Adults with *Plasmodium falciparum* malaria have higher magnitude and quality of circulating T-follicular helper cells compared to children

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Summary

Background Protective malarial antibodies are acquired more rapidly in adults than children, independently of cumulative exposure, however the cellular responses mediating these differences are unknown. CD4 T-follicular helper (Tfh) cells have key roles in inducing antibodies, with Th2-Tfh cell activation associated with antibody development in malaria. Whether Tfh cell activation in malaria is age dependent is unknown and no studies have compared Tfh cell activation in children and adults with malaria.

Methods We undertook a comprehensive study of Tfh cells, along with B cells and antibody induction in children and adults with malaria. Activation and proliferation of circulating Tfh (cTfh) cell subsets was measured *ex vivo* and parasite-specific Tfh cell frequencies and functions studied with Activation Induced Marker (AIM) assays and intracellular cytokine staining.

Findings During acute malaria, the magnitude of cTfh cell activation was higher in adults than in children and occurred across all cTfh cell subsets in adults but was restricted only to the Th1-cTfh subset in children. Further, adults had higher levels of parasite-specific cTfh cells, and cTfh cells which produced more Th2-Tfh associated cytokine IL-4. Consistent with a role of higher Tfh cell activation in rapid immune development in adults, adults had higher activation of B cells during infection and higher induction of antibodies 7 and 28 days after malaria compared to children.

Interpretation Our data provide evidence that age impacts Tfh cell activation during malaria, and that these differences may influence antibody induction after treatment. Findings have important implications for vaccine development in children.

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Abbreviations: cTfh, circulating T-follicular helper; AIM, Activation Induced Marker; OPA, opsonic phagocytosis; GC, germinal centre

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Keywords: Plasmodium falciparum; T-follicular helper cells; immunity; malaria; antibodies

Research in context

Evidence before this study

Immunity to malaria is both cell-mediated and also mediated by antibodies that block parasite replication. In areas of high malaria exposure, these antibodies are acquired in childhood during repeated malaria infections. Antibody development is also influenced by age, with protective immunity and antibodies acquired more rapidly in adults compared to children, independently of cumulative exposure. T-follicular helper (Tfh) cells are the CD4 T cell subset that drives antibody induction. In adults with experimental sub-patent malaria, activation of Th2-Tfh cells, but not other subsets, is correlated with antibody development after parasite treatment. However, in children with naturally acquired malaria, Tfh cell activation is restricted to Th1-Tfh subsets. To date, no studies have directly compared Tfh cell activation in children and adults during symptomatic naturally acquired malaria and it is unknown whether age driven differences in Tfh cells may contribute to age dependent antibody development.

Added value of this study

Here we identified that Tfh cell activation, and the frequency of parasite-specific Tfh cells, is higher in adults compared to children with malaria. B-cell activation and plasmablast frequencies were also higher in adults, suggesting that increased Tfh cell activation is positively associated with antibody development in adults. Indeed, we also report that after a malaria infection, antibody levels increase in adults but not children. These studies for the first time identify a cellular response that is higher in adults that may underpin age dependent acquisition of immunity.

Implications of all the available evidence

Due to the central importance of Tfh cells in antibody development, this cell subset is an attractive target of strategies to boost antibody development following vaccination. Based on our data and prior studies, findings suggest that age may be an important factor in Tfh cell activation. These age dependent differences should be carefully considered in vaccine development for paediatric vaccines for malaria.

Introduction

Plasmodium falciparum malaria caused approximately 229 million infections and 409,000 deaths in 2019.[1] The majority of the malaria disease burden is in children, with immunity to severe and then mild disease acquired after repeated infections in endemic settings.[2] However, immune development is also impacted by the age of the individual, independently of cumulative exposure. For example, seminal studies on migrant populations moving from a malaria free region into a holoendemic setting showed that adults, while initially at increased risk of severe disease, acquired protection from uncomplicated malaria more rapidly than children during subsequent infection.[3,4] Antibodies, which are essential mediators of anti-parasitic immunity and protection from malaria,[5] were induced and/or maintained more rapidly in adults compared to children after the same number of malaria infections.[3,4] Consistent with this finding, anti-parasitic immunity increases with both exposure and age in children in high endemic settings.[6,7] Generation of protective antibodies is the key to the development of highly protective vaccines. However, achieving high efficacy with malaria vaccines in children has been an enduring challenge. Most vaccines evaluated in clinical trials in paediatric populations have shown only low or no efficacy.[8-10] Understanding the cellular determinants underpinning the differences between children and adults in the generation of antibody responses may provide important insights that inform the development of efficacious vaccines in the paediatric age group.

Antibody development is driven by T-follicular helper (Tfh) cells that provide crucial help for B-cells in the germinal centre (GC) to undergo activation, affinity maturation, class-switching, and differentiation into memory B-cells (MBCs) and antibody secreting plasma cells.[11] In humans, circulating CXCR5+ PD-1+ Tfh (cTfh) cells have similar phenotypic, functional, and transcriptional profiles to that of lymphoid Tfh cells (CD45RO+ $CXCR5^{hi}$ $PD\text{-}1^{hi}$+ Bcl-6+).[12,13] Recent studies have confirmed that blood cTfh cells are related to Tfh cells in lymph nodes,[14,15] supporting the analysis of cTfh cell responses from peripheral blood samples to infer phenotypes of lymphoid Tfh. During activation, Tfh cells upregulate the expression of surface molecules, such as inducible co-stimulator (ICOS) and intracellular proliferation marker Ki67, which are necessary for cell development and function.[16,17] Human cTfh cells (CXCR5+ PD-1+) can be further classified into different subsets based on CXCR3 and CCR6 expression (Th1-, Th2-, and Th17-like), with each subset having distinct functional and cytokine profiles.[13] Studies in healthy adults have shown that Th2- (CXCR3- CCR6-

CXCR5+ PD-1+) and Th17-cTfh (CXCR3- CCR6+ CXCR5+ PD-1+) cells were superior to Th1-cTfh (CXCR3 + CCR6- CXCR5+ PD-1+) cells in inducing naÿve B-cells to secrete antibodies in response to polyclonal stimulation with staphylococcal enterotoxin B super antigen.[13] Transcriptional profiles of CXCR3- cTfh cells (Th2- and Th17-like) have relatively stronger resemblance of lymphoid Tfh cells and have been linked to the induction of broadly neutralizing antibodies in HIV infection.[12] Nevertheless, the influence of Tfh cell subsets in antibody production following infection and vaccination appears to be pathogen-specific as Th1-cTfh cells are also associated with antibody induction in multiple viral infections, including influenza, HIV, and SARS-CoV-2.[18-22]

Despite the importance of Tfh cells in antibody induction, studies on cTfh cells during *P. falciparum* malaria in humans are very limited. We have recently shown that during experimental blood-stage infection with *P. falciparum* in human volunteers (malaria-naÿve adults), all cTfh cell subsets are activated during infection, but only Th2-cTfh cell activation during infection (8 days post inoculation) was associated with the induction of functional antibodies after treatment (~30 days after inoculation).[23] In contrast, in Malian children with *P. falciparum* malaria, only Th1-cTfh cells were found to be activated during infection and there was no association between Th1-cTfh cell activation during infection and the induction of anti-malarial IgG following the malaria episode.[24]

Knowledge of the influence of specific Tfh subsets and activation phenotypes in response to malaria on antibody development in both children and adults has relevance to understanding acquisition of immunity both to infection and vaccination. We hypothesised that Tfh cell activation during malaria is more robust in adults compared to children, and that adults have higher induction of functional antibodies after parasite treatment. To test this hypothesis, we comprehensively quantified cTfh and B-cell responses during malaria, and antibody induction after treatment in children and adults from Papua, Indonesia. Unique to this malaria endemic area, both children and adults are susceptible to symptomatic disease because of high migration and transient visitation from non-malaria areas.[25,26] We quantified cTfh cells *ex vivo*, and characterised Tfh subsets distribution based on CCR6 and CXCR3 expression and activation and proliferation by measuring ICOS and Ki67 expression. To quantify malaria specific Tfh cells, we used Activation Induced Marker (AIM) assays, which can sensitively detect Tfh cells that produce only low levels of cytokines following stimulation.[27-29] Together these data inform our understanding of how age impacts immune development to malaria, and have implications for vaccine development in children.

Methods

Ethics statement

Written informed consent was obtained from all study participants or, in the case of children, parents or guardians. Studies were approved by the ethics committees of the Northern Territory Department of Health and Menzies School of Health Research (Darwin, Australia) #HREC 05-16, #HREC 03-64, #HREC 10-1397, QIMR-Berghofer #HREC P3445, the Indonesian National Institute of Health Research and Development (Jakarta, Indonesia) #NIHRD KS.02.01.2.1.4042, and the Oxford Tropical Research Committee (Oxford, United Kingdom) #OXTREC 013-04.

Study site and study participants

Plasma and peripheral blood mononuclear cell (PBMC) samples were obtained from previous clinical trials conducted between 2004 and 2007 in Timika, Papua, Indonesia. Timika is a forested lowland town located in South-Central Papuan province of Indonesia.[25,26] Malaria transmission in lowland Papua is perennial with a prevalence of 28.3% in children <5 years, 46.3% in children aged 5-15 years, and 36.8% in adults >15 years.[25,26] While clinical immunity to malaria is acquired by lowland Papua adults, the region also has a steady 16% annual population growth due to economic migration,[30] with adult recent non-Papuan migrants and visiting or migrating adult indigenous Highland Papuans remaining susceptible to malaria disease due to no/low prior malaria exposure.[30] Consistent with this, within our three patient cohorts, children were a mix of non-Papuan and Low and Highlander ethnicities, while all but one adult were non-Papuan and Highlanders (**Tables 1/2/3**). For the parent clinical trial, children and adults with slide-confirmed malaria and fever or a history of fever within the last 48 hours were enrolled in randomised controlled trials of artemisinin combined therapy.[31] Malaria parasite infection was categorised as mono-infection with *P. falciparum* or *P. vivax* via microscopy. Exclusion criteria included pregnant or lactating women and children with a body weight of 10kg and under. Patients were followed post treatment. In a subset of trial participants, blood samples were collected at enrolment (day 0) for PBMC isolation (<8 mL for children and <20mL for adults), or at enrolment day 0 and day 7 and day 28 post-treatment for plasma isolation. For severe malaria enrolment, *P. falciparum*-infected ≥18 years old adults with moderately severe and severe malaria were included within the same study protocols.[32,33] Severe malaria patients were treated with intravenous quinine or artesunate in accordance with prevailing national policy guidelines upon enrolment at baseline (day 0). Only participants with mono-infection with *P. falciparum* were included in the current study. Healthy endemic controls from the same study site were also included, and were malaria negative

	Children <12			Adult ≥18						
	Healthy	Pf-U	P[1]	Healthy	Pf-U	Pf-Sm	P[1]	P[2]	P[3]	P[4]
n	6	14		8	24	7^				
Male (n, %)	2, 33%	7, 50%	0.6	7, 87.5%	12, 50%	6, 85.7%	0.1	0.9	0.9	0.2
Age (median, [IQR])	7.5 [7-8.75]	6.5 [5-7]	0.16	29 [27-31]	22 [20-27.8]	26 [21.3-27]	**0.02**	<0.01	0.15	0.48
Ethnicity:										
Non-Papuan	2, 33.3%	7, 50%	0.7	2, 25%	0	3, 42.9%	0.06	<**0.01**	0.6	**0.01**
Highland Papuan	2, 33.3%	5, 35.7%		6, 75%	24, 100%	4, 57.1%				
Lowland Papuan	2, 33.3%	2, 14.3%		0	0	0				
Parasitemia (HRP-2 ng/mL, median, [IQR])#	NA	194.1 [29.2-307]	NA	NA	488.5 [55.0-1444]	21,458 [5833-45041]	NA	0.12	NA	**0.01**

