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Repeated *Plasmodium falciparum* infection in humans drives the clonal expansion of an adaptive γδ T cell repertoire

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Abstract

Repeated *Plasmodium falciparum* infections drive the development of clinical immunity to malaria in humans, however, the immunological mechanisms that underpin this response are only partially understood. Here, we investigated the impact of repeated *P. falciparum* infections on human γδ T cells in the context of natural infection in Malian children and adults, as well as serial controlled human malaria infection (CHMI) of U.S. adults, some of whom became clinically immune to malaria. In contrast to the predominant Vδ2$^+$ γδ T cell population in malaria-naive Australian individuals, clonally expanded cytotoxic-Vδ1$_{effector}$ T cells were enriched in the γδ T cell compartment of Malian subjects. Malaria-naïve U.S. adults exposed to four sequential CHMIs defined the precise impact of *P. falciparum* on the γδ T cell repertoire. Specifically, innate-like Vδ2$^+$ γδ T cells exhibited an initial robust polyclonal response to *P. falciparum* infection that was not sustained with repeated infections, whereas Vδ1$^+$ γδ T cell frequencies increased in frequency with repeated infections. Moreover, repeated *P. falciparum* infection drove waves of clonal selection in the Vδ1$^+$ TCR repertoire that coincided with the differentiation of Vδ1$_{naive}$ cells into cytotoxic-Vδ1$_{effector}$ cells. Finally, Vδ1$^+$ T cells of malaria-exposed Malian and U.S. individuals were now licensed for reactivity to *P. falciparum* parasites *in vitro*. Together, our study indicates that repeated *P. falciparum* infection drives the clonal expansion of an adaptive γδ T cell repertoire and establishes a role for Vδ1$^+$ T cells in the human immune response to malaria.

One Sentence Summary:

Malaria drives the adaptive differentiation of the human γδ T cell repertoire.

Introduction

In malaria-endemic regions, non-sterilizing clinical immunity to blood-stage *Plasmodium falciparum* parasites can be acquired, but this typically only occurs after many years of repeated infections (1). However, the mechanisms underlying this protection are only partially understood (2, 3). Recent observational studies in malaria-endemic areas, as well as clinical trials of attenuated *P. falciparum* sporozoite vaccine *Pf*SPZ, have suggested that γδ T cells may contribute to protection from malaria (4–7).

Human γδ T cells are an unconventional T cell population that are thought to play an important role in immunity to microbial pathogens and cancer (8). Unlike conventional αβ T cells, γδ T cells are not restricted by classical MHC or MHC-I-like molecules to recognize antigens (9–11), but instead respond directly to non-peptidic metabolite antigens and other diverse ligands (12, 13). γδ T cells were present in the first jawed vertebrates and

co-evolved with pathogenic organisms for millions of years (10, 14). In humans, the major peripheral blood population of γδ T cells (5–10% of total T cells) express a restricted TCR that consists of paired Vδ2 and Vγ9 chains (15). The Vγ9/Vδ2$^+$ T cell population directly responds to a prenyl-pyrophosphate metabolite (PAg) (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) produced by the microbial non-mevalonate pathway (16). The Vγ9/Vδ2$^+$ TCR repertoire is generated early in gestation and is shaped soon after birth, with a high frequency of public Vγ9 clonotypes (17–19). Innate-like Vγ9/Vδ2$^+$ T cells can expand and comprise up to 40% of T cells during blood-stage malaria infection, a response thought to be driven by recognition of *P. falciparum*-derived HMB-PP (20–22) and they may participate in limiting parasite replication by targeting *P. falciparum* blood-stage parasites through granulysin-dependent cytotoxicity (23) and phagocytosis of antibody-coated iRBCs (24).

In contrast to innate-like Vγ9/Vδ2$^+$ γδ T cells, a diverse biology has been established for γδ T cells that predominantly express the Vδ1$^+$ TCR chain and circulate in blood at low frequency but are a major population in peripheral tissues (25). Firstly, Vδ1$^+$ T cells that form tissue-associated populations in the intraepithelial lymphocyte (IEL) compartment of gut and breast tissue are thought to provide innate-like immune surveillance through host-encoded Natural Killer-receptors (NKRs) (26) and BTN-like (BTNL) 3 proteins (27). Secondly, peripheral blood and liver-resident Vδ1$^+$ T cells possess hallmarks of adaptive T cells and comprise naïve-like (Vδ1$_{naïve}$) and effector (Vδ1$_{effector}$) populations with diverse or highly focused TCR repertoires, respectively (19, 25, 28). Acute cytomegalovirus (CMV) infection has been associated with the selection of a limited set of Vδ2^{neg} γδTCR clonotypes (19, 29). Interestingly, expanded populations of Vδ1$^+$ T cells have been observed in both children and adults with symptomatic *P. falciparum* infection (30–32) and in individuals residing in regions of malaria transmission (33, 34). Despite evidence that γδ T cells contribute to immunity to microbial pathogens, it remains unclear whether *P. falciparum* infection per se or factors associated with malaria transmission in endemic areas are responsible for the expansion of Vδ1$^+$ T cells. Moreover, it is also unclear the impact of repeated *P. falciparum* infection on the phenotype, function and clonality of the γδ T cell compartment.

In this study, we investigated the γδ T cell immune repertoire response to *P. falciparum* malaria in a cohort of children and adults residing in a malaria-endemic region of Mali, and in malaria-naive U.S. adults serially infected with *P. falciparum* via mosquito bite in a controlled setting. We found that repeated *P. falciparum* infections drove the clonal selection and expansion of circulating cytotoxic Vδ1$_{effector}$ T cells that reacted to *P. falciparum* blood-stage parasites.

Results

Heterogeneity in the γδ T cell compartment exists across diverse geographic locations

In general, immune profiles are known to differ between children of high- and low-income countries where the latter typically suffer a disproportionately high burden of infectious disease (35). Here, we compared the circulating γδ T cell repertoire of Malian children (aged 4 – 17 years) who are exposed to intense seasonal malaria transmission (36), with

that of age and gender matched children from Melbourne, Australia (aged 1 – 17 years) (Table S1). We first analyzed Mali samples collected from uninfected subjects at the end of the dry season when malaria transmission is negligible to assess γδ T cell repertoires in a relatively unperturbed state. We found that γδ T cell and Vδ1$^+$ T cell frequencies were significantly higher in Malian children (Fig. 1A and S1A), whereas the frequency of Vγ9/Vδ2$^+$ T cells were similar between both groups (Fig. 1A and B). We then analyzed γδ T cells in Malian adults (aged 21 – 26 years) and adults residing in an area of low malaria transmission in Kenya (aged 26 – 49 years) as well as Australian adults with no history of malaria exposure (aged 20 – 71 years). Vδ1$^+$ T cell frequencies were lower in Kenyan and Australian adults compared to Malian children (Fig. S1B). Next, we assessed γδ T cell effector subsets in Malian children. From birth, Vγ9/Vδ2$^+$ T cells typically form a stable innate-like T cell population composed of a CD27$^+$ CD28$^+$ Granzyme (Gzm) A$^+$ GzmB$^+$ Perforin$^+$ compartment (18, 19, 37). In Malian individuals we found that Vγ9/Vδ2$^+$ T cells had reduced expression of CD27$^+$ CD28$^+$ (Fig. 1C) and lytic Perforin (Fig. 1D), while GzmA was increased (Fig. 1E and F). In contrast, cord blood Vδ1$^+$ T cell population is typically composed of naïve-like CD27^{hi} CX_3CR1^{neg} GzmA/B^{neg} Perforin^{neg} cells ($Vδ1_{naive}$) (28). However, the Vδ1$^+$ compartment in Malian subjects was predominantly composed of CD27^{lo} CX_3CR1^+ GzmA/B$^+$ Perforin$^+$ effector-like cells ($Vδ1_{eff}$) (Fig. 1C–F). Interestingly, a CD16$^+$ Vγ9/Vδ2$^+$ T cell compartment has recently been implicated in antibody-mediated phagocytosis of iRBCs (24). We found that Malian children, when compared to Australian children, tended to have increased frequencies of CD16$^+$ Vδ1$^+$ T cells rather than CD16$^+$ Vγ9/Vδ2$^+$ T cells (Fig. S1C). Together, these data suggest that the composition of the γδ T cell compartment varies significantly across geographic locations. However, it remained unclear whether high malaria transmission and/or factors associated with malaria transmission drive the proportional expansion of Vδ1$^+$ T cells and skewing towards a Vδ1effector phenotype in the Mali cohort.

