before 12-months of age) (1-4). Then after many years of exposure protection against clinical malaria is established, which promotes the transition to asymptomatic infection (usually in adolescence) (5). This temporal dissociation between clinical immunity and immunity to severe malaria has led to the assumption that they must be underpinned by different mechanisms of host defense. In agreement, clinical immunity coincides with control of parasite burden (and is therefore supported by mechanisms of host resistance) whereas immunity to severe malaria is acquired independently of pathogen load and is a form of disease tolerance (1).

One leading hypothesis suggests that broadly neutralising antibodies that recognise variant surface antigens associated with severe malaria (such as group A/DC8 PfEMP1) could prevent severe disease without affecting total pathogen load (2). In this scenario, immunity to severe malaria would depend upon the rapid production of antibodies that can specifically eliminate pathogenic variants. At present, there is limited evidence that such broad cross-reactivity can be achieved or that neutralising antibodies can be produced within the first year of life to inhibit cytoadherence and reduce sequestration in vivo (6). The mechanism that underpins disease tolerance in human malaria therefore remains unclear.

An alternative explanation is that the host response to infection is quickly modified to minimise the harm caused by malaria parasites. It is well known that metabolic adaptations are induced during the blood cycle to increase host fitness (7-9) and control of inflammation might provide an additional path towards disease tolerance (10). In support of this argument, inflammation can be uncoupled from parasite density in children (11) and pathogenic immune responses can be silenced to minimise tissue stress and toxicity in mice (12). Importantly, host control of inflammation as a defense strategy would not rule out a role for variant surface antigens in severe disease. After all, systemic inflammation, tissue damage and hypoxia could create the right conditions for endothelium activation and the selection of pathogenic variants (13). As such, reducing inflammation may minimise the preferential expansion of parasites associated with severe malaria - this would represent a highly effective route to disease tolerance that would not be influenced by parasite strain or genotype.

Host adaptations that are quickly established to reduce disease severity will be hard to identify in an endemic setting due to the difficulty of pinpointing an infant’s first infection of life. On the other hand, controlled human malaria infection (CHMI) (14) offers an unparalleled opportunity to investigate mechanisms of disease tolerance. Naive volunteers are inoculated with live and non-attenuated parasites and their response can be tracked throughout infection and convalescence by repeated sampling; pre-infection samples can be collected to provide all-important baseline measurements; and the immune system is exposed to an enormous antigen load at the peak of infection (more than $10^7$ parasites per litre of blood) (13). Crucially, adults are more susceptible to severe malaria than children during a first-in-life infection (15, 16) and so we can study the most at-risk group of individuals. We therefore developed a human re-challenge model of falciparum malaria to track the development of disease tolerance in real-time across the first three infections of life.
Results

Malaria does not induce fast-acting mechanisms of host resistance

We developed a human re-challenge model of falciparum malaria in which ten healthy adult volunteers were infected two times (4-months apart) by intravenous injection of infected red cells; six volunteers returned for a third infection 8-months later (figure 1A). A blood challenge model was chosen because it standardises the infectious dose, prolongs the period of blood-stage infection (cf. mosquito challenge) and removes liver-stage immunity as a possible confounding factor upon re-challenge (17, 18). Furthermore, volunteers were always inoculated with the same clonal malaria parasite (3D7) to remove parasite genotype as an infection-dependent variable. Importantly, we used a recently mosquito-transmitted line (< 3 blood cycles from liver egress) (19) since mosquitoes have been shown to reset parasite virulence (20). Volunteers attended clinic the day before infection (baseline), every 12-hours from the day after infection until diagnosis (peak of infection) and then 24- and 48-hours after drug treatment. These frequent visits allowed for regular blood sampling to construct a detailed longitudinal time-course of each volunteer’s response to the first three infections of life.

We found that pathogen load and the parasite multiplication rate were comparable between the first, second and third malaria episode (figure 1B-C and supplementary file 1). Whilst epidemiology has shown that anti-parasite immunity does not develop within this time-frame (1) these data nevertheless derive from an endemic setting where each infection is caused by a new parasite genotype. It is remarkable then that in our study (which removes polymorphism as an obstacle to immunity) we find no evidence that volunteers can limit the replication of parasites they have seen previously. Epidemiology has also shown that immunity to clinical malaria is slow to develop, and in most cases is not acquired until adolescence (5). In agreement, we found no significant difference in the number or severity of adverse events (such as headache, fever and fatigue) between first, second and third infection (figure 1D-E). Differential blood counts revealed other hallmark symptoms of clinical malaria, such as lymphopenia and anaemia, were also comparable between infections (figure 1F and figure 1 - supplement 1). These data thus show that healthy adult volunteers do not acquire mechanisms of resistance (to reduce parasite burden) and remain susceptible to clinical malaria in a homologous re-challenge model.

Re-challenge triggers a hardwired emergency myeloid response

The absence of anti-parasite immunity means that any change in the host response to re-challenge must occur independently of pathogen load. Our model therefore provides the ideal setting in which to investigate host adaptations that confer disease tolerance. One route to tolerance may be to reduce systemic inflammation - this correlates with clinical immunity in endemic regions (11) but whether it also coincides with immunity to severe disease is not known. In human challenge studies the immune response detected in whole blood at the peak of infection is largely driven by activated monocytes and neutrophils, which are in transit from the bone marrow (site of activation) (21) to the spleen (target organ) (22). To capture this acute phase response we used whole blood RNA-sequencing and DESeq2 to identify differentially expressed genes at diagnosis (relative to baseline) in first, second and third infection. We found a remarkably similar pattern of interferon-stimulated type I inflammation regardless of infection number (figure 2 - supplement 1). Furthermore, functional
gene enrichment analysis showed that the hierarchy of GO terms was near-identical between infections (figure 2A). This emergency myeloid response has been extensively described in naive hosts infected with *P. falciparum* (13, 23, 24) and *P. vivax* (Bach *et al.*, under review, preprint available at doi.org/10.1101/2021.03.22.21252810) but it was surprising to see no obvious change upon re-challenge. Nevertheless, by analysing each infection independently it was possible that we were missing important quantitative differences and we therefore performed direct pairwise comparisons between first, second and third infection. Initially, we compared each pre-infection time-point to identify season-dependent shifts in baseline gene expression - we found zero differentially expressed genes between infections (adj p < 0.05 and absolute fold change > 1.5). When we then compared each diagnosis time-point to identify adaptations in the host response we again found zero differentially expressed genes (figure 2B); evidently, the first three infections of life trigger a hardwired emergency response that is not influenced by season or previous exposure.

Nonetheless, host control of inflammation may not be transcriptionally regulated and we therefore directly measured systemic inflammation at protein level using a highly multiplexed custom bead array (39 plasma analytes indicative of inflammation, coagulation, oxidative stress & metabolism). By analysing the concentration of each analyte through time we found many of the prototypical products of monocyte and neutrophil activation (such as CXCL10, IL-1RA & MPO) were significantly increased at diagnosis, together with hallmark cytokines associated with innate lymphoid cell (ILC) or T cell activation (IFNγ and IL-21) (figure 2C and figure 2 - supplement 2). Surprisingly, our data seemed to suggest that this response was not attenuated but enhanced upon re-challenge. To directly test this hypothesis we fitted a linear mixed-effects model for each analyte, which showed that the major secreted products of interferon-stimulated inflammation were significantly increased in second and third (compared to first) infection (figure 2D). Collectively, these data demonstrate that *P. falciparum* triggers a hardwired emergency myeloid response throughout the first three infections of life. And crucially, we find no evidence that systemic inflammation can be attenuated to quickly establish disease tolerance.

**Malaria uncouples T cell activation from systemic inflammation**

Our data do not exclude the importance of minimising systemic inflammation to improve clinical outcome but indicate that long-term exposure to parasites is required to restrict an inflammatory myeloid response (12). These data may partially explain why immunity to clinical malaria is not usually established until adolescence. So what other host adaptations could be acquired early in life to promote immunity to severe disease? To answer this question we examined adaptive T cells, which are inherently plastic, proliferative and long-lived, and therefore uniquely placed to quickly and permanently alter the host response to infection. The acute phase response to malaria causes extreme lymphopenia leading to a 30 - 70% loss of circulating T cells at the peak of infection (figure 1 - supplement 1). The majority of these cells are recruited to the inflamed spleen (25) and it is therefore difficult to assess T cell activation and differentiation at diagnosis. Instead, we need to analyse T cell activation after drug treatment when the myeloid response begins to resolve and T cells return to the circulation. At this time-point, analysing T cell phenotypes in whole blood can provide a direct readout of tissue-specific immune responses in human malaria.
Post-treatment blood samples were not available from this cohort of volunteers in their first infection but were collected 6-days after drug treatment of either their second or third malaria episode. We therefore had to recruit new malaria-naive controls to provide time-matched post-treatment samples of first infection and switched to a cross-sectional study design with 11 volunteers infected contemporaneously (3 first infection, 2 second infection and 6 third infection during the VAC063C trial) (see methods and supplementary file 1). Six days after drug treatment (designated T6) lymphopenia had completely resolved and there was no evidence of delayed onset anaemia (figure 3A and figure 3 - supplement 1A). All other clinical symptoms of malaria (inc. fever) also resolved and markers of systemic inflammation had returned almost entirely to baseline - this was observed in every volunteer regardless of infection number (figure 3 - supplement 1B). It was therefore a surprise to find a large transcriptional signature in whole blood at T6, which was equal in size to the emergency myeloid response captured at diagnosis (figure 3 - supplement 2A). These signatures did not overlap and instead the differentially expressed genes at T6 had unique functional enrichment terms relating to cell cycle and nuclear division (figure 3 - supplement 2B-C). Remarkably, this proliferative burst was only observed in volunteers undergoing their first infection of life (figure 3 - supplement 3).