***Table 1*: Cohort Subset 1 participant characteristics.**
Fisher's exact test was used to compare frequency between groups for gender and ethnicity
Mann-Whitney U test was used to compare values between groups for age and parasite biomass
^Missing gender data on one individual in Pf-Sm group
#Missing HRP-2 data for one Pf-U adult
P[1] – comparison of healthy to Pf-U
P[2] – comparison of Pf-U adults to Pf-U children
P[3] – comparison of healthy to Pf-Sm
P[4] – comparison of adult Pf-U to Pf-Sm
Pf-U; uncomplicated *P. falciparum* malaria, Pf-Sm; severe *P. falciparum* malaria

individuals confirmed by PCR with no history of malaria infection in the previous month. For all participants used in this current study, parasite infection was successfully treated and no re-infection or recrudescence was detected at day 28 follow-up. Only severe patients with cerebral malaria were selected. We selected children based on <12 years of age and adults as ≥18 years old to avoid ambiguity of including adolescent aged individuals, and for consistency with previous studies of Tfh cell responses in children with malaria.[24] Three subsets of children and adult participants from these clinical trials and healthy non-infected endemic controls were selected based on plasma and PBMC availability and age only, with no other co-variants or matching considered in subject selection (Cohort Subset 1 PBMCs from children uninfected n=6, uncomplicated malaria n=14, adults uninfected n=8, uncomplicated malaria n=24, severe malaria n=7; Cohort Subset 2 PBMCs from children uncomplicated malaria n=10, adults uncomplicated malaria n=11; Cohort Subset 3 plasma from children n=22, adults n=46 at day 0, day 7 and 28; Table 1/2/3). Available clinical/demographic data (sex, age, ethnicity and parasitemia levels) between children and adult groups presented in Table 1/2/3 was only analysed after sample selection. No sample size/power analysis calculation was performed, and sample size was determined based on previous comparable studies.[24]

Parasitemia

Parasitemia during infection was calculated by blood smear microscopy or via Histidine-rich protein 2 (HRP-2) ELISA. For blood smear microscopy, parasitemia (parasites/μL blood) was calculated from the number of parasites counted by microscopy multiplied with white cell count (cells/μL) and divided with the number of white cells counted by microscopy. For HRP-2, protein was detected with standard ELISA by detecting plasma concentration of HRP-2 protein, as previously described.[34] 96-well flat bottom plate (Maxisorp, Nunc) was coated *P. falciparum*-specific HRP-2 IgM (Immunology Consultant Laboratory) and blocked with 2% bovine serum albumin (BSA) in PBS. Plates were then incubated with plasma samples, and HRP-2 protein was detected by adding *P. falciparum*-specific HRP-2 horseradish peroxidase-conjugated IgG (Immunology Consultant Laboratory) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Scientific). Pooled severe malaria plasma with known HRP-2 concentrations was used as a standard.

Activation induced marker (AIM) assay and cytokine induction by PMA/Ionomycin stimulations

For the detection of antigen-specific cTfh cells using AIM, cryopreserved PBMC samples were thawed in RPMI 1640 (Gibco) containing 10% FCS and 0.02% Benzonase. Following 2 hours of rest at 37°C, 5% CO_2, viable cells were cultured overnight in 96-well plates in 10% FCS/RPMI at 1×10^6 cells per well and stimulated for 18 hours with pRBCS or uninfected red blood cells (uRBCs) at 1:1 ratio of PBMCs:RBCs. After stimulation, supernatants PBMCs were collected for quantification of cytokines using Cytometric Bead Array. Cells were

then stained for flow cytometric assessment as per described in the protocol below.

For cytokine induction following PMA and Ionomycin stimulation, PBMC samples were rested on a separate plate overnight in 10% FCS/RPMI after thawing. After rest, PBMCs were stimulated with PMA (25 ng/mL; Sigma) and Ionomycin (1 μg/mL; Sigma) for 6 hours at 37°C, 5% CO_2. Brefeldin A (10 μg/mL; BD Biosciences) and monensin (10 μg/mL; BD Biosciences) were added after the first 2 hours of stimulation. Cells were then stained for flow cytometric assessment as described in the protocol below. Sample availability was prioritised for AIM assay, followed by PMA and Ionomycin stimulation.

Flow cytometry of PBMC samples

Cryopreserved PBMC samples were preserved in foetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO). Thawing of PBMC samples was done using RPMI 1640 (Gibco) containing 10% FCS and 0.02% Benzonase. Viable cells in each sample were counted using haemocytometer and Trypan blue staining. A minimum of 3 million and 1 million viable cells per sample was required for Tfh and B-cell staining panels, respectively. Sample availability was prioritised for Tfh cell phenotyping, followed by B-cells.

For cTfh cell phenotyping, cells were incubated at 37°C for 2 hours prior to staining. Cells were stained for 15 minutes at room temperature and in the dark. The following surface-labelled antibodies were used: anti-PD-1 PE (BD Biosciences, clone EH12.1, RRID: AB_2033989), anti-CD25 PE/CF594 (BD Biosciences, clone M-A251, RRID: AB_11151919), anti-CD4 PerCP-Cy5.5 (Biolegend, clone RPA-T4, RRID: AB_893322), anti-CXCR5 Pe-Cy7 (Biolegend, clone J252D4, RRID: AB_2562355), anti-CCR6 APC-R700 (BD Biosciences, clone 11A9, RRID: AB_2739092), anti-ICOS APC-Cy7 (Biolegend, clone C398.4A, RRID: AB_2566128), anti-CXCR3 BV421 (BD Biosciences, clone 1C6, RRID: AB_2737653), anti-CD14 V500 (BD Biosciences, clone M5E2, RRID: AB_10611856), anti-CD19 V500 (BD Biosciences, clone HIB19, RRID: AB_10562391), and LIVE/DEAD stain (Invitrogen). Cells were washed with 2% FCS/PBS and intracellular staining was performed using FoxP3 Fix/Perm staining kit (eBiosciences) as per manufacturer's instructions. The following intracellular antibodies were used: anti-Ki67 FITC (BD Biosciences, clone B56, RRID: AB_396302) and anti-FoxP3 AF647 (Biolegend, clone 206D, RRID: AB_439754).

B-cell staining include the following 2 panels of surface-labelled antibodies: 1) anti-IgG FITC (BD Biosciences, clone G18-145, RRID: AB_396121), anti-CD19 PE (Biolegend, clone HIB19, RRID: AB_314238), anti-CD21 PE/CF594 (BD Biosciences, clone B-Ly4, RRID: AB_2738231), anti-IgM PerCP-Cy5.5 (BD Biosciences, clone G20-127, RRID: AB_10611998), anti-IgD Pe-Cy7 (BD Biosciences, clone IA6-2, RRID: AB_10642457), anti-CD38 APC (BD Biosciences, clone HIT2, RRID: AB_398599), anti-CD3 AF700 (Biolegend, clone SK7, RRID: AB_2563420), anti-CD20 APC-Cy7 (Biolegend, clone 2H7, RRID: AB_314262), anti-CD27 BV421 (BD Biosciences, clone M-T271, RRID: AB_11153497), anti-CD14 V500 (BD Biosciences, clone M5E2, RRID: AB_10611856), and LIVE/DEAD stain (Invitrogen) and 2) anti-CD86 AF488 (Biolegend, clone IT2.2, RRID: AB_528881), anti-CD19 PE (Biolegend, clone HIB19, RRID: AB_314238), anti-CD21 PE/CF594 (BD Biosciences, clone B-Ly4, RRID: AB_2738231), anti-CD24 PerCP-Cy5.5 (Biolegend, clone ML5, RRID: AB_10960741), anti-IgD Pe-Cy7 (BD Biosciences, clone IA6-2, RRID: AB_10642457), anti-CD38 APC (BD Biosciences, clone HIT2, RRID: AB_398599), anti-CD20 AF700 (BD Biosciences, 2H7, RRID: AB_1727447), anti-PD-1 APC/Fire750 (Biolegend, clone EH12.2H7, RRID: AB_2616721), anti-CD27 BV421 ((BD Biosciences, clone M-T271, RRID: AB_11153497), anti-CD14 V500 (BD Biosciences, clone M5E2, RRID: AB_10611856), and LIVE/DEAD stain (Invitrogen). cTfh cells from the AIM assay were phenotyped using the following surface-labelled antibodies: anti-CCR7 PerCpCy5.5 (BD Biosciences, clone 150503, RRID: AB_10562553), anti-CD8 BUV496 (BD Biosciences, clone RP8-T8, Catalogue No.: 612942), anti-CD45RA BUV563 (BD Biosciences clone HI100, RRID: AB_2870211), anti-CD69 BUV737 (BD Biosciences, clone FN50, Catalogue No.: 612817), anti-CD3 BUV805 (BD Biosciences, clone SK7, Catalogue No.: 612893), anti-CD38 BV480 (BD Biosciences, clone HIT2, RRID: AB_2739535), anti-CCR6 BV650 (BD Biosciences, clone 11A9, RRID: AB_2738488), anti-CXCR5 BV711 (Biolegend, clone J252D4, RRID: AB_2629526), anti-HLA-DR BV750 (Biolegend, clone L243, RRID: AB_2800802), anti-CD4 BV785 (Biolegend, clone OKT4, RRID: AB_2563242), anti-CXCR3 PE-CF594 (BD Biosciences, clone 1C6/CXCR3, RRID: AB_11153118), anti-PD-1 PECy7 (BD Biosciences, clone EH12.1, RRID: AB_10611585), anti-OX40 APC (BD Biosciences, clone ACT35, RRID: AB_2738230), anti-CD25 APC-R700 (BD Biosciences, clone 2A3, RRID: AB_2744339), anti-ICOS APCCy7 (Biolegend, clone C398.4A, RRID: AB_2566128) . Viability stain was performed using Live/Dead BLUE (Invitrogen). Cells were incubated with all antibodies for 15 minutes at room temperature following 10 minutes of incubation with human Fc Block (BD Biosciences), with the exception of anti-CCR7 staining performed at 37°C for 45 minutes.

For the detection of intracellular cytokines following PMA/ Ionomycin stimulation, cells were incubated with the following surface-labelled antibodies: anti-CD8 BUV496 (BD Biosciences, clone RP8-T8, Catalogue No.: 612942), anti-CD3 BUV805 (BD Biosciences, clone SK7, Catalogue No.: 612893), anti-CCR6 BV650 (BD Biosciences, clone 11A9, RRID: AB_2738488), anti-

CXCR5 BV711 (Biolegend, clone J252D4, RRID: AB_2629526), anti-CD4 BV785 (Biolegend, clone OKT4, RRID: AB_2563242), anti-CXCR3 PE-CF594 (BD Biosciences, clone 1C6/CXCR3, RRID: AB_11153118), and anti-PD-1 PECy7 (BD Biosciences, clone EH12.1, RRID: AB_10611585) for 15 minutes at room temperature. Viability stain was performed using Live/Dead BLUE (Invitrogen). Intracellular staining was done using the BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. Intracellular cytokine antibodies include: anti-IFNγ BUV395 (BD Biosciences, clone B27, RRID: AB_2738277), anti-IL-10 BV421 (Biolegend, clone JES3-9D7, RRID: AB_2632952), anti-TNFα BV750 (BD Biosciences, clone MAb11, RRID: AB_2739709), anti-IL-17α FITC (Biolegend, clone BL168, RRID: AB_961390), anti-IL-21 PE (BD Biosciences, clone 3A3-N2.1, RRID: AB_1645516) and anti-IL-4 APC (Biolegend, clone MP4-25D2, RRID: AB_315131). To maximise surface labelling of CXCR3 and CXCR5 due to their downregulation after exposure to PMA and Ionomycin, anti-CXCR3 and anti-CXCR5 antibodies were additionally included along with PMA and Ionomycin during stimulation, in the presence of human Fc block.