Episodes of febrile malaria associate with fluctuations in Vδ1$^+$ γδ T cell frequencies

To more directly investigate the potential impact of natural malaria infection on the γδ T cell compartment, we conducted a longitudinal analysis of nine Malian children (aged 8 – 14 years) over three malaria seasons (Fig. 1G). These individuals from Mali were exposed to an annual six-month malaria season in which *P. falciparum* transmission is intense and predictable (36). Consistent with this, *P. falciparum* parasite density increased during each malaria season in a subset of five children whose blood smears were examined longitudinally (Fig. S1CD). Moreover, each subject was selected because they experienced two to three febrile malaria episodes over multiple years, as detected by both passive and active clinical surveillance, allowing for longitudinal analyses of γδ T cell dynamics in response to acute symptomatic malaria followed by sustained periods without febrile malaria (Fig. 1G). We then investigated γδ T cell and CD8$^+$ αβ T cell frequencies across consecutive episodes of febrile malaria over three seasons, these analyses pooled T cell frequencies from children who had experienced two or three episodes of febrile malaria (Fig. 1H). γδ T cell and CD8$^+$ αβ T cell frequencies were assessed in blood samples collected on the day febrile malaria was diagnosed and again within 3 – 6 months of diagnosis. We found that Vγ9/Vδ2$^+$ T cell frequencies increased after febrile malaria in year 1 but did not consistently change after febrile malaria in years 2 and 3 (Fig. 1H),

although these observations could be due to the different sampling times in each year. $CD8^+$ T cell frequencies were unchanged after each febrile episode (Fig. 1H). In contrast, $V\delta1^+$ T cell frequencies were consistently decreased upon presentation with febrile malaria and increased after each febrile malaria episode across all three years (Fig. 1H). Moreover, across a sub-set of eight subjects in year 1, we also observed equivalent $CD3^+$ lymphocyte and αβ T cell counts, and all γδ T cell populations expanded in number after febrile malaria (Fig. S1E). We then assessed a subset of children at timepoints without infection before a documented period of asymptomatic *P. falciparum* infection but no febrile malaria episodes (Fig. 1I; pooled from data between month 12 – 19 or 24 – 30). $V\gamma9/V\delta2^+$ and $V\delta1^+$ T cell frequencies did not change significantly across this six to seven-month period. We noted previously that $V\delta1^+$ T cells in Malian children were predominantly composed of $V\delta1_{effector}$ cells (Fig. 1C and D), however, yearly episodes of febrile malaria had little impact on $V\delta1_{effector}$ frequencies and $CD27^+$ $CD28^+$ $V\gamma9/V\delta2^+$ T cell frequencies were reduced in year 3 (Fig. S1F). Together, these data indicate that exposure to seasonal episodes of febrile malaria transiently impacts circulating frequencies of $V\delta1^+$ γδ T cells.

Malian γδTCR repertoires are clonally skewed and change after febrile malaria

We next explored the underlying γδTCR repertoires in Malian children and whether febrile malaria could impact individual clonotypes over time. Initially, we conducted a cross-sectional analysis of blood samples collected subjects during periods of no malaria transmission (subjects 066, 521, 766) and from one subject with febrile malaria (subject 269) and compared these repertoires to those of Australian children (Fig. 2A and S3A). We analyzed both $V\gamma9/V\delta2^+$ ($V\delta2^+$) and non-$V\gamma9/V\delta2$ ($V\delta2^{neg}$) γδ T cell populations, effectively encompassing the total γδ T cell repertoire (Fig. S2). Phenotypically, in both Malian and Australian subjects $V\delta2^+$ γδ T cell populations were composed of effector-like populations of $CD27^+$ $CD28^+$ cells and $V\delta2^{neg}$ γδ T cells were composed $CD27^{lo}$ CX_3CR1^+ effector cells in Malian subjects and $CD27^{hi}$ CX_3CR1^{neg} naïve cells in Australian subjects (Fig. S3B). The $V\gamma9/V\delta2^+$ T cell subset displayed γδ TCR repertoires consistent with those seen in children and adults from Europe (18, 19, 37) (Fig. 2A and Fig. S3A), which are almost exclusively composed of Vδ2–Jδ1 (Fig. S3C) paired to Vγ9–JγP (Fig. S3D), with diverse clonotype composition and common CDR3γ9-JγP sequences shared between individuals (Fig. 2A and Fig. S3C). $V\delta2^{neg}$ γδ TCR repertoires were predominantly composed of Vδ1–Jδ1 sequences (Fig. S3D) that were paired to various Vγ–Jγ1/2 regions (Fig. S3D). These $V\delta2^{neg}$ γδTCR repertoires from Malian children exhibited expanded clonotypes, indicated by an increase in the accumulated frequency of the top 10 clonotypes in comparison to $V\delta2^{neg}$ γδ TCR repertoires in Australian children (Fig. 2B). In support of the skew towards expanded Vδ1 clonotypes, Malian $V\delta2^{neg}$ γδ TCR repertoires also showed a reduced diversity of clonotype composition (Fig. 2C) and a reduced frequency of shared sequences compared to $V\delta2^{neg}$ γδTCR repertoires of Australian individuals (Fig. 2D). These data suggest that the $V\gamma9/V\delta2^+$ T cell repertoires in Malian subjects are highly similar to those of Australian individuals. In contrast, $V\delta2^{neg}$ γδTCR repertoires of Malian individuals showed evidence of reduced clonotype sharing and diversity as a result of expanded private clonotypes.

Next, in a longitudinal analysis we assessed the impact of episodes of acute febrile malaria on γδTCR clonotype composition within the Vγ9/Vδ2^{+} and Vδ2^{neg} γδ T cell populations. Vγ9/Vδ2^{+} clonotypes remained remarkably stable during and after acute febrile malaria (Fig. 2E, Fig. S3E and F). We and others have previously reported on the stability of Vδ2^{neg} and Vδ1^{+} γδTCR clonotypes over several years (18, 19, 28, 29). Here, Vδ2^{neg} γδTCR repertoires displayed changes after acute febrile malaria, characterized by contraction and expansion of existing clonotypes or emergence of new prevalent sequences (Fig. 2F, Fig. S3E and G). These changes impacted the frequency of Vδ1 sequence usage (Fig. S3H), Vγ2 usage (Fig. 2G), the overall repertoire diversity (Fig. 2G) and nucleotide length dynamics (Fig. S3I). To explore the impact of febrile malaria on clonotype composition within Vδ1$_{effector}$ cells, we sorted single cells from the CD27^{lo} CX$_3$CR1^{+} Vδ1$_{effector}$ cell compartment from samples collected over 32 months and three separate acute febrile malaria episodes from subject 179 (Fig. 2H). Interestingly, we noted by flow cytometry that Vδ1/γδTCR antibody staining intensity changed over time, with distinct Vδ1/γδTCR antibody populations emerging after each episode of febrile malaria (Fig. 2H and Fig. S3J). Underpinning these observations, single cell γδTCR sequencing revealed changes in the frequency and identity of individual Vδ1$_{effector}$ clonotypes over time (Fig. 2I and Fig. S3K). Together, these data suggest that Malian individuals have highly stable Vγ9/Vδ2^{+} T cell repertoires that are retained across episodes of febrile malaria and are shared between individuals. In contrast, γδTCR clonotypes in the Vδ1$_{effector}$ compartment were composed of clonotypes that varied in frequency and identity over time.