Myeloid cells are generally terminally differentiated and do not proliferate after their release from the bone marrow; it therefore seemed likely that our whole blood RNA-sequencing data were capturing the return of activated T cells to the circulation. To explore this further we sorted CD4+ T cells with a naive (CCR7\(^{\text{pos}}\) CD45RA\(^{\text{pos}}\), effector / effector memory (EM) (CCR7\(^{\text{neg}}\) CD45RA\(^{\text{neg}}\)) or regulatory (CD25\(^{\text{hi}}\) CD127\(^{\text{neg}}\)) phenotype one day before challenge and six days post-treatment. Analysis of the cell surface markers used for sorting revealed dramatic activation of effector / EM and regulatory subsets in first infection but not reinfection (figure 3B and figure 3 - supplement 4). These data suggested that T cell activation and systemic inflammation could be uncoupled in malaria. To directly test this hypothesis we constructed a pearson correlation matrix and included all plasma analytes that were significantly up- or downregulated at diagnosis or T6 together with the frequency of activated effector and regulatory CD4+ T cells (figure 3C). Also included were hallmark symptoms of clinical malaria such as pyrexia, lymphopenia and anaemia. We found a strong and significant positive correlation between markers of myeloid cell activation (inc. CXCL10, IL-1RA & TNFRII), coagulation (D-Dimer) and maximum core temperature (r > 0.7 and p < 0.05). Moreover, each of these features negatively correlated with lymphocyte counts and haemoglobin. In contrast, activated effector and regulatory CD4+ T cells did not correlate with any feature of the acute phase response (supplementary file 2). The intensity of inflammation therefore scales with clinical symptoms but does not influence CD4+ T cell activation.

**T\(_H\)1 polarisation is a unique feature of first infection**

Naive adults are highly susceptible to severe malaria (15) and our data clearly show that the unique feature of their response to infection is fulminant T cell activation. To explore the transcriptional landscape of activated CD4+ T cells in first infection (compared to third) we undertook RNA-sequencing on the naive, effector / EM and regulatory subsets that we had sorted during the VAC063C trial (figure 3 - supplement 4) and used DESeq2 to identify differentially expressed genes at T6 (versus baseline). As expected, cells with a naive phenotype displayed few transcriptional changes upon their return to circulation; in contrast, we found almost 6000 differentially expressed genes in effector / EM CD4+ T cells (adj p < 0.05 and > 1.5-fold change). Functional gene enrichment
analysis showed that effector cells were proliferative, metabolically reprogrammed and highly activated in first infection, upregulating each of the major costimulatory and inhibitory receptors required to control their fate (figure 4A and figure 4 - supplement 1). Moreover, they specifically upregulated the signature chemokine receptors and transcription factors associated with T\(_{H1}\) polarisation (figure 4B). The cytokines IFN\(_{\gamma}\) and IL-21 were also both strongly induced, which could indicate that infection stimulates double-producers (26) or that activated follicular helper T cells are also released from the spleen (figure 4C).

In third infection IFN\(_{\gamma}\) and IL-21 were once again significantly upregulated at T6 together with TNF and T\(_{H1}\)-associated chemokine receptors (figure 4B-C). CD4\(^+\) T cells are thus clearly responsive to re-challenge despite the lack of evidence for their activation by flow cytometry (figure 3B). Yet remarkably, the transcription factors that drive T\(_{H1}\) differentiation (T-bet and STAT1) were no longer induced, and neither were the costimulatory molecules or inhibitory receptors observed in first infection (figure 4A-B). So what modifies CD4\(^+\) T cell activation to avert T\(_{H1}\) polarisation upon re-challenge? We found no transcriptional evidence that T cells are quiescent or anergic in third infection and crucially the transcription factors that epigenetically enforce exhaustion were not upregulated (figure 4A and figure 4 - supplement 2A-B). Furthermore, there was no evidence for activation induced cell death - this feature was unique to first infection (figure 4 - supplement 2C). There was also no evidence that effector CD4\(^+\) T cells were diverted towards a regulatory fate; for example, hallmarks of T\(_{H1}\) differentiation (such as Eomes and IL-10) were absent (figure 4B-C).

An alternative explanation could be suppression by conventional regulatory CD4\(^+\) T cells yet our data show that Tregs simply mirror the response of effector cells. Their activation is tightly co-regulated (figure 3C) and in first infection Tregs launch a transcriptional programme with remarkable overlap to that of effectors (e.g. they upregulate all signature T\(_{H1}\) genes including T-bet) (figure 4D). In contrast, we could find no transcriptional evidence of Treg activation upon re-challenge. It is well established that regulatory T cells mimic the transcriptional response of the effector cells they are required to regulate (27) and this phenomenon is clearly evident in naive hosts. In fact, by constructing a merged gene ontology network that uses all of the differentially expressed genes identified in effector/EM and regulatory CD4\(^+\) T cells we could show that Tregs do not display any unique functional terms (figure 4E). We therefore conclude that regulatory T cells are redundant during reinfection and that their activation is not required because there is no explosive effector response to keep in check. Instead, it would seem that tolerised hosts launch a specialised adaptive T cell programme that can maintain cytokine production without causing extensive activation, proliferation or T\(_{H1}\) polarisation. Remarkably, we can identify this major shift in the host response in whole blood (figure 4F) and it will therefore be possible to identify tolerised hosts in an endemic setting without the need for complex cell isolation protocols.

**Tolerance minimises effector T cell heterogeneity**

One limitation of bulk RNA-sequencing is that it cannot reveal the heterogeneity of an immune response; this is particularly problematic for CD4\(^+\) T cells, which are inherently diverse and plastic. Furthermore, there is very little power to detect transcriptional changes if only a small proportion of cells are activated, as seemed to be the case in third infection. We therefore used mass cytometry to undertake single cell analysis of T cell activation through first and third infection. Importantly, we
designed an antibody panel that would allow us to interrogate every T cell lineage, and examine the key markers of activation, differentiation and fate (supplementary file 3). We first concatenated all data (from every volunteer and time-point) and used Uniform Manifold Approximation and Projection (UMAP) to visualise the phenotypic diversity of T cells across the dataset. We then used FlowSOM clustering to assign each T cell to one of 49 discrete clusters (figure 5 - supplement 1). As expected most of the diversity was observed within the non-naive CD4+ and CD8+ T cell subsets (figure 5A). Tracking the frequencies of each cluster through time then resolved dynamic changes in the T cell compartment (figure 5 - supplement 2 for CD4+ T cells, supplement 3 for CD8+ T cells and supplement 4 for innate-like T cells). Finally, we performed linear regression on cell count data using EdgeR to identify the differentially abundant clusters at each time-point relative to baseline (FDR < 0.05 and absolute fold-change > 2).

In first infection eighteen T cell clusters increased in abundance at T6 and all had an activated phenotype; these were comprised of twelve adaptive and six innate-like clusters. In third infection, twelve clusters increased in abundance and not one was unique to re-challenge (all were significant in first infection). Remarkably, we found that diversity was specifically reduced in the adaptive T cell response with only six adaptive clusters called as significant in third infection. Moreover, of these six clusters all but one was reduced in size compared to first infection (figure 5B-C). This means that activated CD4+ T cells dominate the response of naive hosts to malaria whereas in tolerised hosts the T cell response largely consists of innate-like cells (figure 5D). So how do the phenotypes of activated CD4+ T cells compare between infections? We observed enormous heterogeneity in first infection with the expansion of nine distinct CD4+ T cell clusters. There were common traits (such as expression of the activation marker CD38 and the memory marker CD45RO) and most had an effector (CCR7neg) phenotype but many clusters displayed unusual or unexpected features (figure 5E). As one example, the largest pool of activated CD4+ T cells had a highly differentiated CD27neg CX3CR1pos cytotoxic phenotype, which is usually observed in autoinflammatory conditions (not infectious disease) (28, 29). Notably, the upregulation of perforin and granzyme B could be seen in CD4+ T cells by RNA-sequencing (figure 4C) and there were many other areas of concordance between the datasets. Most importantly, our protein data confirmed that effector CD4+ T cells were highly activated (CD28hi PD1hi), Tn1 polarised (T-bethi), proliferative (Ki67hi) and accompanied by the activation of Foxp3hi CD39hi T-bet+ regulatory T cells.

In contrast, the unusual CD4+ T cell phenotypes observed in naive hosts were essentially absent in third infection and there was no expansion of activated Tregs (figure 5C). Instead, the majority of expanded CD4+ T cells belonged to a single cluster with an apparently benign effector phenotype (ICOSneg Ki67neg T-bet0 Eomesneg) (figure 5E). These cells are clearly activated (CD38hi Bcl2hi) and had already increased in abundance at diagnosis (figure 5 - supplement 2). A faster response may indicate that these are malaria-specific memory cells. Furthermore, as the dominant activated cluster these CD4+ T cells are likely to be responsible for most of the transcriptional changes in the effector /EM subset in third infection, including cytokine production (figure 5D and figure 4C). To validate these phenotypic observations we used limma to model differential marker expression through time (figure 5 - supplement 5). This analysis confirmed widespread upregulation of activation and differentiation markers in effector / effector memory and regulatory T cells in first infection but an attenuated response to re-challenge (figure 5 - supplement 5 and 6).
Collectively, these data show that tolerance leads to a smaller and less diverse adaptive T cell response. Although at first glance this may seem counterintuitive we argue that this is a more proportional host response designed to support the development of humoral immunity (long-term goal) whilst providing short-term protection against collateral damage. After all, it is essential to prioritise host fitness when infected with a pathogen that can not be cleared and the functionally diverse CD4+ T cell response triggered in naive hosts does not appear to be beneficial (there is no evidence for reduced pathogen load or improved clinical outcome during a first-in-life infection in this study or endemic settings (30)). Moreover, the frequency of CD38hi CD4+ T cells in third infection is not small (it is comparable to typhoid fever (31)) and effector cells have retained critical helper functions (IFNγ and IL-21). It therefore appears that the adaptive response of a naive host is atypical leading to the activation of 20 - 40% of every T cell in circulation (figure 5F) and the generation of cytotoxic effectors previously associated with immune-mediated disease.