All cells were resuspended in 2% FCS/PBS in FACS tube prior to acquisition using Beckman Coulter Gallios flow cytometer. For AIM and intracellular cytokine assay, cells were acquired using AURORA Spectral flow cytometer. Data analysis was done using FlowJo (version 10.7.2). Populations were gated as indicated; cTfh cells (**Supplementary Figure S2**), B-cells (**Supplementary Figure S4/5**). Boolean gates were analysed with Pestle (version 2)/Spice (version 6; J. Nozzi and M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institute of Health available at https://niaid.github.io/spice/).[35]

Cytokine analysis

Cytokine levels in culture supernatants were analysed using Human Cytometric Bead Array (CBA) flex sets (BD Biosciences) according to the manufacturer's instructions and included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-17A, IFNα, IFNγ, TNF, MCP-1, G-CSF and GM-CSF. Samples were acquired on a LSR Fortessa 5 flow cytometer fitted with a high throughput sampler using BD FACS Diva software (version 8.0) and data analysed using BD FCAP Array software (version 3.0).

Antibody responses to PfMSP2-3D7

Levels of different antibody isotypes against recombinant PfMSP2-3D7 were measured using standard enzyme-linked immunosorbent assay (ELISA), as described previously.[36] PfMSP2-3D7 (expressed in *E. coli*) was generated as described,[37] and generously supplied by Robin Anders. MSP2 was used as the antigen because it is an abundant merozoite surface protein, and a major target of immunity.[38-40] It has also been established as a major target of functional antibodies that promote complement fixation and opsonic phagocytosis,[41,42] and antibodies to MSP2 correlate with antibodies to the merozoite surface.[39,43] 96-well flat bottom plate (MaxiSorp, Nunc) was coated with 50 µL of 0.5 µg/mL recombinant PfMSP2-3D7 antigen in PBS overnight at 4°C, followed by washing. All washing between steps was done three times using 0.05% PBS-Tween. Subsequently, plates were blocked using 1% casein-PBS for 2 hours at 37°C. Plasma samples were then added in duplicates at 1:250 dilution in 0.1% casein-PBS, with 2 hours incubation at room temperature (RT). Levels of IgG subclasses and IgM were quantified by adding mouse anti-human IgM (clone HP6083, Life Technologies), IgG1 (clone HP6069, Life Technologies), IgG3 (clone HP6050, Life Technologies), IgG2 (clone HP6002, Life Technologies) and IgG4 antibodies (clone HP025, Life Technologies), followed by goat polyclonal anti-mouse IgG horseradish peroxidase (HRP) (Merck Millipore) at 1/2000 dilution in 0.1% casein-PBS and incubated for 1 hour at RT. Antibody levels were detected using TMB (Sigma) substrate, with enzymatic reactions stopped using 1M HCl. Optical density was measured at 450nm. For detection of complement-fixing antibodies, recombinant C1q at 10 µg/mL (Quidel) was added following incubation with sera. C1q fixation was detected with rabbit anti-C1q antibodies (Dako) and anti-rabbit-HRP (Bio-Rad). Pooled sera from Timika individuals with high antibody responses was used as positive controls and standardised between plates. Malaria naÿve controls obtained from Australian donors were used as negative controls and to define levels of positive responses as greater than average absorbance of malaria naÿve controls plus 3 standard deviations. Additionally, each plate includes blank wells without sample plasma. Absorbance values from blank wells were regarded as background non-specific readings and deducted from test sample readings.

Fcγ Receptor binding assay

Fcγ receptor-binding assay was performed using a plate-based assay, described previously.[44-46] 96-well flat bottom plate (MaxiSorp, Nunc) was coated with 50 µL of 0.5 µg/mL recombinant PfMSP2-3D7 in PBS overnight at 4°C, followed by washing. All washing between steps was done three times using 0.05% PBS-Tween. Subsequently, plates were blocked using 1% BSA for 2 hours at 37°C. Plasma samples were then added in duplicates at 1:100 dilution in PBS-BSA, with 2 hours incubation at RT. 50 µl of biotin-conjugated recombinant soluble FcγRIIa (rsFcγRIIa) H131 ectodomain dimer or rsFcγRIII V158 ectodomain dimer was added at 0.2 and 0.1 µg/ml respectively, followed by 1 hour incubation at 37°C. HRP-conjugated streptavidin (1/10,000) was then

added with 1 hour incubation at 37°C, followed by TMB substrate to measure enzymatic reactivity. The reaction was stopped using 1M sulfuric acid (H_2SO_4). The level of binding was measured as optical density at 450nm.

Opsonic phagocytosis assay using antigen-coated beads and isolated cells

The assay to detect antibody capacity to mediate opsonic phagocytosis by THP-1 promonocyte cell line was previously described.[42,46] Aqueous amine-modified red fluorescent latex beads (2μm size, Sigma) were washed twice with 400µL of PBS and centrifuged at 3000g for 3 minutes. 400uL of 8% glutaraldehyde (diluted in PBS) was added to the beads and incubated on a roller overnight at 4°C. After washing with PBS, 1mg/mL of recombinant PfMSP2-3D7 was added to the mixture and incubated for 4 hours on a vortex. The mixture was then centrifuged, and the pellet was collected as the bound protein fraction. 200μL of ethanolamine was added to the pellet to quench amine groups and the pellet was incubated for 30 minutes on the vortex. The pellet was subsequently washed in PBS and blocked with 1% BSA overnight at 4°C. The antigen-coated beads were stored 4°C in the presence of 0.1% SDS and 0.02% sodium azide. The density of latex beads coated with recombinant PfMSP2-3D7 was standardised to 5×10^7 beads/mL and was opsonized with serum samples (1/10 dilution in 1% Foetal calf serum (FCS)/FACS buffer) for 1 hour in the dark. Samples were then washed three times with 1% FCS/FACS buffer before co-incubation with THP-1 for phagocytosis. THP-1 cells were prepared in media containing RPMI-1640 (Gibco), 10% FCS, 1% glutamine, 1% penicillin-streptomycin (Gibco), and 0,1% 2-mercaptoethanol (Gibco). Phagocytosis was let to occur for 20 minutes at 37°C and samples were subsequently washed with 1% FCS/FACS buffer at 300g for 4 minutes. The proportion of THP-1 cells containing fluorescent-positive beads was measured using flow cytometry (FACS Verse, BD Biosciences) and analysed using FlowJo (version 10.4).

P. falciparum in vitro culture

P. falciparum 3D7 parasites were continuously cultured in human O+ red blood cells (provided by Australian Red Cross Lifeblood) in RPMI medium (Gibco) supplemented with 25 mM HEPES (Gibco), 30 ug/mL gentamicin (Gibco), 370 µM hypoxanthine (Sigma), 5% heat-inactivated human sera and 0.25% AlbuMAX II (Gibco). Parasite cultures were grown at 37°C in 1% O_2, 5% CO_2, 94% N_2 to mid-late trophozoite stage before enrichment in custom magnetic separator with MACS® CS columns (Miltenyi). Purified parasite infected red blood cells (pRBCs) were cryopreserved at 1:2 in Glycerolyte 57 Solution (Baxter HealthCare).

Bioinformatics clustering of cellular responses

The expression of Tfh cell markers PD-1, CD25, CXCR5, CCR6, CXCR3, ICOS, Ki67, and FoxP3 was analysed using CITRUS (cluster identification, characterization, and regression), as previously described.[47] For B-cell subset clustering, the following markers were analysed on panel 1) IgD, CD38, CD27, CD20, CD21, CD27, IgG, and IgM, and panel 2) IgD, CD38, CD20, CD24, CD21, CD27, CD86, and PD-1. CITRUS analysis provides a fully automated and unsupervised algorithm for the identification of phenotypically distinct cell populations using hierarchical clustering based on marker similarity and statistically compares these features between groups using predictive or correlative linearised regression model, thereby adding the advantage of minimising subjectivity or bias along with high reproducibility.[47] Comparison analysis was done between *P. falciparum*-infected children and adults. Flow cytometry data anchored on CD4+ T-cell, CD19 +CD3- B-cell (panel 1), and CD19+ B-cell (panel 2) were imported into CITRUS with gating strategy as described (**Supplementary Figure S2, S4/5**). The Nearest Shrunken Centroid (PAMR) association model with 1-standard error rate (cv.1se) and cluster characterization of abundance were used. A minimum cluster size of 5% was set to consider rare populations. Transformation cofactor was set at 150 along with 10 cross-validation folds, and equal event sampling (5,000 events). The predictive model output on cross-validation error rate and false discovery rate were generated and used to assess the model's predictive power (**Supplementary Figure S6**). We consider a cross-validation error rate of 20% and lower as acceptable, which is 80% predictive accuracy. tSNE dimensional reduction plot and PhenoGraph phenotype clustering (based on cell-surface markers) was generated using the cytofkit package in R.[48,49] Manually gated viable CD19+ B-cell (panel 2) and CD4+ T-cell were imported into cytofkit and analysed using PhenoGraph. PhenoGraph clusters cells into distinct cell populations by creating *k* nearest neighbours network (*k*NN) for each cell, adding weights to the *k*NN network, and partitioning the network into coherent cell populations by using the Louvain algorithm.[48] The following markers were included in the analysis: IgD, CD38, CD20, CD24, CD21, CD27, CD86, and PD-1 for B-cell subset, and PD-1, CD25, CXCR5, CCR6, CXCR3, ICOS, Ki67, and FoxP3 for T-cell subset. Cytofkit settings were set as follow: 5,000 event numbers, autolog transformation, Rphenograph cluster method (k=30), and tSNE visualisation. Phenotype clusters were further annotated and colour-coded manually based on median expression level of markers.

Statistics

Continuous data for multiple group comparisons between healthy control (HC) children and adults and

P. falciparum (Pf) infected children and adults as well as multiple group comparisons between healthy control (HC), uncomplicated *P. falciparum* malaria (UC), and severe *P. falciparum* malaria (SM) in adults were compared using non-parametric Kruskall-Wallis (KW) test followed by Mann-Whitney U test with Benjamini-Hochberg correction. Other single comparisons were tested with Mann-Whitney U test. For comparisons where individual data are matched, such as between uninfected RBCs (uRBCs) and *P. falciparum* infected RBCs (pRBCs) and for longitudinal antibody data, continuous data were compared using non-parametric Wilcoxon paired test. Correlations between cellular subset response and antibody response were analysed using non-parametric Spearman's correlation coefficient test. For linear regression analysis for the association between cTfh cells and parasite biomass, cTfh cells and B cell responses were normalised by log10 transformation and analysed in linear regression models. For comparisons between sex and ethnicities, Fisher's exact test with Freeman-Halton extension was used. All analyses were performed in R (version 4.1.0; version 3.1.4 for CITRUS) or Prism (version 7.03).