Repeated human controlled malaria infection can establish clinical immunity that correlates with increased Vδ1^{+} γδ T cell frequencies

To understand the precise impact of *P. falciparum* infection on the trajectory of γδ T cell development and selection, we assessed γδ T cell subset dynamics in PBMCs collected from five malaria-naïve adults voluntarily exposed to repeated controlled human malaria infection (CHMI). Each volunteer was exposed to the bites of five *Anopheles stephensi* mosquitos infected with *P. falciparum* (strain: NF54) on four separate occasions over 644 days (Fig. 3A). Symptomatic malaria occurs during the blood stage of the *P. falciparum* parasite life cycle, which typically develops after an incubation period of nine to fourteen days (36). Here, we analyzed samples at baseline (malaria naïve), immediately prior to *P. falciparum* infection (day 1; at CHMI1 and 3), and 21 days after infection for all CHMIs (Fig 3A). We did not observe any noticeable leukopenia measured by white blood cell counts (at day 1 or day 28; Fig. S4A) or by clinical tests prior to apheresis (day 21) at the timepoints sampled in this study. Over the course of the four CHMIs, peak parasitemia measured by blood smear did not significantly change (Fig. S4B). We then assessed the instances of febrile malaria and symptomatic disease (ranging from headaches to vomiting; Table S2). Fever was observed at CHMI1 or 2 in all but one individual and the number of symptoms observed in each individual decreased after repeated CHMIs (Fig. 3B). Three individuals had asymptomatic *P. falciparum* infections following CHMI4, while two volunteers remained symptomatic (Fig. 3B). Next, we analyzed γδ and αβ T cell frequencies across all CHMIs. Total αβ T cell frequencies within CD3^{+} T cells showed a non-significant decline with repeated CHMI (Fig. 3C). CD8^{+} αβ T cells increased in frequency and peaked prior to CHMI3 (Fig. 3C), coinciding with an increase in CD8^{+}

T_{naive} cells and $CD8^+$ T_{CM} on day 21 after CHMI2–4 (Fig. S4C). In contrast, γδ T cells frequencies increased across all CHMIs, and this was largely driven by an increase in $V\gamma9/V\delta2^+$ T cells (Fig. 3D). We also found an increase in $V\delta1^+$ T cell frequencies across repeated CHMIs (Fig. 3D). We then analysed the relationship between γδ T cell frequencies and the risk of developing symptomatic malaria. Overall, αβ T cell and $CD8^+$ T cell frequencies were similar in asymptomatic and symptomatic individuals (Fig. S4D). However, volunteers that progressed to asymptomatic malaria with serial CHMIs displayed robust profiles of increasing $V\delta1^+$ and $V\gamma9/V\delta2^+$ T cells frequencies across CHMIs, while symptomatic volunteers retained frequencies of $V\delta1^+$ and $V\gamma9/V\delta2^+$ T cells that were similar to their baseline samples (Fig. 3E). $V\gamma9/V\delta2^+$ T cells frequencies decreased between CHMIs and were not durably maintained at CHMI4 (Fig. 3E). Repeated measures correlations found a significant inverse association between the number of malaria symptoms and $V\delta1^+$ T cell frequencies (P=0.007) (Fig. 3F), but not with $\alpha\beta^+$ (P=0.431), $\gamma\delta^+$ (P=0.109), $CD8^+$ (P=0.391) or $V\gamma9/V\delta2^+$ T cell frequencies (P=0.572) (Fig. S4E). Together, these data from a highly controlled human malaria challenge model confirm that repeated *in vivo P. falciparum* infections drive changes in both $V\delta2^+$ and $V\delta1^+$ T cell frequencies. Increased $V\delta1^+$ T cell frequencies correlated with the development of asymptomatic malaria after CHMI4, while $V\gamma9/V\delta2^+$ T cell frequencies decreased between infections and were not durably maintained after CHMI4 in asymptomatic subjects, suggesting that regulation of $V\gamma9/V\delta2^+$ T cells may contribute to symptom reduction, a hypothesis that is consistent with previous reports in the context of natural infection (38–40).

Repeated *P. falciparum* infection initiates $V\delta1_{naive}$ to $V\delta1_{effector}$ T cell differentiation

Next, we investigated the impact of repeated *P. falciparum* infections on the differentiation of γδ T cell subsets. Although $CD27^{hi}$ $CD28^+$ $V\delta1_{naive}$ T cells were the main population of $V\delta1^+$ γδ T cells in subjects prior to CHMI (malaria naïve), this cell population decreased after repeated *P. falciparum* infections (Fig. 4A). Conversely, $CD27^{lo}$ CX_3CR1^+ $V\delta1_{effector}$ cells became the dominant population within total $V\delta1^+$ T cells (Fig. 4B). The increase in the $CD27^{lo}$ CX_3CR1^+ $V\delta1_{eff}$ector T cell population also correlated with a reduction in malaria symptoms (Fig. S4F). In response to a combination of inflammatory cytokines and HMB-PP stimulation, it has been proposed that $V\gamma9/V\delta2^+$ T cells switch phenotype from $CD27^+$ $CD28^+$ to $CD27^-$ $CD28^-$ (41); however, we found no significant changes in these populations across repeated *P. falciparum* infections (Fig. 4C). As noted earlier, $V\gamma9/V\delta2^+$ T cells can control parasite replication through CD16-mediated antibody-dependent cytotoxicity (24), we found that CD16 expression was upregulated on $V\delta1^+$ T cells, but not $V\gamma9/V\delta2^+$ T cells after repeated *P. falciparum* infections (Fig. 4D). Interestingly, subject 17 displayed a major $CD27^-$ $CD28^-$ $CD16^+$ $V\gamma9/V\delta2^+$ T cell population that persisted over time (Fig. 4C and D). $V\delta1^+$ T cells consistently expressed the T cell activation marker CD38 after each *P. falciparum* infection, while $V\gamma9/V\delta2^+$ T cells only significantly upregulated CD38 after CHMI1 and 2 (Fig. 4E). We previously showed that $V\delta1_{effector}$ cells possess significant cytotoxic potential (19, 28). Here, we found that repeated *P. falciparum* infection drove $V\delta1^+$ T cells to express Gzm A, B, perforin, but not Gzm K (Fig. 4F), whereas $CD8^+$ T cells had no significant increase in Gzm A, B, perforin or Gzm K (Fig. S4G). In keeping with their pre-formed cytotoxic potential, $V\gamma9/V\delta2^+$ T cells retained robust levels of Gzm A, B, K and perforin after repeated *P. falciparum*

infections (Fig. 4F). Our data indicate that *in vivo P. falciparum* infection in humans drives the differentiation of human $V\delta1_{effector}$ γδ T cells.

Repeated *P. falciparum* infections drive diverse waves of γδTCR selection

Next, we sought to understand whether repeated CHMIs impacted γδTCR repertoires. We used the approach described above (Fig. 2) and sorted $V\delta2^{+}$ and $V\delta2^{neg}$ γδ T cell populations from longitudinal timepoints from all five CHMI subjects. We then analyzed the relationship between $CD27^{lo}$ CX_3CR1^{+} $V\delta2^{neg}$ $_{effector}$ cells and $V\delta2^{neg}$ TCR repertoires prior to CHMI1 and at CHMI4 + 21d in subject 2 (Fig. 5A). At baseline, $V\delta2^{neg}$ γδ T cells were predominantly $CD27^{hi}$ CX_3CR1^{neg} and displayed a reasonably diverse γδTCR repertoire (Fig. 5A), but then after repeated CHMIs we observed a shift toward a $CD27^{lo}$ CX_3CR1^{+} effector phenotype (Fig. 5A). Alongside these phenotypic changes, clonotypes found prior to CHMI1 remained stable or contracted over time, and new $V\delta1^{+}$ clonotypes expanded, suggesting the potential recruitment of specific TCR sequences into the γδ T cell immune repertoire after repeated CHMIs (Fig. 5B). Analysis of the $V\delta2^{neg}$ γδ T cell repertoires indicated Vγ9 and Vδ1 chain usage to be the most prevalent (Fig. 5C). Overall, diversity within $V\delta2^{neg}$ or $V\delta2^{+}$ γδ T cell repertoires did not show any significant change (Fig. S5A). Next, we assessed if CDR3 clonotype changes were occurring in $V\delta2^{+}$ TCR repertoires. We found that $V\delta2^{+}$ clonotypes remained stable over time, despite significant changes in the frequencies of the total population (Fig. 5D). Interestingly, in subject 17, the $V\delta2^{+}$ TCR repertoire was already dominated by hyperexpanded CDR3γ and δ sequences at baseline (Fig. S5B), in contrast to all other $V\delta2^{+}$ TCR repertoires in this study. Given the stability of $V\delta2^{+}$ TCR clonotype repertoires at each CHMI, we then assessed the potential for dynamic changes in $V\delta2^{neg}$ γδ T cell repertoires at each CHMI and over time. Analysis of the γδTCR repertoire of subject 4 from baseline and over subsequent CHMI's 1, 3 and 4 indicated dynamic changes in the TCR repertoire, with an increase in low frequency clonotypes at CHMI1 and establishment of a broader immune repertoire over time (Fig. 5E). We then analyzed the 20 most prevalent clonotypes at baseline (subject 4, 10, 17) or at CHMI1 (subject 9; Fig. S5C). We found that prevalent baseline clonotypes declined with each CHMI and we observed waves of new clonotypes that expanded into the most abundant 20 clonotypes after each CHMI (Fig. 5F). In many cases these clonotypes were found at low frequency in the preceding timepoint, suggesting that each CHMI drove rounds of γδTCR selection (Fig. 5F). $V\delta2^{neg}$ γδ T cell repertoire clonotypes possessed few overlapping clonotypes between individuals, while there were many inter-individual overlapping TCRγ sequences in $V\delta2^{+}$ TCR repertoires (Fig. S5D). Although subject 10 and 17 were symptomatic at CHMI4 and did not have a significant increase in $V\delta1^{+}$ T cell frequencies, the repertoire of their $V\delta2^{neg}$ γδTCR repertoire also displayed waves of clonotype selection (Fig. 5F). Together, the Vγ9/$V\delta2^{+}$ T cell response to *P. falciparum* infection displays a highly stable polyclonal immune repertoire over time and infection. In contrast, $V\delta2^{neg}$ γδ T cell repertoires underwent dramatic remodeling of the γδTCR repertoire and displayed waves of clonal selection after each *P. falciparum* infection.