Controlling T cell activation protects host tissues

To directly test whether T cell activation in first infection could be pathological we measured biomarkers of collateral tissue damage, a common histological feature of severe malaria. The blood-stage of infection frequently causes liver injury in naive hosts (32-34) and auto-aggressive human T cells can directly kill hepatocytes through TCR-independent mechanisms (35) - we therefore measured alanine aminotransferase (ALT) to provide a readout of hepatocellular death in our cohort. In first infection, two out of three volunteers had abnormal ALT (more than the upper limit of the reference range) when activated T cells were released from inflamed tissues at T6 (figure 6A). This was accompanied by increased gamma-glutamyl transferase (GGT) and aspartate aminotransferase (AST) leading to moderate or severe adverse events in both volunteers. In contrast, there was little evidence of any deviation from baseline in liver function tests after re-challenge. A meaningful statistical comparison between naive and tolerised hosts was not possible due to the small sample size in first infection (n = 3) and we therefore performed a meta-analysis using a previously published surrogate dataset (33). Specifically, we examined post-treatment ALT measurements in almost 100 volunteers experiencing a first-in-life infection as part of a human challenge study. Importantly, we only included CHMI trials that were directly comparable to our own re-challenge study - that is to say that the same clonal parasite genotype was used (3D7 or the parental NF54 line); parasites had recently been transmitted through the vector; and the same study end-points were applied (treatment at around 10,000 parasites ml⁻¹) (figure 6B). Remarkably, we found that the prevalence of abnormal ALT was reduced from 75% during first infection to 25% upon re-challenge (figure 6C). And in those rare cases where ALT was increased in second or third infection adverse events were mild (not moderate or severe) even though pathogen load was increased (figure 6B). These data thus show that the risk of tissue damage and injury can be significantly reduced in the absence of parasite control. Here then is in vivo evidence that long-lived mechanisms of disease tolerance operate in human malaria and can be acquired after a single infection. Moreover, protection does not require the attenuation of systemic inflammation but instead coincides with host control of T cell activation.
Discussion

It has long been recognised that immunity to severe malaria is acquired early in life, offers protection against all manifestations of severe disease and usually precedes clinical immunity (the transition to asymptomatic infection) by more than a decade (2-5). We also know that immunity to severe malaria does not require improved parasite control (1) and is thus underpinned by acquired mechanisms of disease tolerance. This is an important distinction to make if we want to understand how to reduce malaria mortality. Host control of inflammation could provide a rapid route to disease tolerance but our data show that malaria parasites trigger a hardwired emergency myeloid response across the first three infections of life. In contrast, CD4+ T cell activation is quickly modified to limit the number and diversity of effector cells. Our pilot data from a re-challenge model using P. vivax shows the same attenuation of CD4+ T cells after a single infection (figure 6 - supplement 1). Exposure-dependent control of T cell activation therefore occurs independently of parasite species, pathogen load and systemic inflammation. Importantly, tolerance does not silence the adaptive response to re-challenge (cytokine-producing T-bet+ EM CD4+ T cells are re-activated) but avoids cell fates associated with collateral damage and autoinflammatory disease. Whilst we can’t directly show that this reduces the risk of severe malaria in a human challenge model the epidemiology of P. falciparum clearly shows that disease tolerance can be established within the first three infections of life (1, 36).

So how is the adaptive T cell response to malaria modified so quickly and so dramatically? One possibility is that infection initiates heritable epigenetic programmes that reduce T cell responsiveness. Our data do not provide transcriptional evidence of anergy or exhaustion but this hypothesis needs to be directly tested. An alternative explanation could be the clonal deletion of activated T cells during first infection reducing the number of malaria-specific clones available for re-challenge. Or perhaps, tolerance requires no T cell-intrinsic modifications but an attenuation of the antigen presenting capacity of the spleen. After all, hemozoin-loaded dendritic cells and macrophages have a reduced capacity to stimulate T cell proliferation in vitro (37). Long-term modifications to the tissue environment (12) (including the presence of malaria-specific antibodies) may also impact T cell differentiation and block the development of terminally differentiated pathogenic effectors. Nonetheless, the activation of T-bet+ EM CD4+ T cells (and production of IFNγ and IL-21) may be sufficient to support the gradual acquisition of humoral immunity in tolerised hosts. Perhaps then we are asking the wrong question and should instead examine how malaria can drive such extensive and diverse T cell activation in a naive host.

One possibility is that infection leads to widespread bystander activation of CD4+ T cells that do not recognise malaria antigens. These could be pre-existing memory cells, which can be activated in the absence of TCR signals and costimulation (38, 39). This would certainly explain the speed, magnitude and heterogeneity of T cell activation in our naive volunteers. And furthermore, it may explain the increased susceptibility of adults to severe disease during a first-in-life infection (they have a far larger memory pool than infants and children). Malaria may also cause activation of autoreactive T cells, which would need to be quickly suppressed by activated Tregs in first infection. Central tolerance (which deletes autoreactive clones in the thymus) is only partially effective and there is enormous degeneracy in TCR reactivity (40). Systemic infection with a pathogen carrying more than 5000 protein-coding genes may therefore lead to considerable cross-reactivity between parasite and host. Indeed, hypergammaglobulinemia and autoantibody production are known
features of human malaria (41, 42). We therefore propose that bystander and cross-reactive T cells may account for the majority of T cell activation in naive hosts. And because they can be activated via TCR-independent or low affinity interactions (39, 43) it might be possible to specifically silence their pathogenic response without impeding re-activation of high affinity malaria-specific clones. Support for this idea comes from the observation that P. chabaudi triggers massive polyclonal activation of CD4+ T cells in first infection but not reinfection in mice (44).

Importantly, we have shown that parasite species regulates T cell activation in naive hosts - P. falciparum drives a bigger CD4+ T cell response with more functional diversity than P. vivax even though they trigger a near-identical myeloid response (Bach et al., under review, preprint available at doi.org/10.1101/2021.03.22.21252810). A combination of increased sequestration and T cell activation could therefore underpin the increased virulence of P. falciparum. This could explain why large multi-centre genome-wide association studies have failed to identify SNPs in immune genes that can predict susceptibility to severe malaria (45). Variation in human T cells is almost exclusively driven by non-heritable factors (46). And it may also explain how immunity to severe malaria can be acquired so quickly. Exposure-dependent control of T cell activation could reduce tissue damage, endothelium activation and toxicity to minimise the harm caused by cytoadherence. Crucially, our data show that a single asymptomatic drug-cured infection is sufficient to silence pathogenic T cells. Interventions that reduce the risk of clinical malaria but don’t completely eliminate infection (such as mass drug administration) may thus induce long-lived mechanisms of disease tolerance in endemic settings.
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Data availability

All RNAseq data (whole blood and sorted T cell subsets) have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO SuperSeries accession number GSE172481. CyTOF (mass cytometry) data have been deposited at flowrepository.org and can be accessed through experiment numbers FR-FCM-Z47Z (VAC063C); FR-FCM-Z3HA (VAC069A); and FR-FCM-Z465 (VAC069B).

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A. Homologous rechallenge with *P. falciparum*.

- **First infection**: 10 volunteers, 4 months.
- **Second infection**: 10 volunteers, 8 months.
- **Third infection**: 6 volunteers.

B. Graph showing the max. parasite density (ml⁻¹) over days post-challenge.

C. Bar graph showing max. parasite density (ml⁻¹) for first, second, and third infections.

D. Graph showing the number of adverse events over days post-challenge.

E. Pie charts showing the severity of adverse events for first, second, and third infections.

F. Bar charts showing the percentage of adverse events for each infection type.

- **Lymphopenia**
- **Pyrexia**
- **Anaemia**

Usage note: This image contains data and diagrams related to a study on *P. falciparum* infections and adverse events in volunteers. The data includes parasite density and types of adverse events such as lymphopenia, pyrexia, and anaemia. The study involves homologous rechallenge and examines the responses over different time periods.
Figure 1 | Malaria does not induce fast-acting mechanisms of host resistance. (A) Healthy malaria-naive adults were infected up to three times with *P. falciparum* (clone 3D7) by direct blood challenge. Repeated sampling before, during and after each infection allowed us to track the development of disease tolerance in real-time. (B) Parasite growth curves for first, second and third infection (each line represents a volunteer and lines are colour-coded by infection number). Parasite density in whole blood was measured by qPCR every 12-hours - the grey box represents the lower limit of quantification (20 parasites*ml⁻¹) and the treatment threshold of 10,000 parasites*ml⁻¹ is denoted by the pink line. (C) Maximum parasite density during first, second and third infection; each dot represents a volunteer and bars show the median. No statistically significant difference between infections was observed (Kruskal-Wallis test p = 0.9054). (D-F) Clinical symptoms of malaria were recorded on diary cards (self-reporting) or during clinic visits; these adverse events were graded as mild, moderate, or severe (see methods). (D) Number of adverse events at each time-point throughout infection and up to 48-hours after treatment (each line represents a volunteer and lines are colour-coded by infection number). (E) Severity of adverse events in the 48-hours before and after diagnosis in first, second and third infection. (F) Frequency and severity of lymphopenia, pyrexia and anaemia during first, second and third infection. No statistically significant difference between infections was observed for any adverse event (Kruskal-Wallis test using a significance threshold of 5%). In (B-F) sample size (n) is 10 (for first and second infection) and 6 (third infection).
A divided into sections for clarity:

1. **Graphic A**
   - Represents a scatter plot or violin plot showing various parameters across different conditions or time points.
   - The x-axis represents time or condition, while the y-axis shows a quantitative measure.
   - Different colors represent different categories or groups.
   - Legends and labels help identify the specific parameters being measured.