Role of funding sources

Funders had no role in study design; collection, analysis and interpretation of data; writing of manuscript; and in the decision to submit the paper for publication.

Results

cTfh cell activation and proliferation is higher in adults than children during malarial infection

To investigate cTfh cell responses, we first quantified *ex vivo* cTfh cell frequency and activation (ICOS expression) and proliferation (Ki67 expression) in children and adults with uncomplicated malaria compared to healthy uninfected controls (Table 1). In adults, cTfh cell responses in individuals with severe malaria were also assessed. Parasite biomass determined by plasma HRP-2 levels was comparable between children and adults with uncomplicated malaria (Mann-Whitney U test; p=0.12, Table 1**)**. There was a 66-fold higher parasite biomass in adults with severe malaria compared to uncomplicated malaria (Mann-Whitney U test; p = 0.01, Table 1). Aside from parasite biomass, antibody levels to merozoite antigens are also a strong indicator of exposure.[40,50] Levels of anti-PfMSP2 antibodies within our cohort were not different between children and adults, and between healthy and infected individuals consistent with both children and adults had low levels of baseline immunity and similar prior exposure between children and adults (**Supplementary Figure S1**).

CD4+ T-cell responses in children and adults with acute malaria were first analysed via two methods of unsupervised clustering. CITRUS analysis (cluster identification, characterisation, and regression)[47] identified two distinct clusters (Cluster group 1 and Cluster group 2) that differentiated between children and adults during infection (Figure 1**a**). Frequency of Cluster group 1 was higher in adults and contained cell populations with characteristics of activated cTfh cells with relatively higher expression of CXCR5, PD-1, and ICOS (Figure 1**a/b**). Cluster 2 was more abundant in children and had relatively lower expression of all markers, suggesting non-Tfh (CXCR5- PD-1-) and non-activated (ICOS- Ki67-) type of CD4+ T-cells (Figure 1**a/b**). Phenotype clustering analysis with PhenoGraph[48] did not clearly identify Tfh cell subsets of known phenotypes due to over-clustering (**Supplementary Figure S2a/b**). cTfh cells were further analysed using conventional manual gating in both infected and healthy children and adults (**Supplementary Figure S3**). Total cTfh cells (PD-1+CXCR5+CD4+) were not elevated during symptomatic malaria when compared with healthy controls (Figure 1**c**). When activation (ICOS+) and proliferation (Ki67+) markers were assessed, ICOS+ and Ki67+ cTfh cells were increased in both children and adults during symptomatic malaria compared to healthy controls. Further, adults with malaria had significantly higher frequencies of ICOS+ and Ki67+ cTfh cells compared to children (Figure 1**d/e**), consistent with CITRUS analysis. ICOS+ cTfh cells were considerably lower in healthy compared to infected individuals. However, healthy children had significantly higher ICOS+ expression compared to healthy adults, possibly due to a higher activated baseline state (Figure 1**d**). There was no difference between cTfh cell frequency, or ICOS+ and Ki67+ expression of cTfh cells between adults with uncomplicated compared to severe malaria (**Supplementary Figure S4**).

The activity of Tfh cells is in part regulated by T-follicular regulatory (TfReg) cells, which express CXCR5 and PD-1 along with FoxP3. TfRegs can migrate into the B-cell follicle and control GC responses.[51,52] To investigate if TfReg cells played a role in regulating differences in age dependent cTfh cells during malaria, we quantified circulating TfReg (cTfReg) cells based on FoxP3 expression of CXCR5+ PD-1+ CD4+ T-cells. There was no difference in the frequency of cTfReg cells between age groups nor between healthy and infected individuals (Figure 1**f**), suggesting that increased cTfh cell activation in adults was not balanced by higher cTfReg cells. Further, ethnicity was not associated with frequencies of cTfh, ICOS/Ki67 expression, or TReg frequencies (Kruskal Wallis; all p>0.05).

Activation and proliferation of cTfh is restricted to Th1-Tfh cells in children but not adults

To assess whether *ex vivo* cTfh cell subset composition differed between children and adults during malaria, cTfh cells were categorised into Th1- (CXCR3+ CCR6-),

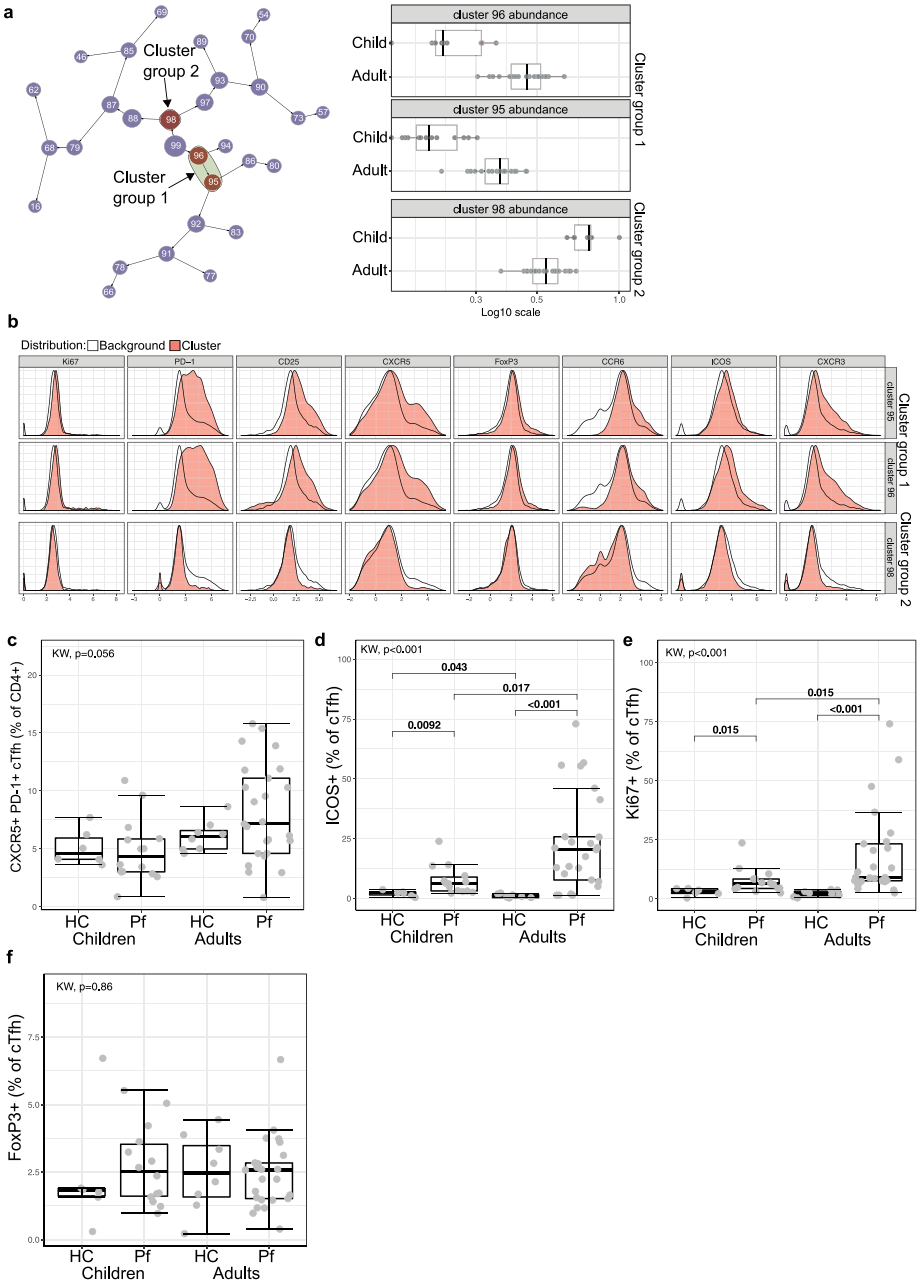

Figure 1. cTfh cell responses during malaria and in healthy individuals. Circulating Tfh cells (PD-1+ CXCR5+) were analysed in children (n=14) and adults (n=24) with P. falciparum (Pf) malaria, and in healthy control (HC) non-infected children (n=6) and adults (n=8). A) CITRUS hierarchical clustering analysis on CD4+ T-cells for comparison between children and adults with malaria. CITRUS is an automated and unsupervised clustering analysis for identification of phenotypically distinct cell clusters between groups. B) Characteristics of expression markers in Cluster group 1 and group 2. C-F) cTfh cell responses were analysed in children and adults with P. falciparum malaria, or healthy controls. C) cTfh cells as a proportion of CD4+ T-cells D) Activated ICOS+ cells and E) proliferating Ki67+ cells as a proportion of cTfh cells. F) Circulating T-follicular regulatory cells (cTfReg, FoxP3+ CXCR5+ PD-1+) were analysed based on FoxP3 expression as a proportion of cTfh cells. Box and whisker plots indicate first and third quartiles for the hinges, median line, and lowest and highest values no further than 1.5 interquartile range from the hinges for whisker lines. All individual data are represented by the grey dots and data beyond whisker lines are treated as outliers. Multiple group comparisons between children and adults, HC and Pf malaria were tested with non-parametric Kruskal-Wallis (KW) test followed by Mann-Whitney U test with Benjamini-Hochberg correction for multiple comparisons.

Th2- (CXCR3- CCR6-) and Th17- (CXCR3- CCR6+) subsets (**Supplementary Figure S3**). In healthy children, Th2-cTfh cells were higher than healthy adults (**Figure 2a**). During *P. falciparum* malaria, adults had higher frequencies of Th2-cTfh cells compared to healthy controls, while there was no change in the composition of cTfh cell subsets in infected compared to uninfected children (**Figure 2a**). In adults with malaria, all cTfh cell subsets were activated, with increased frequencies of ICOS+ cells compared to uninfected healthy controls. In contrast, only Th1-cTfh cells, but not other subsets were activated in children with malaria (**Figure 2b**). Similarly, while frequencies of Ki67+ Tfh cells increased in both Th1-cTfh and Th2-cTfh subsets in adults with malaria, in children there was only increased Ki67+ Th1-cTfh cells (**Figure 2c**). Murine models suggest that severe malaria induces impaired Tfh cell differentiation due to exacerbated Th1-Tfh cell response.[53] However, there was no difference in cTfh cell total subsets, or ICOS expression between uncomplicated and severe malaria (cerebral malaria) in adults, except for Ki67 expression in which uncomplicated infections had higher Th2- and Th17-cTfh cells than severe malaria (**Supplementary Figure S4**). Ethnicity was not associated with frequencies of ICOS or Ki67 expression on any Tfh subset (Kruskal Wallis all $p>0.05$).

Adults have higher frequencies of parasite-specific cTfh cells during malaria

To further investigate the differences in Tfh cells during uncomplicated malaria between children and adults at the antigen-specific level, we used Activation Induced Marker (AIM) assay. AIM assay is a highly sensitive method to detect antigen-specific Tfh cells which produce very low levels of cytokines hampering the ability to detect these cells using intracellular cytokine staining.[27-29] PBMCs from children and adults with acute uncomplicated malaria from a second cohort of individuals (Cohort Subset 2) were stimulated *in vitro* with parasite-infected RBCs (pRBCs), or uninfected RBCs (uRBCs) and antigen-specific cells detected with CD25 and OX40 (**Table 2**; **Figure 3a**). The frequency of CD25+ OX40+ cells was higher in pRBC compared to uRBC stimulated cultures in both children and adults with malaria, indicating parasite-specific cTfh cells were present during malaria. Consistent with a more robust *ex vivo* cTfh cell response in adults, the frequency of parasite-specific cells was higher in adults compared to children (**Figure 3a**). Adults also had a higher frequency of CD25+ OX40+ cTfh cells in uRBC stimulated cultures, consistent with higher levels of activation of cTfh cells *ex vivo* during infection. The memory profiles of parasite-specific cTfh cells was assessed by CCR7 expression (central memory CCR7+ and effector memory CCR7-) in patients with malaria. In both children and adults, the majority of parasite-specific cTfh cells were central memory cells, and the proportion of central or effector memory cells was not different between children and adults (**Figure 3b**). This data suggests that the increased proportion of parasite-specific cTfh cells during infection in adults is not due to differences in memory cell populations.