Previous *P. falciparum* exposure licenses $V\delta1^{+}$ T cells reactivity to blood-stage parasites

Finally, we explored the reactivity of γδ T cell subsets towards *P. falciparum* blood-stage parasites. PBMCs from Australian adults with no history of malaria exposure were co-

cultured with *P. falciparum* infected red blood cells (*Pf*RBC) or trophozoite/schizont extracts (*Pf*TSE) or or intact uninfected RBCs (uRBC) or extracts (uRBCE) as controls. Vδ1$^+$ T cells from Australian adults were unresponsive to *Pf*RBCs or *Pf*TSE, whereas Vγ9/Vδ2$^+$ T cell populations were responsive (Fig. 6A), corroborating prior studies (24, 33, 42). Our *in vivo* results (Fig. 3, 4 and 5) prompted us to re-challenge PBMCs of Australian subjects twice over the 5-day culture period. Upon re-challenge we found that Vδ1$^+$ T cells showed varying levels of proliferation after the second re-stimulation (Fig. 6B) but only in response to *Pf*TSE and not *Pf*RBCs. Previous studies have reported that Vδ1$^+$ T cells from individuals living in malaria endemic regions of Gambia or Tanzania were unresponsive to *Pf*RBC *in vitro* (33, 42, 43). Using PBMCs from two Malian subjects and a malaria-naïve subject after 2 CHMIs (subject 10 at CHMI3+1d), we found that Vδ1$^+$ T cells proliferated in response to *Pf*TSE but not *Pf*RBC after a single stimulation (Fig. 6C and D). This differential responsiveness to *Pf*RBC or *Pf*TSE was not consistently seen in paired Vγ9/Vδ2$^+$ T cell populations or in malaria unexposed Australian subjects (Fig. 6D). These data indicate that prior *P. falciparum* infection primes Vδ1$^+$ T cells for proliferate upon re-challenge with *P. falciparum* parasites.

Discussion

γδ T cells have been implicated in the immune response to pathogenic microbes, including bacteria, viruses and parasites (9). These responses in mice and humans appear to be mediated by innate-like γδ T cell populations, often utilizing semi- or invariant γδTCR repertoires that allow rapid effector responses to be mounted during the acute phases of microbial infection (9). Emerging evidence is currently re-shaping our understanding of the immunobiology of human γδ T cell populations and γδ T cells have the potential for both innate and adaptive properties (44). However, the adaptive-like features of Vδ2^{neg} γδ T cell subsets are only partially understood (19, 28, 29, 45), and the establishment of this arm of the immune response to infectious disease has remained unclear.

Here, we show that repeated *in vivo P. falciparum* infection impacts populations of circulating innate-like Vδ2$^+$ and adaptive-like Vδ1$^+$ γδ T cells. We found that repeated *P. falciparum* infection triggers the differentiation of Vδ1$^+$ T cells from a Vδ1$_{naive}$ phenotype into a distinct Vδ1$_{effector}$ subset, concomitant with dynamic clonotype selection in the γδTCR repertoire with each *P. falciparum* infection. Together, our data indicate that *P. falciparum* infection drives the selection and differentiation of the γδ T cell repertoire.

The association of human γδ T cells and malaria has been largely attributed to the remarkable responsiveness of innate-like Vγ9/Vδ2$^+$ T cells to *P. falciparum* infection (43, 46, 47). In line with this, we found that Vγ9/Vδ2$^+$ T cells were retained after natural infection in Malian subjects and increased in frequency upon exposure to repeated CHMI, an observations that is likely due to encounter with blood stage *P. falciparum* merozoite-derived HMB-PP (15, 24, 48), and possibly also be in response to liver stage infection (4, 7, 49). However, notwithstanding hypotheses that Vγ9/Vδ2$^+$ T cells mount oligoclonal responses to microbial encounters (50, 51), we found that public Vγ9/Vδ2$^+$ TCR repertoires remained highly stable over time despite significant changes in cellular frequency. The composition of these repertoires were very similar to those seen in gestation (52), cord blood, and after birth

(17–19). Moreover, the cellular phenotype of Vγ9/Vδ2$^+$ T cells after repeated *P. falciparum* infection was highly stable. Thus, the γδTCR repertoire of innate-like Vγ9/Vδ2$^+$ T cells appears to allow sustained responsiveness upon *P. falciparum* infection.

In contrast to Vγ9/Vδ2$^+$ T cells, the exact nature of human Vδ2$^-$ γδ T cells, and in particular Vδ1$^+$ T cells, in the immune response to microbial pathogens is poorly defined, with recent studies identifying both innate- (26) and adaptive-like potential for these cells (28). Moreover, how Vδ1$^+$ T cells participate in the complex immune response to *P. falciparum* is largely unknown (30, 33, 34, 53, 54). Current paradigms for conventional memory αβ T cells indicate that T $_{effectors}$ arise from T $_{naïve}$ cells driven by antigen-specific challenge to provide a rapid memory-response upon re-exposure to the same pathogen (55). Whether a similar paradigm applies to human γδ T cells is unclear (56). Here, we demonstrate that Vδ1$_{effector}$ T cells are a major population in Malian children, and that Vδ1$_{naive}$ cells differentiate into Vδ1$_{effectors}$ after repeated *P. falciparum* infections in malaria-naïve adults. Given that Vδ1$_{effector}$ γδ T cells may infiltrate peripheral tissues (25), we speculate that *P. falciparum*-reactive Vδ1$^+$ γδ T cells will subsequently infiltrate the liver (25), and spleen (57). Therefore, *P. falciparum*-reactive Vδ1$^+$ γδ T cells may exert cytotoxic and/or immunoregulatory functions in peripheral tissues during malaria infection and may be possible to explore under certain clinical circumstances (58). Moreover, we also found that γδTCR repertoires undergo dynamic clonotype selection after each *P. falciparum* infection. While only a handful of the antigenic targets are known for Vδ2$^-$ γδTCRs, nearly all identified ligands to date are endogenous host proteins (13, 59). In the case of malaria, it has been proposed that the Vδ1$^+$ T cell response during *P. falciparum* infection is also driven by unknown endogenous host factors, based on the observation that Vδ1$^+$ T cells from malaria-exposed individuals do not respond to *P. falciparum* antigens *in vitro* (33, 42). Our findings, that Vδ1$^+$ T cells from malaria-exposed individuals react to *P. falciparum* lysate *in vitro*, suggests that Vδ1$^+$ T cells may also have the potential to recognize parasite-derived antigens.

In malaria endemic regions, non-sterilizing immunity to symptomatic malaria is gradually acquired with repeated *P. falciparum* infections (60). It is hypothesized that the acquisition of immunity to malaria in humans involves resistance to severe disease followed by resistance to uncomplicated disease (3). Our study provides a window into the dynamic evolution of innate-and adaptive-like γδ T cells in the context of natural *P. falciparum* infection and indicates that these cells may represent an important component of the cellular immune response that contributes to immunity to malaria (4–6). However, we cannot conclude from the current study that there is an association between Vδ1$_{effector}$ T cell expansion and protection from febrile malaria in the context of natural infection, as Malian children who still experience febrile malaria show evidence of Vδ1$_{effector}$ T cell expansion. Our previous analysis of the same cohort in Mali shows that the risk of febrile malaria gradually decreases with age over years of repeated malaria exposures (1). Subjects in the age range (7–14 years) included in the longitudinal portion of the current study are at lower risk of febrile malaria than younger children in the same cohort, but generally, even 7–14-year-olds have yet to acquire immunity that fully protects against febrile malaria from year to year, leaving open the possibility that Vδ1$_{effector}$ T cell expansion with repeated infections may contribute to the gradual acquisition of immunity to malaria in endemic areas. Studies

with larger sample sizes that encompass a broader age range and include more frequent assessments of γδ T cells relative to incident *P. falciparum* infections (both symptomatic and asymptomatic), will be required to assess the relationship between $V\delta1_{effector}$ T cells and the risk of febrile malaria in the context of natural infection.