2. **Textual Description**
   - Details the conditions, measurements, or groups being compared.
   - Reference specific sections or figures to contextualize the data.

B. **Graphic B**
   - Circular or radial plots displaying data across different conditions or time points.
   - The plots are color-coded to differentiate between conditions.
   - Annotations indicate significant differences or trends.

3. **Textual Description**
   - Provides context or explanation for the circular plots.
   - Explains the significance of the data presented in the circular plots.

C. **Graphic C**
   - Bar charts or similar visualizations showing comparisons across conditions.
   - Axes are labeled to indicate the parameters being measured.
   - Different colors or patterns represent different groups.

4. **Textual Description**
   - Describes the bar charts, highlighting key findings or trends.
   - Compares results across conditions or groups.

D. **Graphic D**
   - Multiple box plots showing distributions of data across conditions.
   - Each box plot represents a different parameter or condition.
   - Highlight bars in a different color code to indicate significance.

5. **Textual Description**
   - Analyzes the box plots, noting outliers, medians, and interquartile ranges.
   - Compares distributions across conditions to identify significant changes.

Overall, the document appears to be an analysis of gene expression or protein levels across different conditions or time points, with detailed visualizations to illustrate the findings.
Figure 2 | Re-challenge triggers a hardwired emergency myeloid response. (A) RNA-sequencing was used to identify differentially expressed genes in whole blood at diagnosis versus baseline (adj p < 0.05 and > 1.5 fold-change). ClueGO was then used for functional gene enrichment analysis and placed significant GO terms into functional groups by relatedness (see methods). Shown are the leading GO terms from 15 non-redundant groups with the lowest adj p value in first infection. We plotted the same GO terms in second and third infection. Note that each infection was analysed independently. (B) Radar plots (or 3-way volcano plots) show the number of differentially expressed genes in whole blood between infections. Direct pairwise comparisons between second and first, third and first and third and second infection were performed - the left plot compares all baseline samples and the right plot the diagnosis time-points. Dashed lines represent the centre-point for each volcano plot and the position of each dot relative to this line shows up- or downregulation. There were no differentially expressed genes in any of the six pairwise comparisons (adj p < 0.05 and > 1.5 fold-change). (C) 39 plasma analytes were quantified before and during each infection using a highly multiplexed bead-based array. The log2 fold-change of each analyte is shown relative to baseline on day 6 and 8 post-challenge (c6 and c8, respectively) and at diagnosis. Analytes are ordered by log2 fold-change in second infection. (D) Mixed-effects modelling was used to identify plasma analytes that vary significantly between infections at diagnosis. Multiple test correction was performed using the Benjamini-Hochberg method and analytes that were significant in both second and third infection (versus first infection) are shown (adj p < 0.05). Box (median and IQR) and whisker (1.5x upper or lower IQR) plots are shown with outliers as dots. In (A-B) n = 10 (first infection), 9 (second infection) and 6 (third infection). v1040 was excluded from RNA-sequencing analysis in second infection because their baseline sample failed QC. In (C-D) n = 9 (first and second infection) and 5 (third infection). v1040 was excluded from plasma analysis because all samples failed QC (see methods).
Figure 3 | Malaria uncouples T cell activation from systemic inflammation. (A) Lymphocytes were quantified in whole blood 6-days post-challenge (c6), at diagnosis, 6-days after treatment (T6) and at two memory time-points (45 and 90-days post-challenge). Counts were normalised to baseline measurements (taken one day before challenge). Each line represents a volunteer and lines are colour-coded by infection number. A statistically significant deviation from baseline was observed only at diagnosis (Wilcoxon matched-pairs signed rank test, *** p < 0.001). (B) The percentage of activated CD38hi CD4+ T cells was analysed by flow cytometry at baseline (grey dots) and T6 (coloured dots). Data are shown for naive, effector/effector memory (EM) and regulatory subsets in volunteers undergoing their first, second or third infection of life (see figure 3 - supplement 4 for gating strategies). (C) Pearson correlation matrix of the log2 transformed fold-change of lymphocytes, haemoglobin and differentially abundant plasma analytes. Fold-change was calculated either at diagnosis or T6 (relative to baseline) according to when this was largest for each feature. Also included is maximum parasite density, maximum core temperature (up to 48-hours post-treatment) and the percentage of activated effector and regulatory CD4+ T cells (at T6). The order of features was determined by hierarchical clustering. In (A-B) n = 11 (3 first infection, 2 second infection and 6 third infection) and in (C) n = 10 (3 first infection, 2 second infection and 5 third infection). v1040 was excluded from plasma analysis because all samples failed QC.
CXCR4
CXCR5
CXCR3
CXCR6
CCR5
RORA
RORC
STAT3
STAT4
STAT6
GATA3
EOMES
BCL6
STAT1
[TBET]

log2FC
2
-2
0

BCL2
GPR174
MR1
LAIR1
[PD1]
LAG3
KLRG1
ICOS
[TCF1]

[HELIOS]

CD38
MKI67
HLA-DR
CTLA4
ICOS
KLRG1
LAG3
[PD1]
LAIR1
MR1
GPR174
BCL2
TOX2
NR4A1
TOX

first
third

exhaustion
activation

effector molecules

log2FC
2
-2
0

BCL2

first infection
third infection

shared GO term
enriched in effector / EM CD4+ T cells
enriched in regulatory T cells

T cell activation
interleukin-1-mediated signaling pathway
regulation of cellular response to stress
cellular response to cytokine stimulus
nuclear division
oxidative phosphorylation
chromatin remodeling
receptor ligand activity

figure four
**Figure 4** | Tₜ₁ polarisation is a unique feature of first infection. (A-C) During the VAC063C study RNA-sequencing was used to identify differentially expressed genes in effector/effector memory (EM) CD4⁺ T cells 6-days after parasite clearance (T6 versus baseline) in first and third infection (adj p < 0.05 and > 1.5 fold-change). Heatmaps show the log2 fold-change of (A) markers of T cell activation and exhaustion and (B) the master transcription factors that shape T cell fate (plus associated chemokine receptors). The stacked circular bar chart in (C) shows the log2 fold-change of cytokines and cytotoxic effectors upregulated in first or third infection. Note that IL10 is not differentially expressed in either infection. (D) RNA-sequencing was used to identify differentially expressed genes in regulatory CD4⁺ T cells at T6 (versus baseline) in first and third infection (adj p < 0.05 and > 1.5 fold-change). Shown are markers of Treg activation and differentiation plus key suppressor molecules. (E) Differentially expressed genes in effector/EM and regulatory CD4⁺ T cells were combined for GO analysis of T6 in first infection. ClueGO was used to construct a merged functional gene ontology network. Each node represents a GO term and nodes are coloured according to whether their associated genes were majoritively (> 60%) derived from effector or regulatory cells. GO terms that were shared between T cell subsets are coloured grey - nine leading GO terms (each from a unique functional group) that were shared are labelled (see methods). (F) RNA-sequencing was used to identify differentially expressed genes in whole blood at diagnosis and T6 (relative to baseline) in first and third infection (adj p < 0.05 and > 1.5 fold-change). Signature genes of T cell activation, Tₜ₁ differentiation and cytokine production are shown. In (A-E) n = 2 or 3 for first infection (T6 and baseline, respectively) and 6 for third infection. v313 was excluded at T6 because this sample failed QC. In (F) n = 3 for first infection and 6 for third infection. Square brackets indicate that common gene names were used.
Figure 5 | Tolerance minimises effector T cell heterogeneity. Whole blood was preserved within 30-minutes of blood draw at baseline, diagnosis, T6 and 45-days post-challenge (memory time-point) during the VAC063C study. Samples were stained with a T cell focussed antibody panel (see supplementary file 3) and acquired on a Helios mass cytometer. After exclusion of normalisation beads and doublets we gated on CD45<sup>pos</sup> CD3<sup>pos</sup> T cells for UMAP projections and FlowSOM clustering. (A) Data from all volunteers and time-points was concatenated to generate a UMAP projection showing the global structure of our dataset. FlowSOM identified 49 discrete T cell clusters and each is given a unique colour. The major T cell subsets are labelled according to expression of lineage, memory and activation markers. (B) UMAP projection showing the T cell clusters that are differentially abundant at T6 in first (left) and third infection (right) (FDR < 0.05 and > 2 fold-change). Clusters that are not significant are shown in black. (C) The mean frequency of each T cell cluster that is differentially abundant at T6 in first and/or third infection is shown as a proportion of all CD45<sup>pos</sup> CD3<sup>pos</sup> T cells. (D) Pies show the relative size of each differentially abundant cluster at T6 in first (top) and third (bottom) infection. (E) Heatmap showing the normalised median expression values of all markers used for clustering in each of the 18 T cell clusters that were differentially abundant at T6. Colour codes to the left of the heatmap indicate cluster identity and show whether clusters were significant in first infection or first and third infection. Note that no cluster was unique to third infection. (F) Stacked bar chart showing the frequency of activated (CD38<sup>hi</sup> Bcl2<sup>lo</sup>) T cell clusters at each time-point. In (A-F) n = 3 for first infection and 6 for third infection.
A

![Graph showing ALT levels over time](image)