At the cTfh subset level, there were higher frequencies of CD25+ OX40+ cells following pRBC stimulation compared to uRBC stimulation in Th1-cTfh cells and Th2-cTfh cells for both children and adults, and in Th17-cTfh cells for adults (**Figure 3c**). Consistent with more robust cTfh cell response in adults, the frequencies of parasite-specific cells were higher in adults compared to children across all subsets. The existence of parasite-specific cTfh cells in both Th1- and Th2-cTfh cell subsets in children suggests children do not have a deficiency in parasite-specific Th2-cTfh cells, but instead lack the capacity to activate this subset during infection (**Figure 2a/b/c**). To assess whether differences in parasite-specific cTfh cell responses during infection between children and adults were underpinned by the cytokine milieu of the cultured cells, levels of IFNγ, IL-10, IL-6, IL-1b, IL-4, IL-5, IL-17A, TNF, IL-12p40, monocyte chemotactic protein 1 (MCP-1), IL-2, IL-8, G-CSF, GM-CSF and IL-13 were measured in culture supernatants from AIM assays. Differences between uRBC verses pRBC in children were minimal or undetected, however higher IFNγ, IL-10 and MCP-1 levels were seen in adults following pRBC stimulations (**Supplementary Figure S5**).

To further assess functional differences between cTfh cells during infection between children and adults, a small number of individuals with available cells were stimulated with PMA/Ionomycin and IL-21, IL-4, IL-10, IFNγ, IL-17A and TNF production were assessed by intracellular staining. Despite limited sample numbers, there was evidence that cTfh-derived IL-4 production during infection was higher in adults compared to children (Mann-Whitney U test; $p=0.057$) consistent with a more robust Th2-cTfh response in adults (**Figure 3d**). Further, the total cytokine profile of cTfh cells indicated that adults had increased polyfunctionality of cTfh cells during infection, with an increased complexity of cytokine production and higher frequencies of cells co-producing 2 or more cytokines (**Figure 3e/f**). Taken together, these data indicate that the magnitude of parasite specific Tfh cells is higher in adults in malaria, and that Tfh cells in adults during infection have higher polyfunctionality and produce higher levels of Th2-Tfh associated cytokine IL-4, consistent with increased quality responses.

cTfh activation is correlated with higher frequency of activated B-cells in adults during malaria

To investigate whether increased Tfh activation during infection and increased frequencies of parasite specific

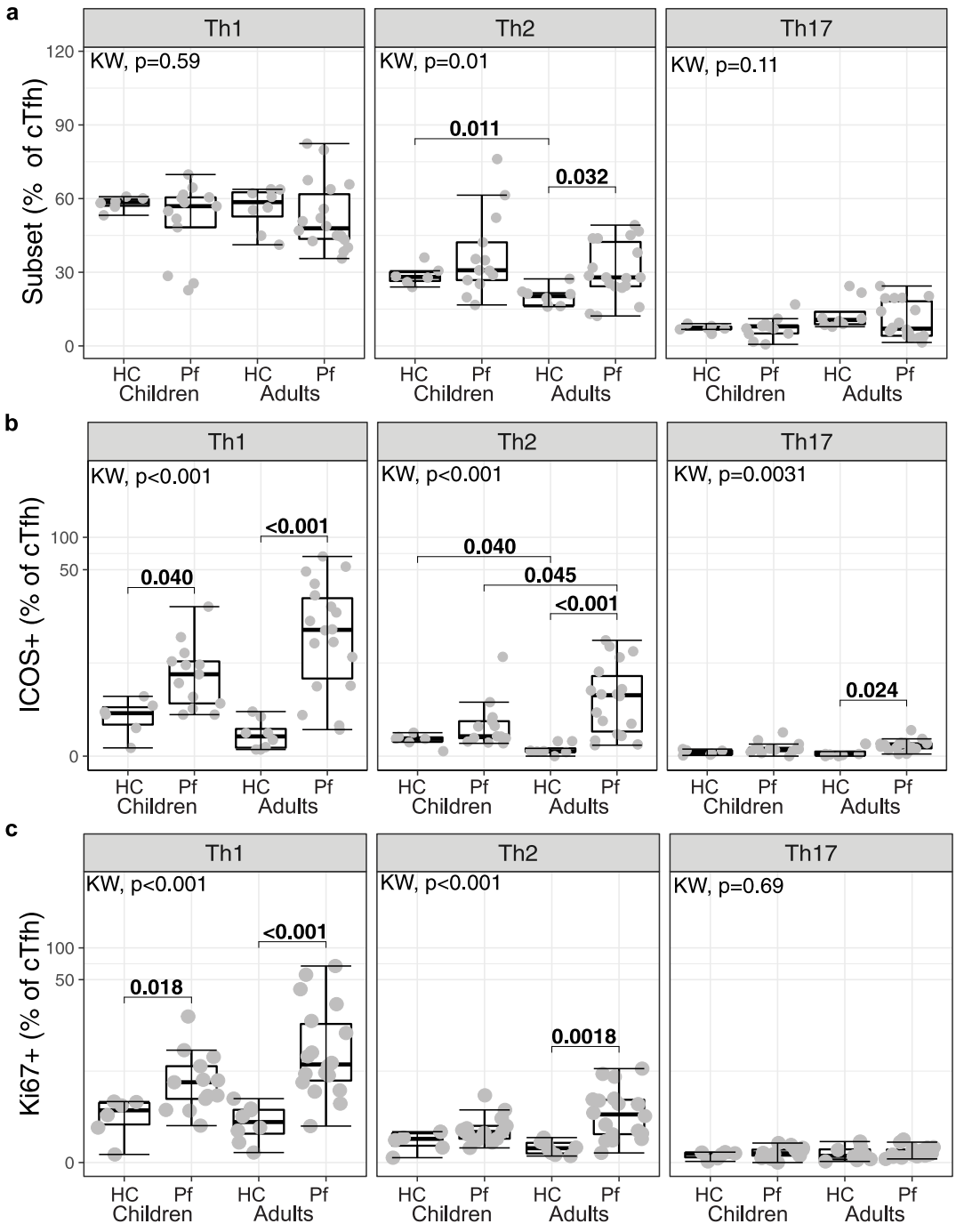

Figure 2. cTfh cell subset responses during malaria and in healthy individuals. Subsets of cTfh cells were analysed in children (n=13) and adults (n=18) with P. falciparum (Pf) malaria, and in healthy control (HC) non-infected children (n=6) and adults (n=8), based on CXCR3 and CCR6 expression, grouped into the following: Th1- (CXCR3+ CCR6-), Th2- (CXCR3- CCR6-) and Th17-like (CXCR3- CCR6+) cell subsets. **A)** cTfh cell subsets as a proportion cTfh cells. **B)** Activation ICOS+ cells as a proportion of cTfh cells. **C)** Proliferating Ki67+ cells as a proportion of cTfh cells. Box and whisker plots indicate first and third quartiles for the hinges, median line, and lowest and highest values no further than 1.5 interquartile range from the hinges for whisker lines. All individual data are represented by the grey dots and data beyond whisker lines are treated as outliers. Multiple group comparisons between children and adults, HC and Pf malaria were tested with non-parametric Kruskal-Wallis (KW) test followed by Mann-Whitney U test with Benjamini-Hochberg correction for multiple comparisons.

	Children <12 (Pf-U))	Adults ≥18 (Pf-U)	P-value
n	10	11	
Male (n, %)	2	5	0.3*
Age (median, [IQR])	6 [5-7.8]	24 [20.5-29.5]	<0.01#
Ethnicity:			
Non-Papuan	1, 10%	3, 27%	0.6*
Highland Papuan	9, 90%	8, 73%	
Lowland Papuan	0	0	
Parasitemia (parasites/μL blood, median, [IQR])	5782 [1500-24215]	633 [331-5813]	0.10#

***Table 2*: Cohort Subset 2 participant characteristics.**
* Fisher's exact test. Comparison between age groups and ethnicity.
Pf-U; uncomplicated *P. falciparum* malaria
Mann-Whitney U test

Tfh cells had a positive impact on antibody development, we first investigated B cell responses in Cohort Subset 1 study participants where sufficient cell numbers were available for analysis (n; healthy children = 8, infected children = 10, healthy adults = 7, infected adults = 20) (**Supplementary Table S1**). PhenoGraph analysis[48] grouped cells into known CD19+ B-cell subsets, and detected a cell cluster with CD38+ CD20- expression that differed between groups (**Figure 4a/b/c**). These cells were most similar to antibody secreting plasma cells. Manual gating of antibody-secreting plasma cell as $CD38^{hi}$ $CD27^{hi}$ CD20- B-cells, indicated this subset was also higher in adults compared to children, but this did not remain significant following multiple comparison corrections (Mann-Whitney U test; p=0.045, Benjamini-Hochberg; p=0.09, **Figure 4e**). Unsupervised clustering using CITRUS generated a predictive model with high cross-validation error (~40-50%) and high false discovery rate, indicating low and unreliable predictive power analysis, likely due to limited sample size in the B-cell panel compared to the T-cell panel (**Supplementary Figure S8**).[47,54] Manual gating of B-cells based on IgD, CD21, and CD27 expression ,[55] showed that the overall composition of switched and unswitched B cell subsets was similar between children and adults and not changed during malaria (**Supplementary Figure S9**). Similarly, a regulatory subset of human B-cells (Breg) identified by the expression of $CD24^{hi}$ $CD38^{hi}$ CD19+, which suppress T-helper cell activity,[56,57] did not change with malaria (**Supplementary Figure S9**). To assess B cell activation, expression of PD-1 and CD86 was measured. CD86 expression did not change with malaria but was higher in uninfected and infected adults compared to children, consistent with higher cTfh cell activation in adults (**Figure 4e**). However, there was a significantly increased expression of PD-1 activation marker in adults with malaria compared to healthy controls and infected children (**Figure 4e**).

Associations between cTfh, B cells and antibodies and parasite burden

To link Tfh cell activation to B cell activation we first calculated correlations between cell responses. We also explored the relationship between Tfh and B-cell responses and antibody levels in healthy and infected individuals (**Supplementary Figure S1**). Amongst both healthy and infected individuals, plasma cells and CD86 expressing B-cells were correlated with activating (ICOS+) and proliferating (Ki67+) Th1- and Th2-cTfh, but not cTfh cells alone (**Figure 5a**). PD-1 expressing B-cells did not correlate with any of the cTfh cells (**Figure 5a**). IgG1, IgG2, IgG3, IgM and functional antibody responses during acute infection were not correlated with any of the cellular response, likely due to kinetics of Tfh activation and antibody induction. There was a weak correlation between IgG4 levels and Ki67+ Th2-cTfh cells, however it should be noted that the overall magnitude of IgG4 was very low (mean OD 0.011).