The findings from our CHMI study suggest that initial $V\gamma9/V\delta2^+$ T cell activation may contribute to the early priming and activation of naïve $V\delta1^+$ γδ T cells, potentially involving the capacity of $V\gamma9/V\delta2^+$ T cells to phagocytose and present parasite antigens (22, 24). Moreover, we noted a reduction in $V\gamma9/V\delta2^+$ T cells at the fourth CHMI, consistent with prior studies showing that loss and dysfunction of $V\delta2^+$ T cells is associated with clinical immunity to malaria (40). How the emerging population of $V\delta1^+$ γδ T cells may contribute to protection from symptomatic malaria is unclear. While the regulatory functions of γδ T cells in response to infectious diseases remains poorly understood, there is mounting evidence that these cells may play a role in regulating inflammation in the context of cancer (61, 62). Therefore, it seems plausible that repeated febrile malaria episodes could drive the expansion of a regulatory population of $V\delta1^+$ γδ T cells that dampen inflammation through IL-10 (31), TGF-β1 (63) or other mechanisms (64).

There are several limitations of this study. First, the Mali cohort was conducted in a small rural village where the population is predominantly of a single ethnic group, limiting the generalizability of our findings. Nonetheless, we observed lower frequencies of $CD16^+$ $V\gamma9/V\delta2^+$ T cells in the Mali cohort relative to studies in Uganda and Brazil (24, 65). Thus, it will be of interest to further investigate the impact of genetics and/or environmental factors underlying regional differences. Secondly, the number of subjects included in the CHMI study was relatively small, precluding a rigorous analysis of the factors that underlie the inter-individual variability we observed in γδ T cell responses.

In summary, our study shows that both innate and adaptive-like properties of the human γδ T cell repertoire are driven by *P. falciparum* infection *in vivo*. $V\delta2^+$ γδ T cells mount a rapid innate-like polyclonal immune response to acute *P. falciparum* infection. Alongside these innate-like $V\delta2^+$ γδ T cell responses, repeated *P. falciparum* infection established clonally selected populations of adaptive-like $V\delta1_{effector}$ γδ T cells. Together, our study suggests the importance of future studies exploring the role of the γδ T cell repertoire in contributing to the establishment of clinical immunity to malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data are available in Data File S1. The T cell receptor (TCR) sequence data that support the findings of this study have been deposited in the Open Science Framework (OSF) and is accessible from https://osf.io/7rdm9/ and https://osf.io/3qvmh/.

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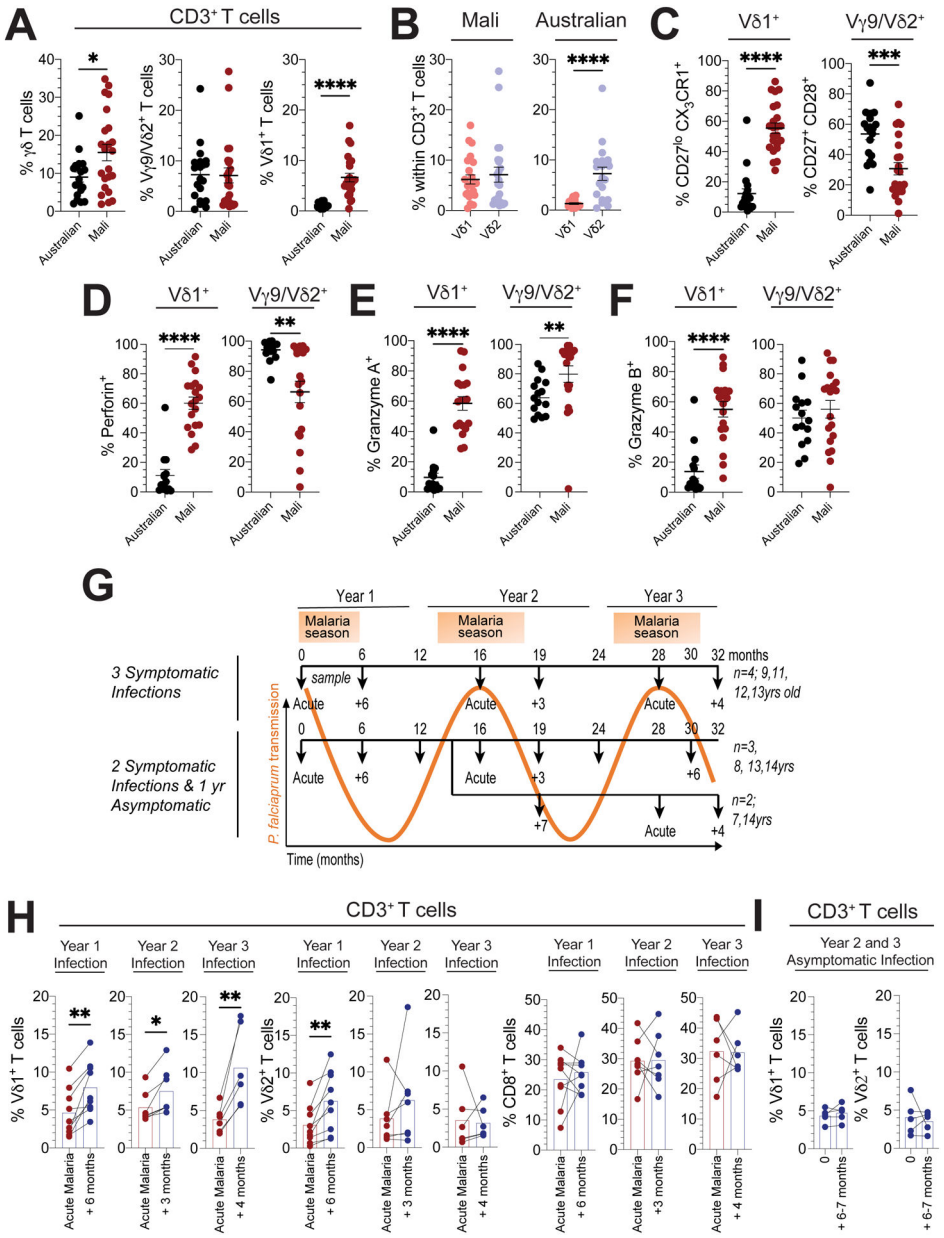

Figure 1. Increased Vδ1$^+$ γδ T cells frequencies in Malian subjects exposed to *P. falciparum* infection.

In age and gender matched Malian (n=23) or Australian subjects (n=20): **A.** Frequencies of total γδ, Vγ9/Vδ2$^+$ and Vδ1$^+$ T cells within CD3$^+$ T cells, **B.** Frequencies of Vγ9/Vδ2$^+$ and Vδ1$^+$ T cells in total CD3$^+$ T cells, **C.** Frequencies of CD27^{lo} CX$_3$CR1$^+$ cells within Vδ1$^+$ or CD27$^+$ CD28$^+$ cells within Vγ9/Vδ2$^+$ T cells, **D.** Frequencies of perforin$^+$, **E.** Gzm A +, **F.** Gzm B$^+$ cells within Vδ1$^+$ or Vγ9/Vδ2$^+$ T cells (**D**, **E** and **F**: Malian n=19 and Australian n=15). **G.** Schematic of samples and malaria exposure for Malian subjects included in the longitudinal arm of our study. Subjects are stratified based on presentation with a confirmed febrile malaria episode in all three years (n=4) or two febrile episodes (n=5), with either one episode in year 2 (n=3) or 3 (n=2). **H.** Frequencies of Vδ1$^+$,

Vγ9/Vδ2$^+$ and CD8$^+$ T cells in total CD3$^+$ T cells during febrile malaria and 3–6 months following treatment over the 3-year seasonal transmission periods. Year 1 (n=9), Year 2 (n=7) and Year 3 (n=6). **I.** Frequencies of Vδ1$^+$ and Vγ9/Vδ2$^+$ T cells within CD3$^+$ T cells over a 6-month period without a febrile malaria episode (n=5; 12 – 18 months, n=2, or 24 – 30 months, n=3). Bars show the mean and error bars indicate means ± SEM. Normality was tested using the Shapiro-Wilk test.; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; p-values were determined by Mann-Whitney test (**a - f**) and Wilcoxon matched-pairs signed rank test (**h, i**).