B

| data source      | number of volunteers | abnormal ALT | severity | peak parasite density |
|------------------|----------------------|--------------|----------|-----------------------|
| naive            |                      |              |          |                       |
| Reuling et al.   | 95                   | 70           | 40       | 16                    | 14 |
| VAC063C          | 3                    | 2            | 0        | 0                     | 2  |
| Total            | 98                   | 72           | 40       | 16                    | 16 |
| tolerised        |                      |              |          |                       |
| VAC063C          | 8                    | 2            | 2        | 0                     | 0  |

| source | abnormal ALT | severity | peak parasite density |
|--------|--------------|----------|-----------------------|
|        | mild | moderate | severe |                   |
| naive  |      |          |        |               |
|     |      |          |        |               |
| tolerised |      |          |        |               |

a studies: EHMI-3, LSA-3, EHMI-8B, EHMI-9, ZonMw2, TIP5 and CHMI-trans1
b plasma concentration exceeds the upper limit of the reference range
c weighted mean number of parasites per ml whole blood
Figure 6 | Controlling T cell activation protects host tissues. (A) Blood chemistry measured the concentration of alanine aminotransferase (ALT) throughout infection and convalescence in the VAC063C study (baseline, 6-days post challenge (c6), diagnosis, 6 to 18-days after treatment (T6-T18) and 45/90-days post-challenge (memory time-point 1 and 2, respectively)). Each line represents one volunteer and lines are colour-coded by infection number. (B) A surrogate dataset from Reuling et al. (33) was used to extract information on the frequency and severity of abnormal ALT during a first-in-life infection (up to 6-days post-treatment). All volunteers were infected with P. falciparum (3D7 or NF54) as part of a CHMI trial that used equivalent end-points to our own study (see methods). In every case, abnormal ALT was scored using the same adaptation of the WHO adverse event grading system. Data from 95 volunteers in Reuling et al. and the 3 first infection volunteers in VAC063C were pooled for analysis. (C) Frequency and severity of liver injury during first infection (naive hosts) versus re-challenge (tolerised hosts). Barnard's test was used to statistically determine whether an abnormal ALT reading was more prevalent during a first-in-life infection compared to second or third infection (a p value below 0.05 was considered significant). In (A) n = 11 (3 first infection, 2 second infection and 6 third infection) and in (B-C) n = 98 (first infection) and 8 (re-challenge).
Methods

Clinical trial design

All volunteers were healthy, malaria naive adults aged between 18 and 50 years and were enrolled in up to three CHMI studies - VAC063A (November 2017), VAC063B (March 2018) and VAC063C (November 2018). The VAC063 protocol encompassed VAC063A and VAC063B, and was an open label, non-randomised phase I/IIa clinical trial evaluating vaccine efficacy of the recombinant blood-stage malaria protein RH5.1 in AS01B adjuvant (GSK) (47). The VAC063C trial was designed to investigate the durability of any protective anti-parasite immunity measured in control (non-vaccinated) volunteers upon homologous re-challenge (Themistocleous Y et al., in preparation). We collected and analysed whole blood samples from 8 control volunteers in VAC063A (all receiving their first infection), 10 control volunteers in VAC063B (8 receiving their second infection and 2 their first infection) and 11 control volunteers in VAC063C (6 receiving their third infection, 2 receiving their second infection and 3 their first infection) (refer to supplementary file 1). The VAC063 and VAC063C trials were sponsored by the University of Oxford. VAC063 received ethical approval from the UK NHS Research Ethics Service (Oxfordshire Research Ethics Committee A, reference 16/SC/0345) and was registered on ClinicalTrials.gov (reference NCT02927145). VAC063C received ethical approval from the UK NHS Research Ethics Service (South Central - Oxford A, reference 18/SC/0521) and was also registered on ClinicalTrials.gov (reference NCT03906474). All CHMI studies were conducted in the UK at the Centre for Vaccinology and Tropical Medicine (University of Oxford) and were conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH Guidelines for Good Clinical Practice. Volunteers signed written consent forms and consent was checked prior to each CHMI. Details of volunteer recruitment, consent, inclusion/exclusion criteria and group allocation can be found in Minassian et al. (47).

In each CHMI study, all volunteers were infected by direct intravenous inoculation of \textit{P. falciparum} (clone 3D7) blood-stage parasites. The inoculum was thawed and prepared under strict aseptic conditions exactly as described (47, 48) and volunteers received between 452 and 857 infected red cells in a total volume of 5 ml normal saline. Starting one day after infection volunteers attended clinic for assessment and blood sampling every 12-hours, and parasite density was measured in real time by qPCR (target gene = 18S ribosomal RNA). In VAC063A thick blood films were also evaluated at each time-point by experienced microscopists and diagnosis required volunteers to fulfil two out of three criteria: a positive thick blood film (one viable parasite in 200 fields) and/or qPCR data showing at least 500 parasites per ml and/or symptoms consistent with malaria. In VAC063B and C microscopy was removed as a diagnostic tool to reduce the number of volunteers diagnosed prematurely without impacting volunteer safety. The new criteria for diagnosis were: asymptomatic with any available qPCR result above 10,000 parasites per ml or symptomatic with any available qPCR data above 5000 parasites per ml. At diagnosis volunteers were treated with artemether and lumefantrine (Riamet) except in cases where its use was contraindicated and atovaquone and proguanil (Malarone) were given instead. In our analysis, we refer to the blood-draw immediately before drug-treatment as the diagnosis time-point.

Clinical symptoms of malaria (pyrexia, malaise, fatigue, arthralgia, back pain, headache, myalgia, chills, rigor, sweats, headache, nausea, vomiting and diarrhoea) were either recorded by the
volunteers on diary cards or during clinic visits. All symptoms were recorded as adverse events and assigned a severity score: 0 - absent; 1 - transient or mild discomfort (no medical intervention required); 2 - mild to moderate limitation in activity (no or minimal medical intervention required); 3 - severe limitation in activity requiring assistance (may require medical intervention). Pyrexia was scored as follows: absent ($\leq 37.5^\circ$C), mild (37.6 - 38.2$^\circ$C), moderate (38.3 - 38.9$^\circ$C) and severe ($\geq 39^\circ$C). In addition, full blood counts and blood chemistry were evaluated at the Churchill and John Radcliffe Hospital in Oxford providing 5-part differential white cell counts and quantification of electrolytes, urea, creatinine, bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and albumin.

**Meta-analysis of liver injury**

A surrogate dataset from Reuling et al. (33) was used to assess the risk of liver injury during a first-in-life infection compared to re-challenge. We extracted data from every CHMI study that used the 3D7 *P. falciparum* clone (or its parental NF54 line), initiated infection via mosquito bite or direct blood challenge and that had a treatment threshold based on thick smear positivity (estimated to be at least 5,000 parasites per ml). This produced data for 95 volunteers across 7 CHMI studies. Notably, Reuling et al. used longitudinal data to show that liver function test (LFT) abnormalities peaked up to 6-days post-treatment in line with our own T6 time-point. And furthermore, in every CHMI study (including our own) LFT abnormalities were graded using the same adaptation of the WHO adverse event grading system. An LFT reading $> 1.0$ but $< 2.5$ times the upper limit of normal was graded as mild; a reading $> 2.5$ but $< 5.0$ times the upper limit was graded moderate; and a reading $> 5.0$ the upper limit was graded severe. For ALT, the upper limit of normal was 35 or 45 units per litre for female and male volunteers, respectively. Data from the 95 volunteers in Reuling et al. and the 3 primary controls in our VAC063C study were pooled for analysis and we calculated a weighted peak parasite density across the entire cohort by using $a$, the mean number of parasites per ml and $b$, the number of volunteers in each of the 8 CHMI studies. To statistically test whether an abnormal ALT reading was more prevalent in the 98 volunteers experiencing their first malaria infection compared to the 8 volunteers undergoing re-challenge (either second or third infection) we used Barnard’s test, which examines the association of two independent categorical variables in a 2x2 contingency table. A $p$ value below 0.05 was considered significant.

**Processing whole blood for RNA and plasma**

Venous blood was drawn into K$_2$EDTA-coated vacutainers (Becton Dickinson #367835). To preserve RNA for whole blood transcriptome analysis 1 ml of blood was mixed thoroughly with 2 ml Tempus reagent (ThermoFisher #4342792) and samples were stored at -80°C. Note that no more than 2-hours passed between blood draw and RNA preservation. To obtain platelet-depleted plasma 3 ml of blood was divided into two 2 ml Eppendorf tubes and centrifuged at 1,000 xg for 10-minutes at 4°C to pellet cellular components. Working on ice, plasma was then carefully transferred to a new tube and spun again – this time at 2,000 xg for 15-minutes at 4°C to pellet platelets. Cell-free, platelet-depleted plasma was then aliquoted into new tubes, snap frozen on dry ice and stored at -80°C.
**Whole blood RNA-sequencing**

Whole blood RNA was extracted using the Tempus Spin RNA isolation reagent kit (ThermoFisher #4380204) according to the manufacturer’s instructions. To account for the reduced starting volume and to maintain Tempus stabilizing reagent at the correct final concentration we added just 1 ml PBS to each sample after thawing. Diluted samples were then centrifuged at 3000 x g for 30-minutes at 4°C to pellet nucleic acids. Pellets were resuspended in RNA purification resuspension solution and centrifuged on a silica column to remove non-nucleic acid contaminants. After washing, the column was incubated for 2-minutes at 70°C before eluting nucleic acids. 40 µl eluate was then subjected to DNA digestion using the RNA Clean and Concentrator-5 kit (Zymo Research #R1013). Purified RNA was eluted in 30 µl DNase/RNase-free water, quantified using a Qubit Fluorometer (HS RNA assay kit, ThermoFisher #Q32852) and RNA integrity assessed using an Agilent Bioanalyzer 2100 (RNA 6000 nano kit Agilent #5067-1511). The average RNA integrity number (RIN) was 9 (98% of samples > 8, lowest RIN 7). Libraries were prepared by Edinburgh Genomics (Edinburgh, UK) using the TruSeq Stranded mRNA library prep kit (Illumina #20020595). Stranded library preparation allows transcript expression to be estimated more accurately; in particular it is more effective in quantifying anti-sense gene expression, properly assigning transcripts to putative coding genes and to resolve ambiguity in reads from overlapping genes. Libraries were sequenced using the NovaSeq 6,000 Illumina platform yielding 50 bp paired end (PE) reads. These short reads are sufficient to accurately capture gene expression thanks to the well annotated human transcriptome. The average number of reads per sample passing QC across all samples was 8.45 x 10^7.