To investigate a role for parasitemia on Tfh and B cell activation, the correlation between parasitemia and cell subsets were assessed amongst infected children and adults with uncomplicated malaria. Parasite burden was positively correlated with both the activation (ICOS+) and proliferation (Ki67+) of Th2-cTfh cells, but not other cell subsets (**Figure 5b**). In linear regression modelling, this positive association between parasite burden and Th2-cTfh cell activation/proliferation remained after controlling for age group for ICOS+ expression but not Ki67+ expression (Linear regression; age group adjusted p=0.045 for ICOS+ Th2-Tfh, and p=0.53 for Ki67+ Th2-Tfh).

Malaria antibodies increase in adults but not children after a malaria episode

To assess whether the differences in cTfh cell responses between adults and children may impact antibody development we tested antibody responses in an additional

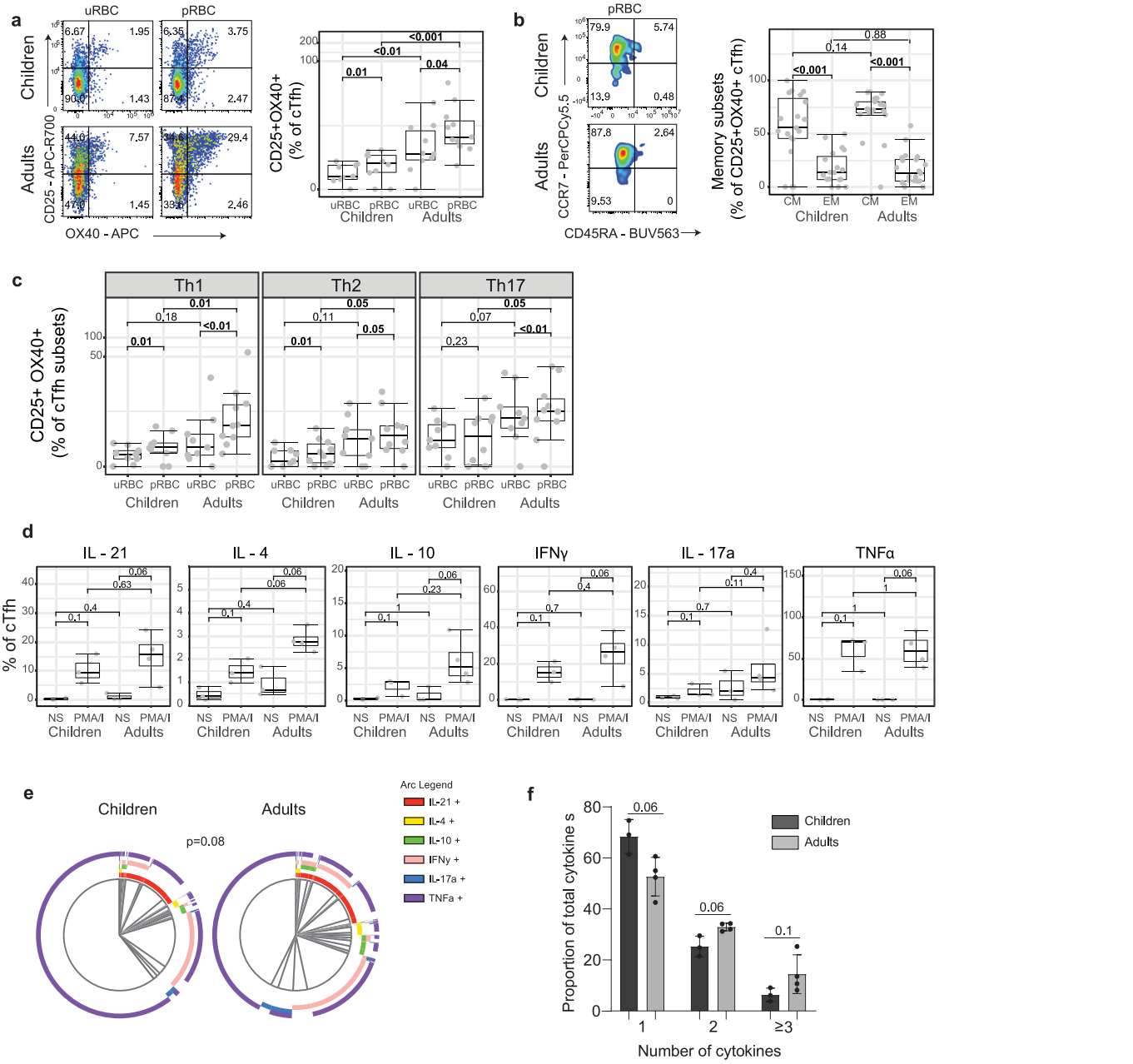

Figure 3. Parasite-specific cTfh cell responses during malaria and cytokine production. **A)** PBMCs from children (n=10) and adults (n=11) with acute malaria were stimulated *in vitro* with *P. falciparum* infected RBCs (pRBCs) or uninfected RBCs (uRBCs) and parasite-specific responses detected by upregulation of CD25 and OX40. CD25+ OX40+ cells were assessed as a proportion of cTfh cells. **B)** Memory phenotypes of parasite-specific cTfh cells were analysed based on CCR7 and CD45RA expression as central memory (CM - CCR7+ CD45RA-) and effector memory (EM - CCR7- CD45RA-). The CM and EM cells were analysed as a proportion of parasite-specific cTfh cells. **C)** CD25+OX40+ as a proportion of each cTfh subset (Th1 CXCR3+ CCR6-, Th2 CXCR3- CCR6- and Th17 CXCR3- CCR6+). **D)** PBMCs from children (n=3) and adults (n=4) were stimulated with PMA/Ionomycin (PMA/I) or unstimulated (NS) and IL-21, IL-4, IL-10, IFNγ, IL-17(and TNFα from Tfh cells detected via intracellular staining. **E)** Co-production of cytokines was analysed by Boolean gating in SPICE. **F)** **c**Tfh cells producing 1, 2, or $\geq$3 cytokines during malaria. Box and whisker plots indicate first and third quartiles for the hinges, median line, and lowest and highest values no further than 1.5 interquartile range from the hinges for whisker lines. All individual data are represented by the grey dots and data beyond whisker lines are treated as outliers. Paired data for comparisons between uRBC and pRBC, CM and EM, and NS and PMA/I were tested with Wilcoxon signed-rank test while unpaired data for differences between children and adults was tested with Mann-Whitney U test.

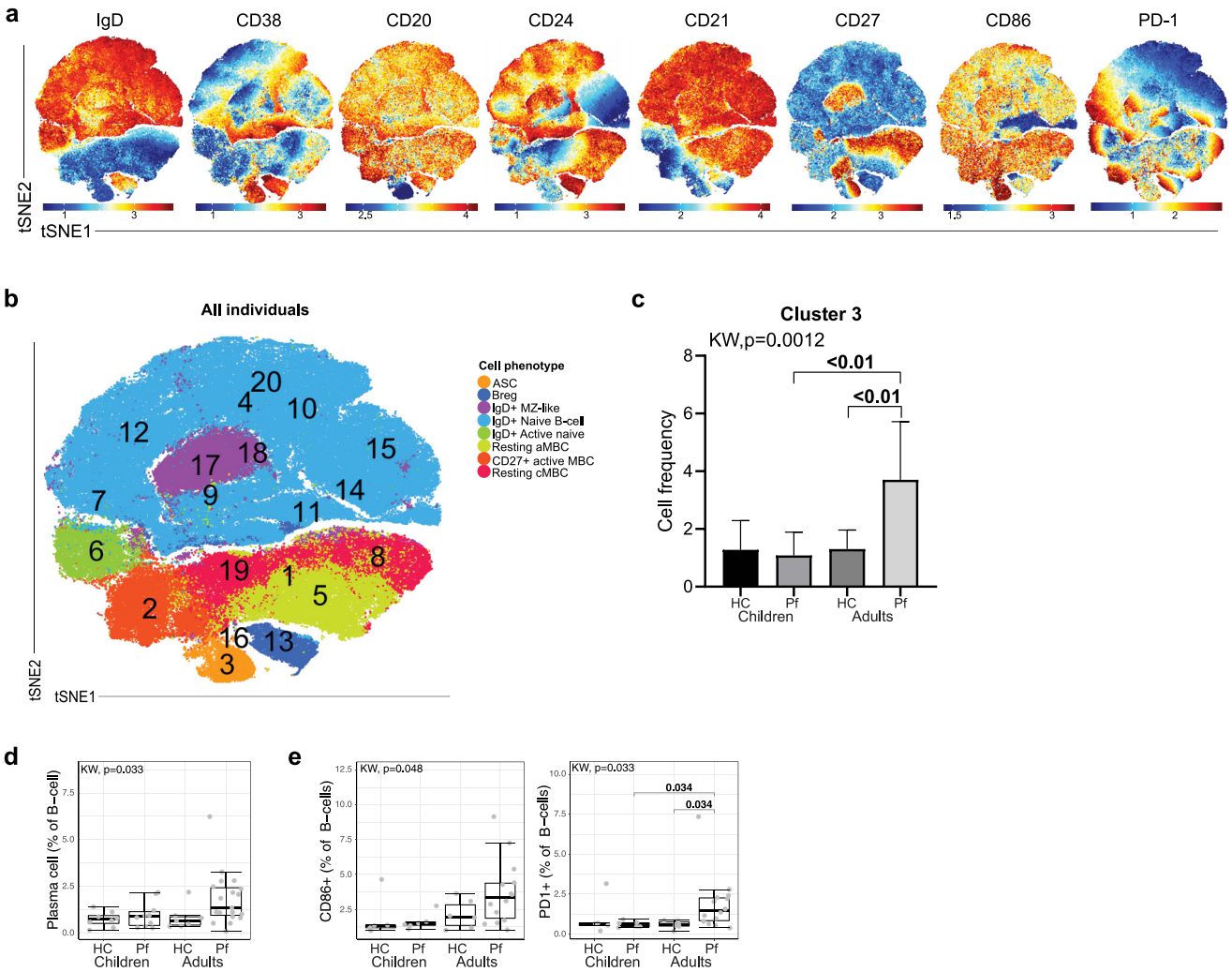

Figure 4. B-cell responses during malaria and in healthy individuals. B-cell subsets were analysed in children (n=10) and adults (n=19) with P. falciparum (Pf) malaria, and in healthy control (HC) non-infected children (n=8) and adults (n=7). **A)** tSNE plot on different expression markers, coloured according to expression intensity. **B)** tSNE plot with cell phenotype clusters generated by PhenoGraph analysis. Cell phenotypes are colour-coded and annotated manually. tSNE plots (**A** and **B**) indicate merged sample analysis from children and adults, healthy and infected. **C)** Cell frequency comparison between groups from cluster #3, characterised as CD19+ CD38+ CD20- plasma cell. **D)** CD38^{hi} CD27^{hi} CD20- plasma cell as a proportion of B-cells. **E)** CD86+ and PD-1+ expression as a proportion of CD19+ B-cells. P. falciparum malaria (Pf), healthy controls (HC). Box and whisker plots indicate first and third quartiles for the hinges, median line, and lowest and highest values no further than 1.5 interquartile range from the hinges for whisker lines. All individual data are represented by the grey dots and data beyond whisker lines are treated as outliers. Multiple group comparisons between children and adults, HC and Pf malaria were tested with non-parametric Kruskal-Wallis (KW) test followed by Mann-Whitney U test with Benjamini-Hochberg correction for multiple comparisons.