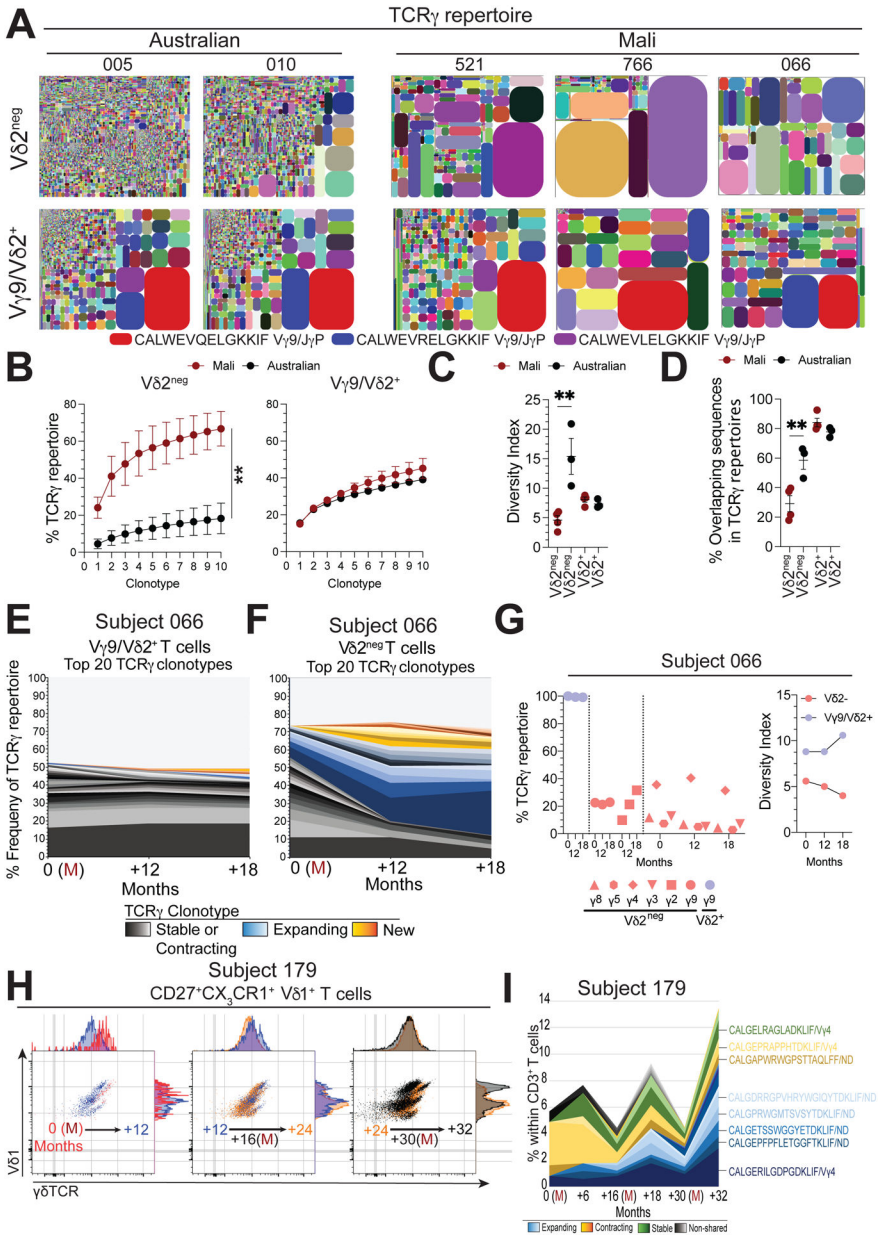

Figure 2. γδTCR repertoires in Malian subjects evolve over time.

A. TCR**γ** clonotype tree plot analysis of Vδ2^{neg} and V**γ**9/Vδ2^{+} T cell populations from Australian children or Malian children during stable periods without malaria transmission. Tree plots show unique clonotypes (coloured segments) and their proportion within the total repertoire (size), in general coloured clonotypes do not match between plots unless indicated. **B.** Pooled accumulated frequency curves of the top 10 most prevalent clonotypes in Vδ2^{neg} or V**γ**9/Vδ2^{+} TCR repertoires (Australian, n=3; Mali, n=4). **C.** Diversity index of Vδ2^{neg} and Vδ2^{+} **γ**δ T cell repertoires in Malian (n=4) or Australian (n=3) subjects. **D.** Frequency of shared CDR3**γ** (a.a.) sequences in Vδ2^{neg} and V**γ**9/Vδ2^{+} T cell repertoires (Australian, n=3; Mali, n=4). **E.** Longitudinal tracking of the 20 most abundant TCR**γ** clonotypes in V**γ**9/Vδ2^{+} and **F.** Vδ2^{neg} T cell repertoires over time in subject 066. (M)

indicates acute febrile malaria. **G.** Longitudinal analysis of Vγ chain usage and diversity index for $V\delta2^{neg}$ (red) and $V\gamma9/V\delta2^{+}$ (blue) T cell repertoires from subject 066. **H.** γδTCR expression patterns within ($CD27^{lo}$ CX_3CR1^{+}) $V\delta1^{+}_{effector}$ populations in donor 179. Each flow cytometry plot has two time points overlaid, indicated by an arrow, together covering three febrile *P. falciparum* infections (months 0, 17 and 30). **I.** TCRδ clonotypes sequencing relative to total $CD3^{+}$ T cells from subject 179. Error bars indicate means ± SEM. Normality was tested using the Shapiro-Wilk test.; *P < 0.05; **P < 0.01; ***P < 0.001; p-values were determined by two-way ANOVA with Sidaks post hoc testing (**b**) and one-way ANOVA with Holm-Sidaks post hoc testing (**c, d**).

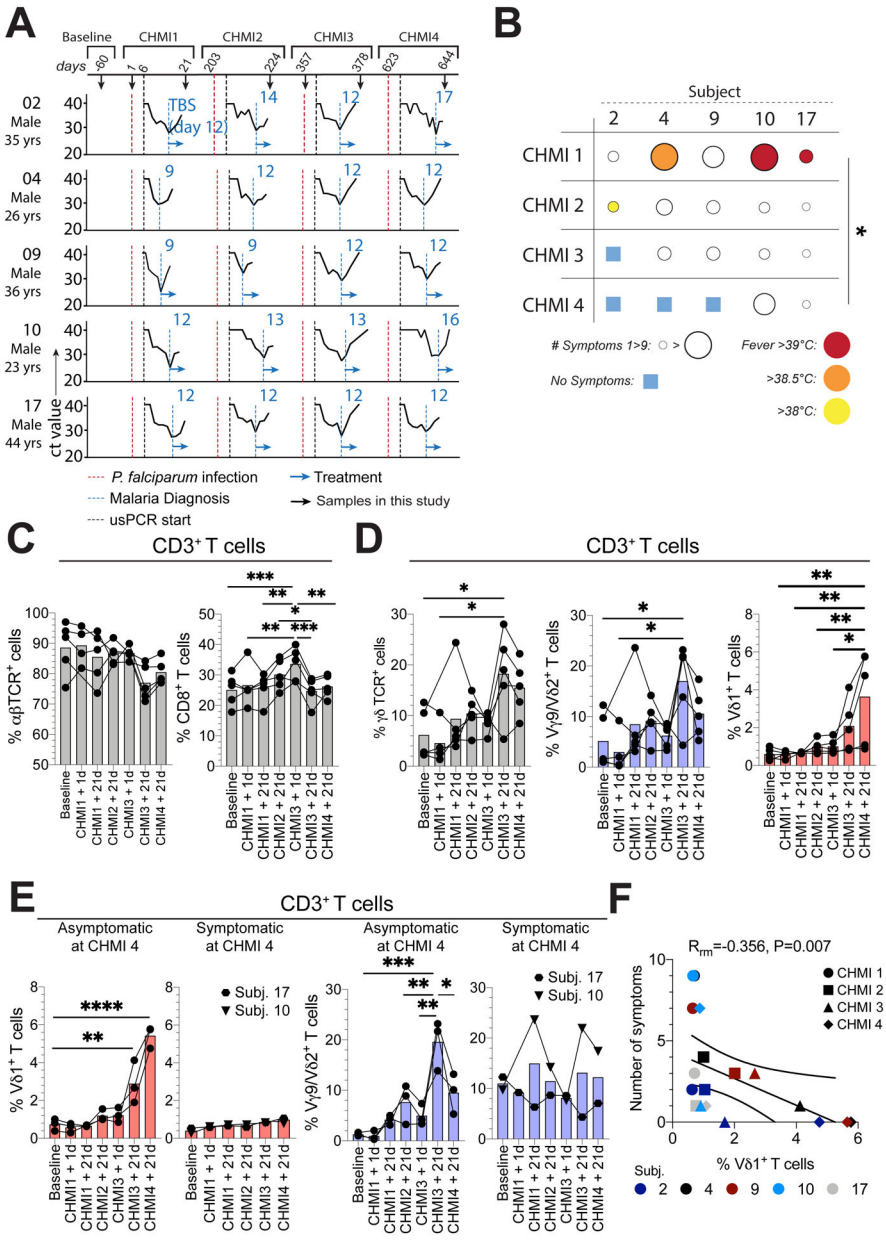

Figure 3. Repeated controlled *P. falciparum* infection drive clinical immunity to malaria and increased frequencies of γδ T cells.