**Data analysis of whole blood RNA-sequencing**

Quality and content of FASTQ files, which contain raw PE sequencing reads, were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). A single sample (volunteer 1040 baseline second infection) failed quality control and we therefore excluded all RNAseq data from this volunteer’s second infection from our analysis. TruSeq primer sequences were removed using cutadapt v1.9, reads were aligned to the Ensembl release 96 Homo sapiens transcript set with bowtie2 v2.2.7 (49) (parameters: —very-sensitive -p 30 —no-mixed —no-discordant —no-unal) and reads that mapped to globin transcripts were discarded (on average 11.7% of mapped reads, range 1.6 – 38.1%). We opted for bioinformatic globin depletion as it is highly sensitive and reproducible (50) whereas depleting globin during RNA preparation can compromise the amount and quality of RNA recovered (51). Following these steps, the average alignment rate to the human transcriptome across all samples was 88%. A matrix of normalised counts for each transcript was obtained from sorted, indexed bam files using samtools idxstats (http://www.htslib.org/doc/samtools.html). Transcript counts were imported into the R/Bioconductor environment (v3.6) and differential gene expression analyses (pairwise group comparisons) performed using functions within the DESeq2 package (52). lfcShrink was applied to the output of each pairwise comparison (type normal). Lists of differentially expressed transcripts were analysed using R (v3.6) - non-coding transcripts were removed and multiple transcripts annotated to the same gene were consolidated by keeping the transcript with the highest absolute fold-change. Only protein-coding transcripts with an adjusted p value (adj p) < 0.05 and a fold-change > 1.5 were considered differentially expressed. Radar plots and heatmaps were generated using the ggplot2 package (53). The gene lists used for cell cycle analysis were manually compiled from published datasets (54, 55) and we allocated genes to a single phase based on cell cycle transcriptional network analysis (56, 57).
Flow-sorting CD4+ T cells

We flow-sorted CD4+ T cell subsets for ex vivo RNA-sequencing from 3 ml whole blood collected in K2EDTA Vacutainers. After red cell lysis (erythrocyte lysis buffer, ebioscience #00-4300-54) leukocytes were washed in flow buffer (PBS supplemented with 2% fetal bovine serum (FBS Performance Plus, ThermoFisher #16000044, heat inactivated & filtered 0.22 μm) and 5 mM EDTA (Life Technologies #AM9260G)). We then blocked Fc receptors (human TruStain FoC, BioLegend #422302) and stained leukocytes for 20-minutes at 4°C (8-colour panel: CD3 (clone OKT3), CD4 (clone OKT4), CD127 (clone A019D5), CD25 (clone M-A251), CCR7 (clone G043H7), CD45RA (clone HI100), CD38 (clone HIT2) and HLA-DR (clone L243)). After washing leukocytes were kept in cold flow buffer and filtered through a 40 μm cell strainer (Scientific Labs Supply #352340). We used a FACS-Aria Illu or Fusion cell sorter (BD Biosciences, FACS Diva v8 software, 70 μm nozzle, sort setting “purity”) to simultaneously sort 10,000 of each of the following CD4+ T cell subsets - naive (CD3pos CD4pos CD127pos CCR7pos CD45RApos), effector/effector memory (EM) (CD3pos CD4pos CD127pos CCR7neg CD45RAneg) and regulatory (CD3pos CD4pos CD25hi CD127neg). T cells were sorted directly into RNase free sterile 1.5 ml screw cap tubes (ThermoFisher #11529924) containing 1 ml Trizol Reagent (ThermoFisher #15596026). Tubes were incubated for 5-minutes at room temperature and stored at -80°C. At the same time, we sorted naive T cells into flow buffer and reacquired them to check sort purity, which was above 95% for every sample analysed in this study. Flow data acquired during sorting was analysed using FlowJo v9 software.

CD4+ T cell RNA-sequencing

RNA was extracted using a modified phenol-chloroform protocol (58) with 1-Bromo-3-chloropropane (Sigma-Aldrich #B62404) and Isopropanol (Acros Organics #423835000). Total RNA was quantified and assessed for quality and integrity by Bioanalyser (RNA Pico 6000 Chip, Agilent #5067-1513). All sequenced samples had a RIN value above 8. cDNA was generated from 1 ng total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio #634894) and amplified using 12 cycles of PCR. Amplified cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter #A63880), quantified on a Qubit Fluorometer (dsDNA HS assay kit, ThermoFisher #Q32851) and quality assessed by Bioanalyser (DNA HS Kit, Agilent #5067-4626). Libraries were then constructed from 150 pg of cDNA using the Nextera XT DNA Library Preparation Kit (Illumina #FC-131-1024) according to the manufacturer’s instructions. Libraries were quantified by Qubit (dsDNA HS assay) and fragment size distribution was assessed by Bioanalyser (DNA HS Kit). Using this information, samples were combined to create equimolar library pools that were sequenced on a NextSeq 550 Illumina platform to yield 75 bp PE reads; the average number of PE reads per sample passing QC across all samples was 2.74 x 10^7.

Data analysis of CD4+ T cell RNA-sequencing

Quality and content of FASTQ files were assessed using FastQC and reads were aligned to the Ensembl release 96 Homo sapiens transcript set with bowtie2 v2.2.7 (49) (parameters: —very-sensitive -p 30 —no-mixed —no-discordant —no-unal). Our median alignment rate was 66.4% after removing primer and adapter traces (SMART-Seq v4 primers, polyG, polyT and polyN) with cutadapt v1.9. The sorted, indexed bam files were then used to obtain a matrix of normalised counts for each transcript using samtools idxstats. Transcript counts were imported into the R/Bioconductor
environment (v3.6) and differential gene expression analyses (pairwise group comparisons) performed using functions within the DESeq2 package (52). IfcShrink was applied to the output of each comparison (type normal). Lists of differentially expressed transcripts were filtered by removing all non-coding transcripts and retaining only those with adj p < 0.05 and fold-change > 1.5. Multiple transcripts annotated to the same gene were consolidated by keeping the transcript with the highest absolute fold-change. Heatmaps and circular stacked bar plots were generated using the ggplot2 package.

**Functional gene enrichment analysis using clueGO**

Lists of differentially expressed genes were imported into clueGO v2.5.7 (59, 60). ClueGO identified significantly enriched GO terms (GO Biological Process and GO Molecular Function) associated with these genes and placed them into a functionally organised non-redundant gene ontology network based on the following parameters: adj p cutoff = 0.01; correction method used = Bonferroni step down; min. GO level = 5; max. GO level = 11; number of genes = 3; min. percentage = 5.0; GO fusion = true; sharing group percentage = 40.0; merge redundant groups with > 40.0% overlap; kappa score threshold = 0.4; and evidence codes used [All]. Each of the functional groups was assigned a unique colour and a network was then generated using an edge-weighted spring-embedded layout based on kappa score. Groups were named by the leading GO term (lowest adj p with min. GO level 5 or 6). Merged networks were constructed by inputting two lists of differentially expressed genes. For each GO term information on what fraction of associated genes were derived from each list was retained. Any GO term containing > 60% associated genes from a single list was considered to be enriched in that group, otherwise GO terms were considered to be shared.

**Multiplexed plasma analyte analysis**

The concentration of 39 analytes was measured in plasma samples collected at baseline, during infection, diagnosis, 6-days after drug treatment (T6) and at a memory timepoint (28- or 45-days post-challenge). Plasma was thawed on ice and centrifuged at 1000 xg for 1-minute to remove potential protein aggregates. We customised 4 LEGENDplex panels (BioLegend) and performed each assay on filter plates according to the manufacturer's instructions. Samples and standards were acquired on a LSRFortessa flow cytometer (BD Biosciences). FCS files were processed using LEGENDplex software (version 7.1), which automatically interpolates a standard curve using the plate-specific standards and calculates analyte concentrations for each sample. All samples from v1040 were excluded after failing QC and downstream data analysis was performed in R v3.6. To determine which plasma analytes varied significantly through time we used the lme4 package to fit a linear mixed-effects model for each analyte. All available time-points were included and models were fitted using log2 transformed plasma analyte concentration as dependent variable, time-point as a categorical fixed effect and volunteer as a random effect. A Kenward-Roger approximation was used to calculate p values (using the pbkrtest package), which were adjusted for multiple testing (Benjamini-Hochberg method). Results were visualised with ggplot2 (53) and only those with at least a 1.5 fold-change from baseline are shown. To determine which plasma analytes varied between infections at diagnosis we used lme4 to fit a mixed-effects model for each analyte in the form of analyte ~ timepoint + infection_number + (1 | volunteer) using log2 transformed concentrations. Time-point and infection number were included as categorical fixed effects and volunteer as a random effect. Linear hypothesis testing via pairwise comparisons was performed using multcomp's glht
function and adjusted for multiple testing (Benjamini and Hochberg). Only analytes that were significant in both second and third infection (versus first infection) are shown (adj p < 0.05 and fold-change > 1.5).

**Pearson correlation analysis**

The fold-change of each significant plasma analyte (shown in figure 3 - supplement 1B), the number of circulating lymphocytes and haemoglobin concentration were calculated at diagnosis or T6 (relative to baseline) according to their largest absolute fold-change. The percentage of activated effector and regulatory CD4+ T cells, the maximum parasite density and maximum core temperature (at any time-point up to 48-hours post-treatment) were also included in the analysis. All data were log2 transformed and Pearson correlation was performed using the corrplot function. Correlation coefficients were then used for hierarchical clustering by Euclidean distance.