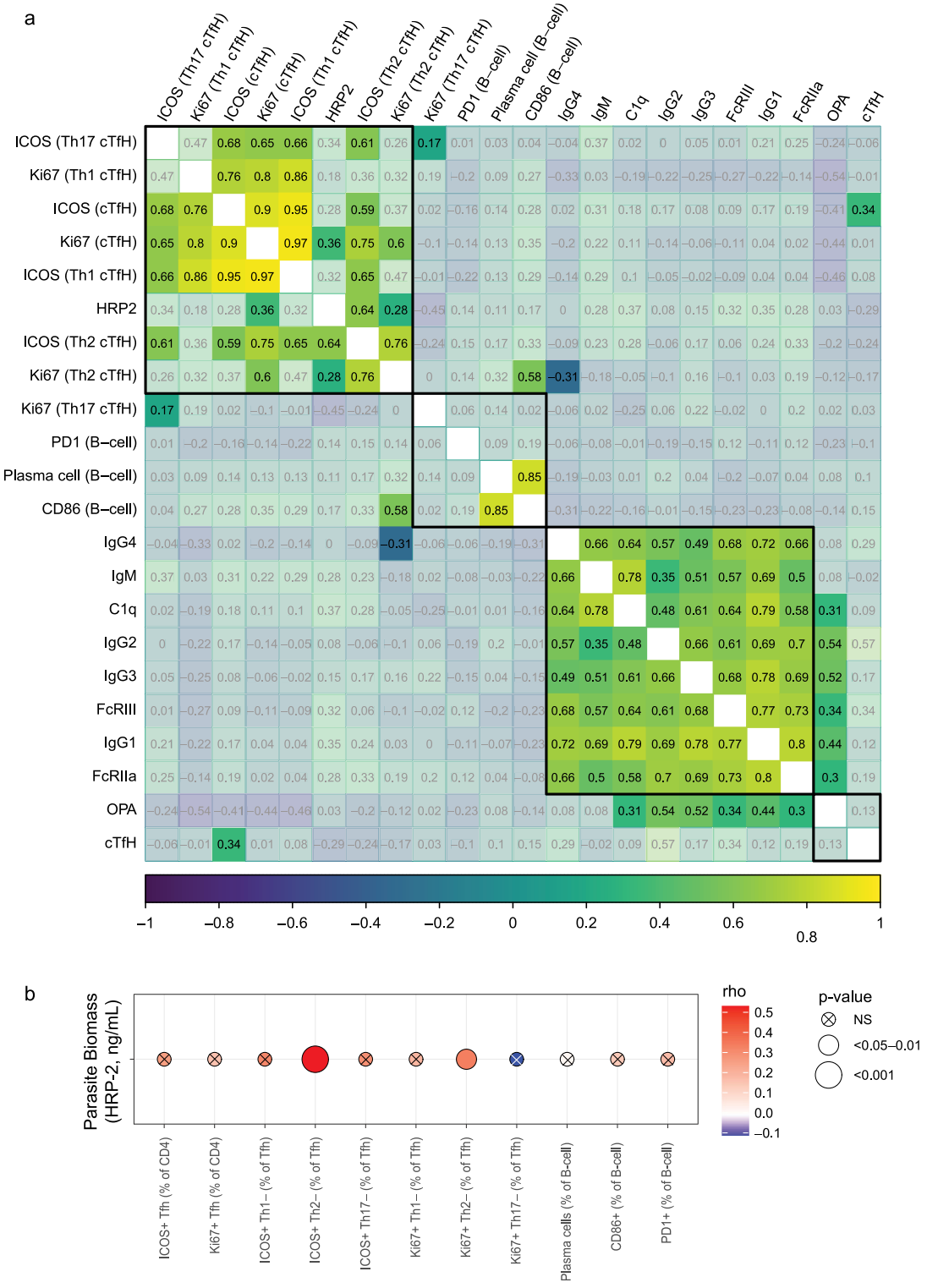

Figure 5. Correlations between cTfh and B-cell subsets and antibodies during malaria. **A)** Circulating Tfh cell, B-cell subsets, and antibody response correlations were analysed from all study subjects, with malarial infection and healthy controls (n=48). Numbers in the box indicate Spearman's correlation value. Uncoloured boxes indicate non-statistically significant Spearman's correlation, $p>0.05$. **B)** Correlations between cTfh and B-cell subsets and parasite biomass (HRP2 ng/ul) in infected children and adults (n=38).

	Children <12			Adults ≥18			P-value
Day	**0 (Pf-U)**	**7**	**28**	**0 (Pf-U)**	**7**	**28**	
n	22	21	13	46	46	46	
Male (n, %)	8, 36.3%	8, 38.1%	2, 15.4%	31, 67.4%	31, 67.4%	31, 67.4%	**p<0.05***
Age (median, [IQR])	7 [7-8.8]	7 [7-8]	7 [7-8]	30 [25-36.8]	30 [25-36.8]	30 [25-36.8]	p<0.01#
Ethnicity:							
Non-Papuan	7, 31.8%	8, 38.1%	6, 46.2%	25, 54.3%	25, 54.3%	25, 54.3%	p[1]>0.05*
Highland Papuan	15, 68.2%	13, 61.9%	7, 53.8%	20, 43.5%	20, 43.5%	20, 43.5%	
Lowland Papuan	0	0	0	1, 2.2%	1, 2.2%	1, 2.2%	
Parasitemia (HRP-2 ng/mL, median, [IQR])	20.2 [2.0-93.4]	NA	NA	23.6 [4.5-36.9]	NA	NA	0.93#

***Table 3*: Longitudinal cohort characteristics (Cohort Subset 3).**
p[1] – comparison of children to adults
Pf-U; uncomplicated *P. falciparum* malaria
* Fisher's exact test. Comparison between age groups at each time point (P>0.05 for all comparisons).
# Mann-Whitney U test

cohort of individuals during malaria and at 7 and 28 days post-treatment where plasma but no PBMCs were available for analysis (Cohort Subset 3, Table 3). Parasite biomass during infection was not different between children and adults (Mann-Whitney U test; p=0.68), and overall patient characteristics were comparable to individuals tested for cTfh and B-cell responses during infection (**Supplementary Table S2**). During symptomatic malaria (day 0), the magnitude of all antibody responses was comparable between age groups (**Supplementary Figure S10**). However, after treatment, adults but not children had an increase in antibody responses (Figure 6). In adults, all responses except for opsonic phagocytic ability (OPA) were significantly increased by day 7, and IgG1, IgG2, IgM, C1q, and FcγRIIa binding remained significantly elevated at day 28 compared to acute infection (Figure 6). In contrast to the boosting of antibody responses after treatment in adults, there was no significant increase in antibodies in children at day 7 or day 28 after malaria infection (Figure 6). Magnitude of antibody responses were comparable between female and male individuals (Mann-Whitney U test; p>0.05 for all antibody comparisons). Data is consistent with more robust activation of Tfh cells, B cells and antibody secreting cells during malaria in adults driving higher antibody development following treatment.

Discussion

A positive impact of increasing age on acquisition of malaria immunity has been reported in a number of settings,[3,4,6,7] yet the cellular drivers of this are unknown. We found that during malaria infection, the magnitude of cTfh cell activation was higher in adults, and activation occurs across all cTfh subsets while in children cTfh cell activation was restricted to the Th1-cTfh subset, consistent with previous studies.[24] Further, adults had higher levels of parasite-specific cTfh cells, and cTfh cells which produced more Th2-Tfh associated cytokine IL-4. Consistent with a role of higher Tfh cell activation in rapid immune development in adults, adults had higher activation of B cells during infection and higher induction of antibodies, seven and 28 days after malaria treatment compared to children. Our data reveals important differences in cTfh cell responses between children and with malaria, with adults having more robust Tfh cell responses during malaria. Linking this robust activation to increased B cell responses, and higher antibody induction in adults after parasite treatment, our data support the notion that age drives changes in the magnitude and quality of cTfh cells activation during malaria, and these changes may impact the acquisition of antibody to malaria.

In the analysis of circulating Tfh cells during infection, both the magnitude of cTfh cell activation, measured by both ICOS and Ki67 expression, and quality (specific subsets of activation) were higher in adults with malaria compared to children. In adults, cTfh cell activation during malaria was observed in all subsets, including Th2-cTfh cells. In contrast, cTfh cell activation was restricted to Th1-cTfh cells in children, as shown previously with children in Mali.[24] In addition to age, parasite biomass contributed to Th2-cTfh cell activation and proliferation, with a weak but positive correlation between parasite biomass and ICOS+/Ki67+ Th2-cTfh cells. This finding is in contrast to our previous results where total parasite burden was not associated with cTfh cell activation during experimental challenge with human malaria,[23] possibly indicating differences between subclinical and clinical infections. Further, in contrast to the consistent differences between children and adult responses, disease severity appears to only have minor impacts of cTfh cell activation in malaria in adults. This finding is inconsistent to findings in mice,[53] possibly due to the underlying