A. Controlled human malaria infection (CHMI) study subjects, samples, parasite ultra-sensitive PCR (usPCR) detection curves and diagnosis by blood smear. **B.** Symptomology and fever analysis of each subject during each CHMI. **C-E**. Within total $CD3^+$ T cells: **C.** Total $\alpha\beta^+$ and $CD8^+$ T cell frequencies. **D.** Total $\gamma\delta^+$, $V\gamma9/V\delta2^+$ (blue), and $V\delta1^+$ (red) γδ T cells frequencies and **E.** $V\delta1^+$ and $V\gamma9/V\delta2^+$ frequencies in individuals that were asymptomatic or symptomatic at CHMI4. **F.** Repeated measure correlation between $V\delta1^+$ frequencies within total $CD3^+$ T cells and the number of symptoms each individual suffered at each CHMI. Bars show the mean. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001;

p-values were determined by Kruskall-Wallis test (**b**), linear mixed effects modelling with Bonferroni's correction (**c, d, e**) and repeated measures correlation (**f**).

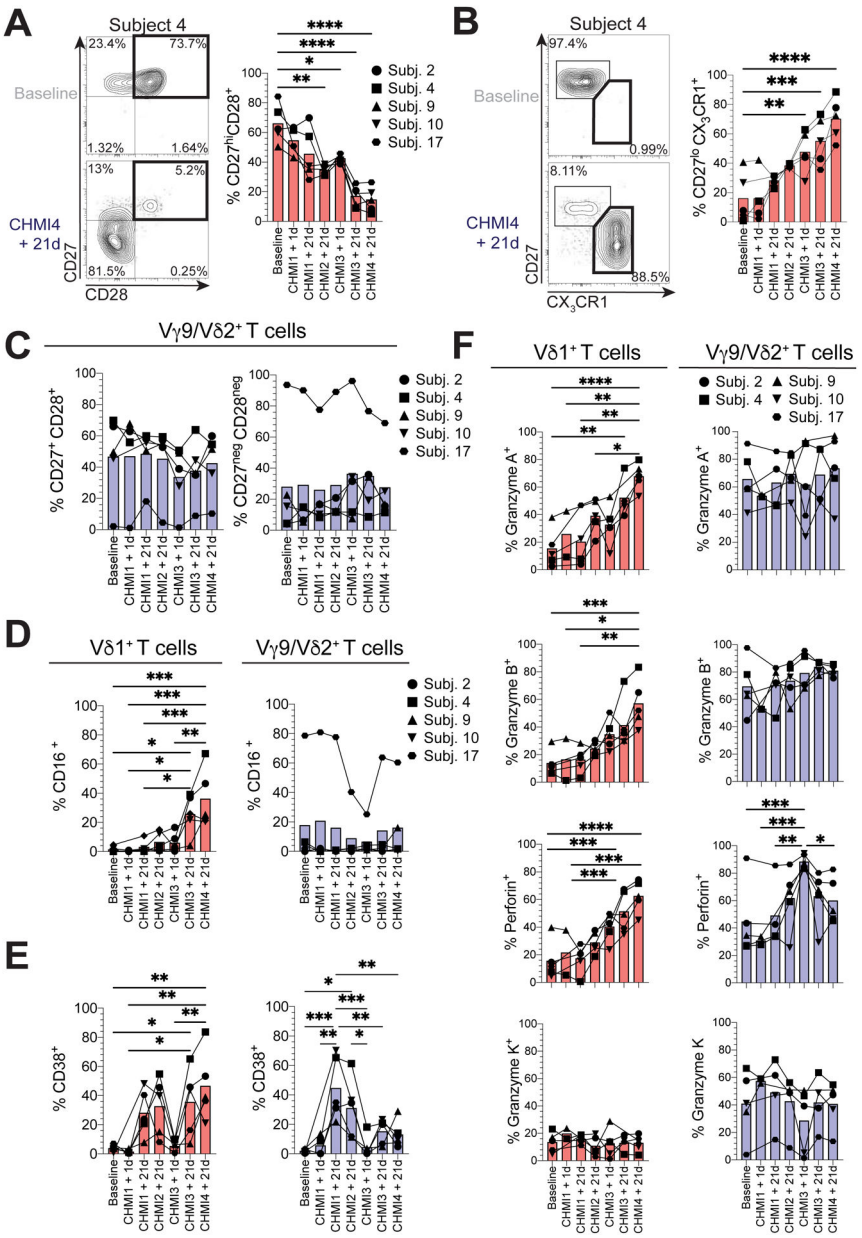

Figure 4. Repeated *P. falciparum* infection drives the differentiation of cytotoxic Vδ1$_{effector}$ T cells.

A. Representative flow cytometry plot and graph showing the frequencies of CD27^{+} CD28^{+} cells in Vδ1^{+} T cells after repeated CHMIs (n=5). **B.** Representative flow cytometry plot and graph showing the differentiation of CD27^{lo} CX$_3$CR1^{+} Vδ1$^{+}_{effector}$ cells after repeated CHMIs (n=5). **C.** Frequencies of CD27^{+} CD28^{+} and CD27^{neg} CD28^{neg} cells within Vγ9/Vδ2^{+} T cells. **D-F.** Within Vδ1^{+} (red) and Vγ9/Vδ2^{+} (blue) T cells, the frequencies of: **D.** CD16^{+}, **E.** CD38^{+}, **F.** Gzm A^{+}, B^{+}, K^{+} and perforin^{+} cells. Bars show the mean. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; p-values were determined by linear mixed effects modelling with Bonferroni's correction.