**CyTOF sample acquisition**

Venous blood was collected in K$_2$EDTA-coated vacutainers, stabilised within 30-minutes of blood draw in whole blood preservation buffer (Cytodelics #hWBCS002) and stored at -80°C. For antibody staining, samples were thawed at 37°C in a water bath, fixed for 15-minutes and red blood cells lysed using the whole blood preservation kit (Cytodelics #hC002). Cells were permeabilised with Maxpar barcode perm buffer (Fluidigm #201057) and each sample was barcoded using Cell-ID 20-plex palladium barcodes (Fluidigm #201060). Samples were then pooled and stained with our T cell focussed surface antibody panel (see supplementary file 3) for 30-minutes. Following washing, cells were fixed and permeabilised with the Maxpar nuclear antigen staining buffer set (Fluidigm #201063) and incubated with the nuclear antibody mix for 45-minutes. After washing, cells were fixed for 10-minutes in 1.6% formaldehyde diluted in PBS (ThermoFisher #28906). Cells were then washed again, resuspended at a concentration of 3 x 10$^6$ cells per ml in 72.5 nM Cell-ID Intercalator Ir solution (Fluidigm #201192A) and stored overnight at 4°C. Samples were acquired the next day on a freshly tuned Helios mass cytometer (Fluidigm, acquisition rate 300-500 events/second) using the WB injector and 10% EQ Four Element Calibration Beads (140Ce, 151Eu, 165Ho, and 175Lu; Fluidigm #201078).

**CyTOF data analysis**

The Fluidigm CyTOF software (version 6.7) generated FCS files, which were normalised (61) and debarcoded (62) using the R package CATALYST (63). Samples were compensated using single stained beads (64). After exclusion of normalisation beads and doublets we gated on CD45$^{pos}$ CD3$^{pos}$ T cells using the Cytobank web portal (Beckman Coulter, www.cytobank.org). We then inspected the intensity distribution of each channel and removed channels of low variance (CD3, CD16, CD45, CD69, CXCR5, GATA3, RORγt, TCRγδ, and Tim3). The remaining 30 markers were used for both UMAP projections and FlowSOM clustering.

UMAP (65) was used to generate a 2D projection of this high-dimensional dataset. Here phenotypic similarity of cells within and between populations is preserved in the Euclidean distance of the projection. We used its R implementation in the scater package (66), which in turn relies on uwot (github.com/jlmelville/uwot). Features were scaled to unit variance and the 15 nearest neighbours
were considered for embedding. UMAP coordinates were then exported for visualisation using ggplot2 (53). FlowSOM (67) uses self-organising maps (SOM) to efficiently categorise cytometry data into non-overlapping cell populations and was performed using CATALYST (63) (default parameters, target: 100 clusters, 50 metaclusters). After manual inspection we merged two phenotypically similar clusters to avoid overclustering (68) and ended up with 49 discrete T cell clusters. The R/Bioconductor package ComplexHeatmap (69) was used to visualise T cell phenotypes; the arcsine transformed signal intensity of each marker was independently scaled using a 0-1 transformation across all 49 clusters.

For differential cluster abundance analysis we used the workflow laid out by Nowicka et al. (70). FlowSOM cluster cell counts were modelled linearly with time-point as a dependent categorical variable and volunteer as a fixed effect using the diffcyt (71) implementation of edgeR (72). The edgeR functions automatically normalise cluster counts for the total number of cells and improve statistical power by sharing information on cluster count variance between clusters. Pairwise comparisons were performed relative to baseline, and clusters with an FDR < 0.05 and absolute fold change > 2 were deemed to vary significantly through time. We assessed differential cluster abundance independently for volunteers receiving either their first or third infection. We also assessed whether marker expression significantly varies through time in the major T cell subsets. To do this, we merged clusters of the same lineage according to their expression of CD4, CD8, CD56, V62 and V\(\gamma\)7.2. CD4\(^+\) and CD8\(^+\) T cells were then split into naive, effector, effector memory and central memory T cells based on their expression of the markers CD45RA, CD45RO, CD57 and CCR7. All regulatory T cells (CD25\(^+\)CD127\(^{neg}\)) were merged into a single cluster as were all double negative, gamma delta, MAIT and NK T cells. Linear models derived from the limma package, which is optimised for continuous data, were then used to independently assess differential marker expression relative to baseline using pairwise comparisons with moderated t-tests; a shift in median expression of at least 10% and an FDR < 0.05 were required for significance. Results were visualised using ComplexHeatmap (69) with row-wise z-score transformed marker intensities shown for each subset.

To analyse the T cell response to Plasmodium vivax data from two previously conducted clinical trials were used. In VAC069A six healthy malaria-naive adults were recruited to test the infectivity and safety of a new cryopreserved stablitate containing a novel clone of \(P.\) vivax (PvW1); this stablitate had been carefully prepared for use in CHMI by blood challenge (Minassian et al., under review). In VAC069B three volunteers returned for a homologus re-challenge (8-months after VAC069A) and two additional malaria-naive adults received their first infection with PvW1. In both CHMI trials treatment was initiated once two diagnostic conditions were fulfilled: parasitaemia above 5,000 parasite genome copies per ml; parasitaemia above 10,000 genome copies per ml; positive thick blood smear and/or symptoms consistent with malaria. Treatment usually consisted of artmether and lumefantrine (Riamet) or atovaquone and proguanil (Malarone) if Riamet was contraindicated. Blood sampling, sample processing and CyTOF data analysis were all performed analogously to VAC063C. VAC069 was sponsored by the University of Oxford, received ethical approval from UK NHS Research Ethics Service (South Central - Hampshire A reference 18/SC/0577) and was registered on ClinicalTrials.gov (NCT03797989). The trial was conducted in line with the current version of the Declaration of Helsinki 2008 and conformed with the ICH Guidelines for Good Clinical Practice.
A

lymphocyte counts (diagnosis vs baseline)

first second third

0 0.5 1.0 1.5 2.0

B

proportion (%)

eosinophils

lymphocytes

monocytes

neutrophils

baseline diagnosis baseline diagnosis baseline diagnosis

figure one - supplement 1
Figure 1 - supplement 1 | Lymphocyte recruitment to inflamed tissues is a shared response to the first three infections of life. (A) Lymphocytes were quantified in whole blood at diagnosis and counts were normalised to baseline measurements (taken one day before challenge). Normalised counts are shown for each volunteer (dots) and bars show the median fold-change during first, second and third infection. No statistically significant difference between infections was observed (Kruskal-Wallis test $p = 0.4483$). (B) Proportion of lymphocytes, monocytes, neutrophils and eosinophils in whole blood at baseline and diagnosis. The mean frequency is shown for each time-point and infection. In (A-B) $n = 10$ (first and second infection) and $n = 6$ (third infection).
A figure two - supplement 1
Figure 2 - supplement 1 | Interferon-stimulated inflammation is a shared response to the first three infections of life. (A) RNA-sequencing was used to identify differentially expressed genes in whole blood at diagnosis versus baseline (adj p < 0.05 and > 1.5 fold-change). Note that first, second and third infection were analysed independently. Volcano plots show all differentially expressed genes (coloured dots) and the dashed lines represent the significance/fold-change cut-offs (genes that are not significant are shown in grey). The top 10 differentially expressed genes (lowest adj p) in each infection are labelled. (B) The log2 fold-change of signature genes associated with interferon signaling and type I inflammation are shown at diagnosis (versus baseline) in first, second and third infection. Square brackets indicate that common gene names have been used. In (A-B) n = 10 (first infection), 9 (second infection) and 6 (third infection). v1040 was excluded from RNA-sequencing analysis in second infection because their baseline sample failed QC.
**figure two - supplement 2**
Figure 2 - supplement 2 l Systemic inflammation peaks at diagnosis. Mixed-effects modelling was used to identify plasma analytes that vary significantly at diagnosis across the entire dataset (all volunteers and all infections). Kenward Roger approximation was used to calculate p values and multiple test correction was performed using the Benjamini-Hochberg method (*adj p < 0.05, **adj p < 0.005, ***adj p < 0.0005). Box (median and IQR) and whisker (1.5x upper or lower IQR) plots are shown with outliers as dots. n = 9 (first and second infection) and 5 (third infection). v1040 was excluded from plasma analysis because all samples failed QC.
Severity of adverse events:
- Absent
- Mild
- Moderate
- Severe

A

Diagnosis

B

Log2 concentration

Figure three - supplement 1
**Figure 3 - supplement 1** | The acute phase response resolves 6-days after parasite clearance. (A)

Frequency and severity of lymphopenia, anaemia and thrombocytopenia at diagnosis and T6 across the VAC063C cohort. (B) Mixed-effects modelling was used to identify plasma analytes that vary significantly at diagnosis and/or T6 across the entire dataset (all volunteers regardless of infection number). Kenward Roger approximation was used to calculate p values and multiple test correction was performed using the Benjamini-Hochberg method; significance (adj p < 0.05) is indicated by a coloured box and whiskers (purple at diagnosis and turquoise at T6). In (A) n = 11 (3 first infection, 2 second infection and 6 third infection) and in (B) n = 10 (3 first infection, 2 second infection and 5 third infection). v1040 was excluded from plasma analysis because all samples failed QC.
**Figure Three - Supplement 2**

- **A**: Heatmap showing gene expression levels with log2FC and -log10 padj values.
- **B**: another heatmap with similar details.
- **C**: Gene ontology (GO) term enrichment analysis, with terms such as:
  - nuclear division
  - mitotic spindle organisation
  - response to interferon γ
  - activation of innate immune response
  - response to type 1 interferon
  - regulation of defense response
  - positive regulation of programmed cell death
  - cytokine secretion
  - regulation of leukocyte cell-cell adhesion
  - DNA replication
  - deoxyribonuclease activity
  - chromatin remodelling
  - DNA recombination