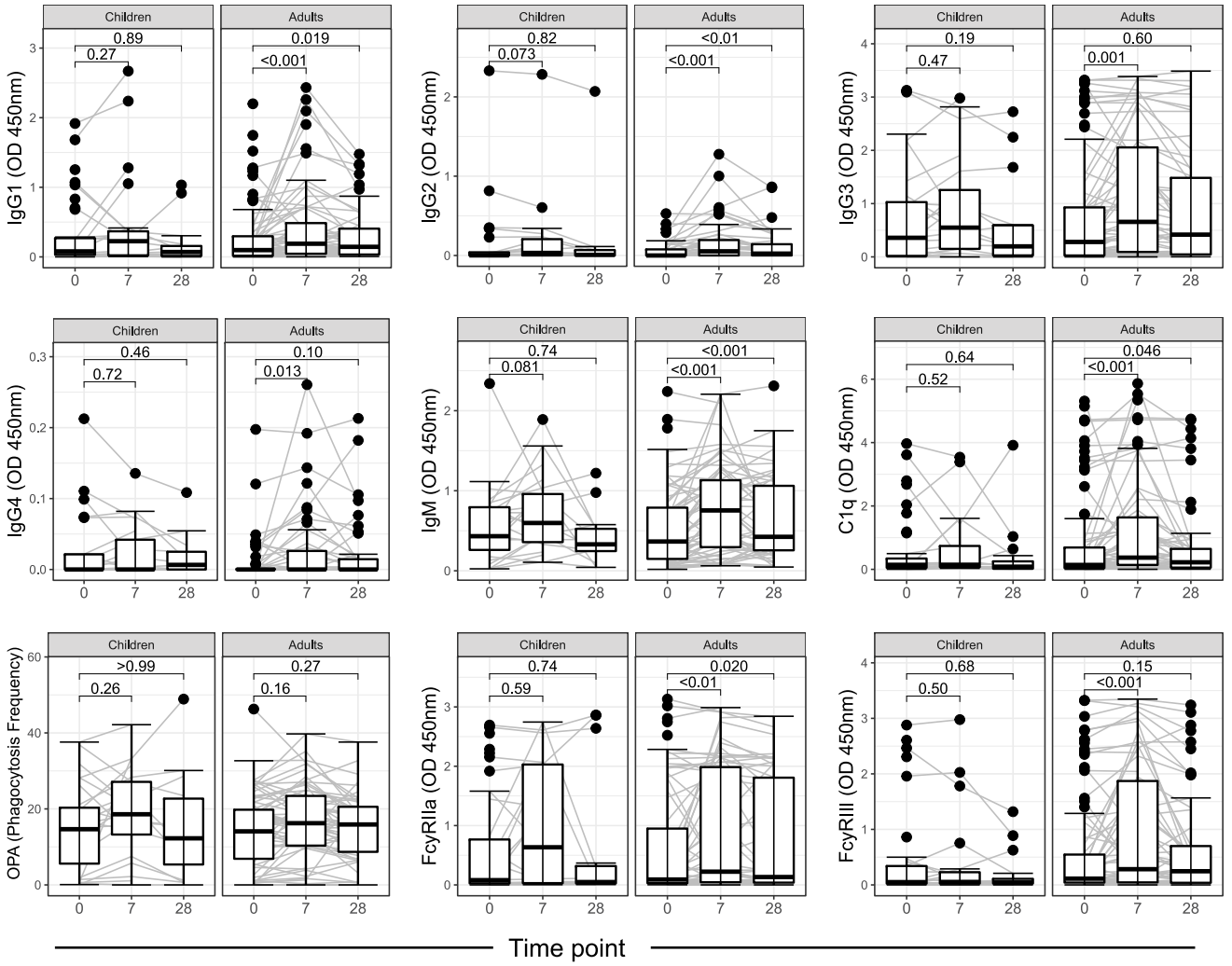

Figure 6. Induction of IgG, IgM and functional antibodies during malaria and following treatment. Magnitude of IgG subclasses, IgM and functional antibodies to PfMSP2 during P. falciparum symptomatic malaria (day 0) (children n=22, adults n=46), and day 7 (children n=21, adults n=46) and 28 (children n=13, adults n=46) after treatment in children and adults from the longitudinal study cohort. P-values indicate paired Wilcoxon signed-rank test comparing between symptomatic malaria (day 0) and post-treatment time points (day 7 and 28), tested on samples with matched time points. OPA – opsonic phagocytosis. Box and whisker plots indicate first and third quartiles for the hinges, median line, lowest and highest values no further than 1.5 interquartile range from the hinges for whisker lines, and outliers for the black dots. All individual data are represented by connected grey lines.

differences in disease pathology and/or immune responses between human and mice infection. Increased cTfh cell responses during malaria in adults compared to children was confirmed at the antigen-specific level using AIM assay, which is a highly sensitive method of detecting antigen-specific Tfh cells following antigen stimulation which often produce low levels of cytokines.[27-29] Supporting more robust activation of cTfh cells during infection in adults, adults with malaria had higher frequencies of malaria-specific cells including in both Th1- and Th2-cTfh subsets compared to children. Interestingly, parasite-specific cTfh cells were detected in children in both Th1- and Th2-subsets, suggesting that the restriction of activation in children to Th1-cTfh cells is not due to the lack of parasite-specific responses per se. Consistent with a more robust Th2-cTfh cell responses in adults, adult cTfh cells produced higher amounts of IL-4, a canonical Th2-cytokine. Additionally, adult cTfh cells were highly polyfunctional. Polyfunctional T-cells which produce high levels of a range of different cytokines have been linked to increased levels of protection in a number of settings.[58-61]

We have recently shown that functional antibody induced ~30 days following experimental *P. falciparum* infection, is associated with Th2-cTfh cell activation 8 days after infection.[23] Consistent with this positive role of Th2-cTfh cells in antibody development, both B cell activation and plasma cells were increased in adults compared to children, and activation of B cells as measured by PD-1 and CD86 expression was associated with all activated cTfh cell subsets. While excessive plasma cell induction may have detrimental impacts on GC responses in *Plasmodium* infection,[62] plasma cell expansion is also positively associated with antibody development in other settings such as influenza vaccination.[18] Th2-cTfh cells and B cell activation was not strongly linked to antibody levels during malaria, consistent with previous studies which show a kinetic delay in the association between Tfh cell activation during infection and vaccination and subsequent antibody induction.[18,23] For example, we have recently shown that Th2-cTfh cell activation 8 days after parasite infection is associated with antibody induction after treatment, ~30 days after infection.[23] Similarly, activation of Th1-cTfh cells 7 days following influenza vaccination is associated with antibody responses 28 days after vaccination.[18] While we were unable to directly link cTfh cell activation with antibody development due to the lack of follow-up samples, we were able to show in a separate cohort from the same study site that anti-malarial antibodies increased in adults but not children following anti-malarial treatment. Acquired immunity to malaria is largely mediated by cytophilic antibodies (IgG1/3 and IgM) which limit parasite burden via multiple mechanisms including antibody-mediated complement fixation and antibody interactions with Fcγ receptors to promote phagocytosis.[36,39,42,46,63,64] Here, we show that cytophilic and functional antibody, with the exception of OPA, were induced only in adults following acute malaria. The difference between OPA and all other antibody responses tested may be underpinned by different sensitivities of the ELISA compared to the cell-based assays, with OPA measuring opsonic phagocytosis by THP-1 cell lines which is largely mediated via FcγRI,[65] and may only be supported at very low opsonization titres. Non-cytophilic IgG2 and IgG4 antibodies, while far lower in magnitude, were also increased in adults but not children after a malaria infection. In mice models of infection, reduced T-bet expression in Tfh cells (which drives Th1-like phenotypes and functions), increases the induction of non-cytophilic antibodies (IgG1 in mice), however this is dependent on the context of infection.[66] Similarly, T-bet deficient Tfh cells during helminth infection drive increased induction of non-cytophilic antibodies,[67] together suggesting Th1-Tfh cells block the induction of non-cytophilic antibodies. However, whether Th2-like Tfh are specifically required to drive non-cytophilic responses is unknown. Here, while we observed a weak association between proliferating Th2-cTfh cells and IgG4 (non-cytophilic) antibodies during malaria, overall IgG4 antibody levels are very low, and the dominant antibodies developed against malaria infection are cytophilic IgG1 and IgG3. Taken together, the data presented here are consistent with the hypothesis that the ability of adults to mount a higher quantity and quality of cTfh cell activation leads to more robust antibody development and immunity. More studies are required to understand the influence of total magnitude of the Tfh responses, compared to the balance of Th1 and Th2-Tfh cells in the induction of specific antibody subclasses and functions.

The mechanism leading to higher magnitude and quality of cTfh cell activation in adults with malaria compared to children is unknown. Indeed, both age intrinsic factors, prior malaria infections and/or age associated confounders not accounted for in our study may underpin our findings. While little is known regarding the development of Tfh cells with age and differences between Tfh cell responses in children and adults, multiple layers of the human immune system mature with age which may impact Tfh cell activation during malaria.[68] For example, IL-12 is required for early commitment of naÿve T-cells to Tfh cell development,[69] and IL-12 production in response to TLR stimulation is lower in neonatal compared to adult cells.[70] Tfh cell intrinsic mechanisms may also be important, and neonatal Tfh cells have expression signatures indicative of intermediate or pre-GC Tfh cells, suggesting infant Tfh cells may have impaired Tfh cell development capacity compared to adults.[71,72] Subsequent studies are underway to identify key innate cell subsets that mediate Tfh cell activation during malaria in humans, and identify age dependent differences in these key subsets that may explain our findings.

Our findings have important implications for vaccine development, suggesting that strategies to target Tfh cells may improve vaccine efficacy. Recent studies have indicated that adjuvant selection can modify Tfh cell response to malaria vaccination in adults.[73] Glucopyranosyl lipid adjuvant-stable emulsion with P27A experimental malarial vaccine enhanced the quantity, but not composition of cTfh cells and resulted in increased antibody responses compared to alum adjuvants.[73] In contrast, co-administration of RTS,S with viral vectors resulted in increased skewing of cTfh cell responses to Th1-like cells and reduced antibody function and vaccine efficacy.[74] Given that age and exposure also appears to be an important element in vaccine induced responses to malaria, with vaccine efficacy lower in infants compared to young children and in high transmission settings,[8,75] understanding mechanisms underpinning Tfh cell responses across all ages and prior exposures is important for optimized vaccine development for at risk populations. Indeed, in mice, Tfh cell responses to aluminium adjuvanted vaccines are reduced in infants compared to adults,[76,77] however this reduced response can be overcome by MF59 oil-in-water adjuvant vaccines.[78] Given that infants and children are the target populations of anti-malarial vaccines, our data highlight the importance of understanding Tfh cell development and response to vaccination strategies in target populations.

Considering limitations of our study, we are unable to definitively identify whether the differences in adults are specifically due to age intrinsic factors or prior exposure since the complete history of previous malarial infection in our cohort is unknown. However, all but one of the adults in this study were migrant individuals and indigenous Highland Papuans whom are likely to have had low previous malaria exposures.[32] Further, children and adults had comparable levels of parasite biomass and antibodies at baseline during acute malaria, suggesting similar levels of immunity during presentation. Repeated malarial episodes resulted in the gradual increase of antigen-specific B-cell frequency and antibody titre in children and adult individuals,[79] and antibody levels to merozoite antigens are strongly associated with exposure,[40,50] therefore the comparable antibody levels at presentation is consistent with comparable exposure histories. Nevertheless, an impact of prior exposure may have influenced our findings, and previous studies have reported that the frequency of cTfh cells is positively associated with the number of previous malaria episodes in adults with uncomplicated *P. vivax* malaria.[80] It is also possible that other age associated confounders not accounted for here such as co-infections, nutritional status or host genetics, may be driving the associations seen in our study, rather than age per se. Further, our cohort did not include young infants, and had a relatively small sample size and matched longitudinal sampling between sera and PBMC samples was also not available, hence we were not able to specifically assess cellular and humoral responses within the same patients over time. While we observed no differences in Tfh cell activation in adults with uncomplicated malaria, compared to severe malaria, our study was restricted to cerebral malaria presentation. Finally, we were unable to link directly Tfh cell activation with immunity from malaria during subsequent infection, due to lack of follow up in our cohorts.

In conclusion, our data identify Tfh cells as a key cellular subset driving differences in immune development between adults and children. During malarial infection, Tfh cells in adults have increased activation across all subsets compared to children within whom activation is restricted to Th1-Tfh cells. These findings are consistent with the more robust induction of anti-malarial antibody responses following treatment in adults. Our findings provide new mechanistic insights to understand the differences in immune responses to malaria between children and adults. Together, these results have significant implications for development of malaria vaccines, particularly in children, suggesting that children may require additional or modified targeting of Tfh cells to facilitate the generation of more effective immunity and higher vaccine efficacy.

Contributors

DAO, JGB, GM, MJB designed research study; DAO, JRL, JAC, MSFS, DA, SDO, ADC conducted experiments; DAO, GM, MSFS, JRL, AV, MJB analysed data; BW and MH contributed to experimental design; EK, NMA, RNP conducted and supervised the clinical studies and sampling; DAO, MSFS generated figures; DAO, JRL, MSFS, MJB verified underlying data. DAO, GM, MSFS, JRL, MJB led manuscript preparation with feedback from all authors. All authors have read and approved the final version of the manuscript.

Data sharing statement

Deidentified data from this manuscript is available from the corresponding author upon reasonable request and upon completion of appropriate data sharing agreements.

Declaration of Competing Interest

BDW and PMH are listed on patent application Agents, Binding Assays and Method for Probing Antibody Function, PCT WO/2017/054033. RP is a pro bono member of the Access and Delivery Advisory Committee for Medicines for Malaria Venture, the Novartis Malaria Council and the G6PD diagnostic Expert Advisory Committee for PATH. All other authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103784.

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