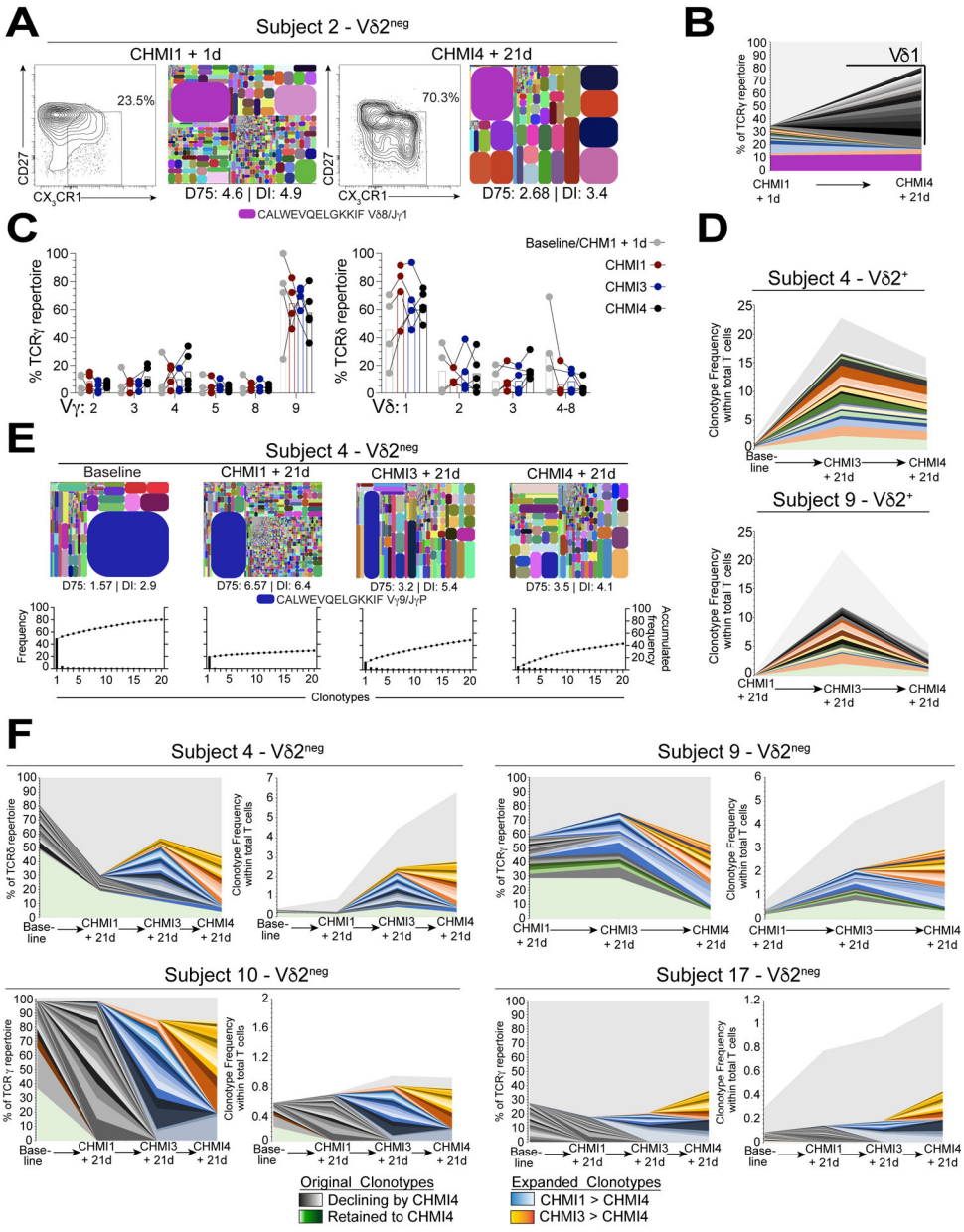

Figure 5. Repeated *P. falciparum* infection drives waves of Vδ1 TCR clonotype selection.

A. Flow cytometry plots showing frequencies of $CD27^{lo}$ CX_3CR1^+ $V\delta2^{neg}$ γδ T cells after repeated CHMI challenge in subject 2. TCRδ tree plots of the corresponding total $V\delta2^{neg}$ γδ T cells and DI are given for each tree plot. **B.** Increase in new Vδ1 sequences between baseline and CHMI4 within the top 20 clonotypes in TCRγ from subject 2. **C.** Vγ and Vδ usage in $V\delta2^{neg}$ T cell repertoires from baseline to CHMI 4 (n=4–5). **D.** Longitudinal tracking of the top 20 CDR3γ clonotypes in $V\delta2^+$ T cell repertoires as a frequency of total $CD3^+$ T cell populations. **E.** TCRγ tree plots showing $V\delta2^{neg}$ TCR repertoires at baseline and after repeated CHMIs in subject 4. The D75 and DI metrics are indicated. The graphs show the accumulated frequency of the top 20 clonotypes for each repertoire. **F.** Longitudinal tracking of the top 20 CDR3γ clonotypes in $V\delta2^{neg}$ TCR repertoires from

subject 4, 9, 10, 17; displayed as a proportion of the total TCRγ repertoire (left) or within the total $CD3^+$ T cell population (right).

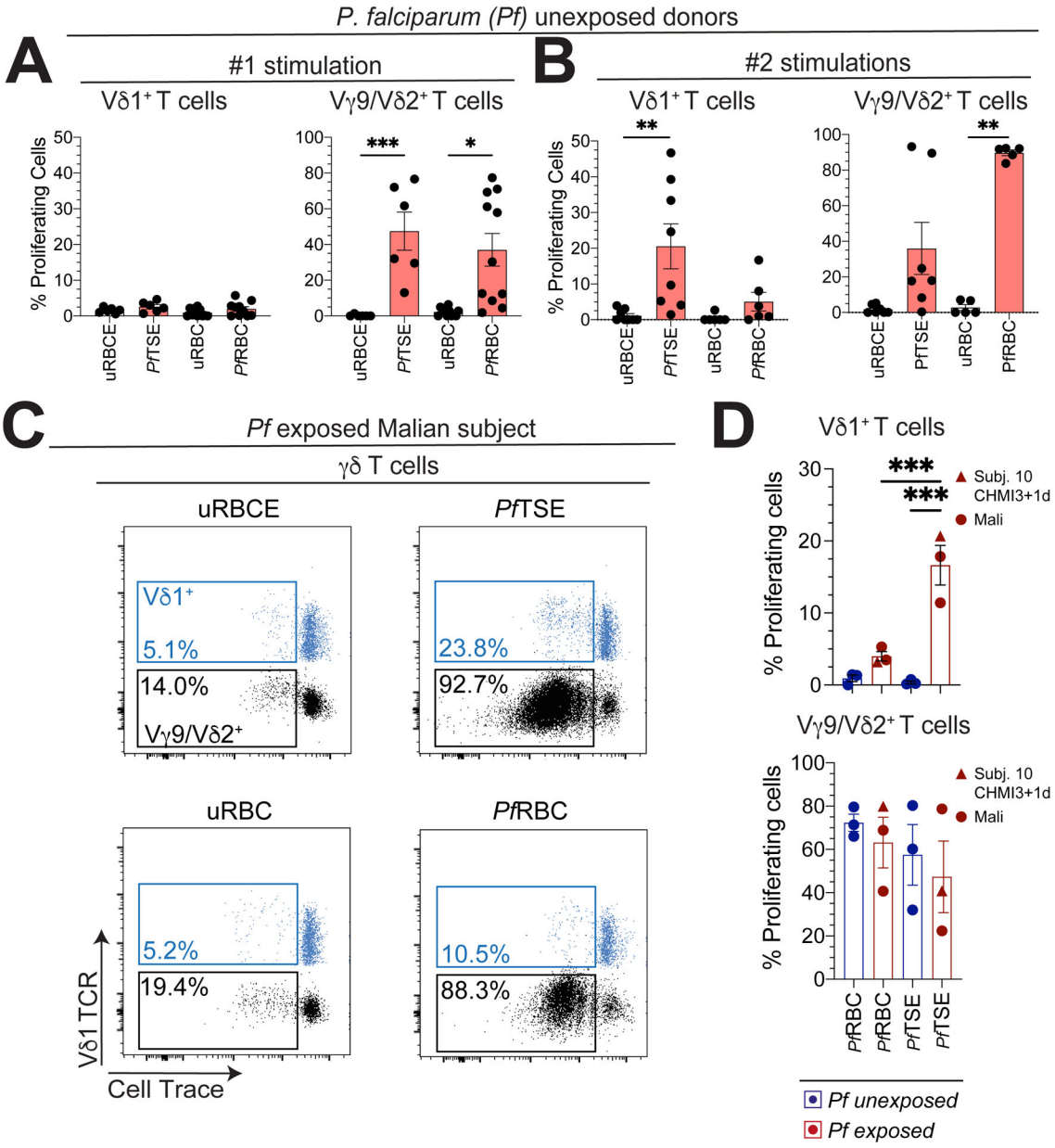

Figure 6. Previous *P. falciparum* exposure licenses Vδ1$^+$ T cells for parasite reactivity.

Vδ1$^+$ and Vγ9/Vδ2$^+$ T cells were assessed for proliferation in Australian adult donors with no history of malaria, PBMCs were labelled with cell trace and incubated for 6 days with **A.** One or **B.** two stimulations (at day 0 and 3 of culture) with *P. falciparum* trophozoite/early schizont extract (*Pf*TSE) or infected red blood cells (RBCs) and uninfected RBCs (uRBC) or extract (uRBCE) (uRBCE/PfTSE: n=6; uRBC/PfRBC: n=10). **C.** Representative flow cytometry plots show Vδ1$^+$ (blue) and Vδ2$^+$ (black) T cells assessed for proliferation in the PBMCs from a Malian subject after co-culture with *Pf*TSE. *Pf*RBCs, uRBC or uRBCE controls. **D.** Graphs show the proliferation of Vδ1$^+$ and Vγ9/Vδ2$^+$ T cells from two Malian subjects with a history of repeated prior exposure to *P. falciparum* malaria, subject 10 at CHMI3 + 1d and three independent Australian donors with no history of

malaria exposure. Each data point represents the proportion of proliferating cells in cultures exposed to *Pf*RBCs or *Pf*TSE minus the response to uRBC or uRBCE controls. Bars show the mean ± SEM. Normality was tested using the Shapiro-Wilk test.; **P < 0.01; p-values were determined by one-way ANOVA with Holm-Sidak's post hoc testing (**d**).