The figure illustrates gene expression changes and enrichment of specific biological processes.
Figure 3 - supplement 2 | The transcriptional signature in whole blood at T6 does not overlap with diagnosis. (A) RNA-sequencing was used to identify differentially expressed genes in whole blood at diagnosis (left) and T6 (right) during first infection (versus baseline, adj p < 0.05 and > 1.5 fold-change). Volcano plots show all differentially expressed genes (coloured dots) and the dashed lines represent the significance/fold-change cut-offs (genes that are not significant are shown in grey). The top 10 differentially expressed genes (lowest adj p) at each time-point are labelled. (B) Direct pairwise comparison between T6 and diagnosis - the 5 genes with the lowest adj p value are labelled in both directions. (C) Differentially expressed genes at T6 and diagnosis were combined for GO analysis of first infection and clueGO was used to construct a merged functional gene ontology network. Each node represents a GO term and nodes are coloured according to whether their associated genes were majoritively (> 60%) derived from the diagnosis or T6 time-point. GO terms that were shared between time-points are coloured grey (see methods). Seven leading GO terms (each from a unique functional group) are labelled for each time-point. In (A-C) n = 3 (all first infection).
figure three - supplement 3
Figure 3 - supplement 3 | The acute phase response is followed by a signature of cell proliferation in naive hosts. Whole blood RNA-sequencing was used to identify differentially expressed genes at diagnosis and T6 (versus baseline) during first and third infection (adj p < 0.05 and > 1.5 fold-change). The log2 fold-change of key genes associated with each phase of the cell cycle is shown. n = 3 (first infection) and 6 (third infection).
Figure 3 - supplement 4 | Gating strategy for sorting CD4+ T cells during the VAC063C trial. CD4+ T cell subsets were sorted *ex vivo* (within 2-hours of blood draw) into TRIzol for downstream RNA-sequencing - cells with a naive, effector/effector memory (EM) and regulatory phenotype were gated as shown at baseline and T6. Note that we did not use CD38 for sorting but subsequently used this marker to assess the level of activation within each subset at both time-points.
Figure 4 - supplement 1 | Effector CD4+ T cells are proliferative and metabolically reprogrammed during first infection. (A-B) Genes that were differentially expressed in effector/effecter memory (EM) CD4+ T cells at T6 of first infection (versus baseline, adj p < 0.05 and fold-change > 1.5) were used to create a non-redundant functional gene ontology network in clueGO. Each node represents a GO term and node size is determined by adj p. Related GO terms that share > 40% of genes are connected by a line and organised into functional groups (each given a unique colour). The leading GO term from each of the 13 largest groups is labelled and (B) shows their proportion of total GO terms. The outer ring on the pie indicates whether groups were up- or downregulated (red or blue, respectively). (C) Transcriptional regulation of fatty acid β-oxidation, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (oxphos) in effector/EM CD4+ T cells during first infection. The circular bar charts show the log2 fold-change of each major enzyme involved in fatty acid β-oxidation and the TCA cycle at T6 (shown in reaction order, clockwise). The vertical bar chart shows the proportion of oxphos enzymatic subunits that are transcriptionally upregulated at T6 - all subunits required to form complex I to IV in the electron transport chain and ATP synthase are shown. The key molecules that connect these metabolic pathways are labelled. In (A-C) n = 2 for T6 and 3 for baseline (v313 was excluded at T6 because this sample failed QC).
Figure 4 - supplement 2 | CD4⁺ T cells are not anergic, exhausted or deleted in tolerised hosts. (A-C) RNA-sequencing was used to identify differentially expressed genes in naive, effector/effector memory (EM) and regulatory CD4⁺ T cells 6-days after parasite clearance (T6 versus baseline) in first and third infection (adj p < 0.05 and > 1.5 fold-change). Heatmaps show the log2 fold-change of (A) transcription factors downstream of T cell receptor signaling, (B) hallmark genes indicating T cell anergy or quiescence and (C) markers of clonal deletion. In (A-C) n = 2 or 3 for first infection (T6 and baseline, respectively) and 6 for third infection. v313 was excluded at T6 because this sample failed QC.
Figure 5 - supplement 1 | Phenotype of T cell clusters. Heatmap showing the normalised median expression values of all markers used for clustering in each of the 49 T cell clusters. Names were assigned manually using activation, lineage and memory markers to broadly categorise each T cell cluster; when more than one cluster was placed into the same category (e.g. activated EM CD4) clusters were given an accessory label to highlight their unique phenotype or property (e.g. T-bet<sup>(c)</sup>).
Figure five - supplement 2
**Figure 5 - supplement 2** | Frequency of each CD4+ T cell cluster during and after infection. Every cluster is shown as a proportion of all CD45pos CD3pos T cells at each time-point. Clusters that were differentially abundant in first and/or third infection are shown first (ordered by size, big to small) and clusters that did not vary significantly through time are displayed thereafter (ordered by size, small to big). Box (median and IQR) and whisker (1.5x upper or lower IQR) plots are shown (with outliers as dots) and significance (FDR < 0.05 and > 2 fold-change) is indicated by a coloured box. n = 3 (first infection) and 6 (third infection).
figure five - supplement 3
Figure 5 - supplement 3 | Frequency of each CD8+ T cell cluster during and after infection. Every cluster is shown as a proportion of all CD45pos CD3pos T cells at each time-point. Clusters that were significantly increased during infection are shown first followed by clusters that were significantly decreased (ordered by size, big to small). Thereafter, clusters that did not vary significantly through time are displayed (ordered by size, small to big). Box (median and IQR) and whisker (1.5x upper or lower IQR) plots are shown (with outliers as dots) and significance (FDR < 0.05 and > 2 fold-change) is indicated by a coloured box. n = 3 (first infection) and 6 (third infection).
Figure 5 - supplement 4 | Frequency of each innate-like T cell cluster during and after infection. Every cluster is shown as a proportion of all CD45^{pos} CD3^{pos} T cells at each time-point. Clusters that were significantly increased during infection are shown first followed by clusters that were significantly decreased (ordered by size, big to small). Thereafter, clusters that did not vary significantly through time are displayed (ordered by size, small to big). Box (median and IQR) and whisker (1.5x upper or lower IQR) plots are shown (with outliers as dots) and significance (FDR < 0.05 and > 2 fold-change) is indicated by a coloured box. n = 3 (first infection) and 6 (third infection).
Figure 5 - supplement 5 | Differential marker expression reveals attenuation of the adaptive T cell response to re-challenge. We assessed whether marker expression significantly varies through time in all major T cell subsets. First, T cell clusters belonging to the same lineage were merged and then CD4+ and CD8+ T cells were split into naive, effector, effector memory (EM) and central memory (CM) subsets. Next, linear models were used to independently assess differential marker expression in each subset at each time-point (relative to baseline); a shift in median expression of at least 10% and an FDR < 0.05 were required for significance. Shown are all subset/marker pairs that were called as significant at T6 and data are presented as row-wise z-score marker intensities. Colour codes to the left of the heatmap indicate whether markers were differentially expressed during first infection, third infection or both infections.
Baseline | Diagnosis | T6 | Memory
---|---|---|---
Gamma delta | MAIT

Activation (% lineage)

First infection
Third infection
Figure 5 - supplement 6 | Innate-like T cells are highly activated in tolerised hosts. Bar charts display the frequency of activated (CD38hi Bcl2lo) gamma delta and MAIT clusters at each time-point. Data are shown as a proportion of the entire lineage (Vδ2pos for gamma delta and Vγ7.2pos for MAIT). n = 3 (first infection) and 6 (third infection).
Activation of T cells during different infections. Figure A shows the activation of CD4+ and CD8+ T cells, double negative, gamma delta, MAIT, and Treg cells during the first and second infections. Figure B includes additional markers such as v21, v11, v09, v07, v06, v05, v03, and v02. The color coding is as follows: CD4+ T cells (red), CD8+ T cells (blue), double negative (green), gamma delta (yellow), MAIT (orange), and Treg (pink).
Figure 6 - supplement 1 | *Plasmodium vivax* induces tolerance after a single infection. Six healthy malaria-naive adults were infected with *P. vivax* (clone PvW1) by direct blood challenge and three volunteers returned for a homologous re-challenge 8-months later. These CHMI trials were called VAC069A and VAC069B, respectively. Two additional healthy malaria-naive volunteers were infected with PvW1 (for the first time) during VAC069B. In both trials, whole blood was preserved within 30-minutes of blood draw at baseline, diagnosis and 6-days post-treatment (T6) - samples were stained with a T cell focussed antibody panel and acquired on a Helios mass cytometer. After exclusion of normalisation beads and doublets we gated on CD45\(^{\text{pos}}\) CD3\(^{\text{pos}}\) T cells for FlowSOM clustering, which was performed independently for VAC069A and VAC069B. (A) Bar chart showing the frequency of activated (CD38\(^{\text{hi}}\) Bcl2\(^{\text{lo}}\)) CD4\(^{+}\) T cell clusters at T6. (B) Stacked bar chart showing the frequency of all activated (CD38\(^{\text{hi}}\) Bcl2\(^{\text{lo}}\)) T cell clusters at T6. In (A-B) data are shown as a proportion of all CD45\(^{\text{pos}}\) CD3\(^{\text{pos}}\) T cells and n = 8 (first infection) or 3 (second infection).
Supplementary files

**Supplementary file 1** | Demographics of volunteers infected and re-challenged with *Plasmodium falciparum* (3D7); includes genetic and non-genetic variables known to influence human immune variation *in vitro*. Also shown is the parasite multiplication rate (detailed methodology for the modelling of qPCR data can be found in Minassian *et al.* (47)) and maximum core body temperature measured in each volunteer during each infection. There were no statistically significant differences between first, second and third infection (Kruskal-Wallis test, $p = 0.3287$ for parasite multiplication rate and $p = 0.9949$ for core temperature).

**Supplementary file 2** | Pearson correlation coefficient and p value measuring the relationship between inflammation, coagulation, symptoms, parasites and T cell activation. These data underpin the matrix shown in figure 3C.

**Supplementary file 3** | Mass cytometry antibody panel for T cell fate and function in falciparum malaria; includes information on antibody clone and heavy metal conjugate.
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