Heterogeneity of the human immune response to malaria infection and vaccination driven by latent cytomegalovirus infection

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Summary

Background Human immune responses to infection and vaccination are heterogenous, driven by multiple factors including genetics, environmental exposures and personal infection histories. For malaria caused by *Plasmodium falciparum* parasites, host factors that impact on humoral immunity are poorly understood.

Methods We investigated the role of latent cytomegalovirus (CMV) on the host immune response to malaria using samples obtained from individuals in previously conducted Phase 1 trials of blood stage *P. falciparum* Controlled Human Malaria Infection (CHMI) and in a MSP1 vaccine clinical trial. Induced antibody and functions of antibodies, as well as CD4 T cell responses were quantified.

Findings CMV seropositivity was associated with reduced induction of parasite specific antibodies following malaria infection and vaccination. During infection, reduced antibody induction was associated with modifications to the T -follicular helper (Tfh) cell compartment. CMV seropositivity was associated with a skew towards Tfh1 cell subsets before and after malaria infection, and reduced activation of Tfh2 cells. Protective Tfh2 cell activation was only associated with antibody development in individuals who were CMV seronegative, and a higher proportion of Tfh1 cells was associated with lower antibody development in individuals who were CMV seropositive. During MSP1 vaccination, reduced antibody induction in individuals who were CMV seropositive was associated with CD4 T cell expression of terminal differentiation marker CD57.

Interpretation These findings suggest that CMV seropositivity may be negatively associated with malaria antibody development. Further studies in larger cohorts, particularly in malaria endemic regions are required to investigate whether CMV infection may modify immunity to malaria gained during infection or vaccination in children.

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Keywords: Cytomegalovirus; Malaria; Antibodies; CD4 T cells

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Research in context

Evidence before this study

Immune responses to infection and vaccination are heterogeneous, with diversity driven by genetics, environment and personal histories of exposure and can be detected on the individual and population levels. For immune responses to malaria, caused by *Plasmodium falciparum*, rates of immune acquisition vary in endemic settings, and differ between populations. For example, immunity induced by vaccination in endemic areas is consistently lower than Phase 1 trials in high income countries. One factor that can influence human immune responses is cytomegalovirus (CMV), a herpes virus that establishes persistent latent infection and is associated with immunomodulation. CMV serostatus has been associated with modulation of the immune response induced to vaccines targeting viruses. CMV infection occurs early in life, and is universal in malaria endemic countries, however, the impact of CMV serostatus on the immune response to malaria infection or vaccination is unknown.

Added value of this study

We identified CMV associated differences in the development of humoral immunity as well as composition of T cells during malaria infection and vaccination. CMV seropositivity was associated with reduced induction of functional antibodies. The reduced induction of antibodies was correlated with CMV associated changes in the CD4 T cell compartment. Findings show for the first time that CMV serostatus modulates the immune response to malaria infection and vaccination.

Implications of all the available evidence

CMV is universally acquired early in life in malaria endemic areas, however in high income countries may only reach 50% seroprevalence. Our data suggests that differences in CMV seroprevalence should be taken into account in malaria vaccine development. Further studies are required to understand the impact of CMV serostatus on malaria immunity to infection and vaccination in children in endemic areas.

Introduction

The human immune response to infection and vaccination is heterogenous. Responses range from robust responses that resolve infection, to sub-optimal responses that may lead to severe disease or fail to generate long-term protection. This heterogeneity reflects the interplay of genetics, environment, and personal exposure histories. For malaria caused by *P. falciparum* parasites, infection in endemic areas ranges from asymptomatic parasitaemia to severe disease and death.[1] At the population level, the largest disease burden occurs in children under the age of five, who develop immunity after repeated infection throughout childhood. However, the rate of immune development is heterogeneous, with some children rapidly gaining protection while others experience numerous symptomatic episodes, despite similar exposure.[2] Heterogeneity is also seen in responses to malaria vaccination, both between individuals within malaria endemic areas, and between populations across geographical locations.[3–5] Of note, responses to *P. falciparum* malaria vaccines are consistently lower in low- and middle-income countries (LMIC) compared to those reported during Phase 1 trials in high income countries. For example, antibodies to circumsporozoite protein (CSP) following experimental vaccination with radiation-attenuated whole sporozoite parasite vaccine (PfSPZ) were significantly lower in Tanzanian and Malian adults, compared to adults in the USA.[6] Further, CSP antibodies induced in Kenyan adults with the licenced malaria subunit vaccine RTS,S are generally lower than those seen following vaccination of adults in the USA.[7–10] As such, dissecting factors that modify immune responses to *P. falciparum* may inform development of second-generation vaccines with better protection, or other avenues for malaria control.

Immunity to malaria generated by infection or vaccination is mediated by antibodies that prevent parasite replication, largely via Fc mediated mechanisms.[11,12] Antibody induction is supported by the $CD4^+$ T cell compartment, particularly T follicular $CD4^+$ T (Tfh) cells, which activate B cells to drive germinal centre activation and antibody development,[13] including during malaria.[14] In human infection, Tfh cell subsets can be identified based on CXCR3 and CCR6 chemokine expression, and specific Tfh cell subsets are associated with the development of antibodies following infection and vaccination in a context dependent manner.[15] We have previously evaluated the role of Tfh cells in antibody development during *P. falciparum* malaria, using the powerful controlled human malaria infection (CHMI) model that allows the longitudinal analysis of the immune response to a single infection without the confounding effects of prior malaria exposure and heterogeneity in timing of infection that occur when investigating immunity in individuals with naturalexposure.[16] We showed that early activation of Tfh2 cells was associated with induction of functional antibodies, and Tfh1 cells were associated with short lived plasmablasts which have negative roles in germinal centre formation.[17,18] While age has been shown to influence Tfh2 and Tfh1 proportions and activation,[19,20] other host factors that may influence Tfh cells during malaria are unknown.

One of the multiple factors known to modify the human immune response is cytomegalovirus (CMV)

infection, a ubiquitous beta-herpes virus which establishes a life-long persistence and results in major remodelling of the immune response.[21] In monozygotic twins with discordant CMV serostatus, over 50% of immune parameters tested were influenced by CMV seropositivity,[22] with impacts across the immune landscape, including monocytes, NK cells, and T cells.[23] Within the T cell compartment, CMV seropositivity has been associated with the expansion of memory cells that lose expression of costimulatory receptors CD27 and CD28, and gain expression of markers of terminal differentiation including CD57.[24] While these changes are most striking in $CD8^+$ T cells, they also occur within $CD4^+$ T cells.[25] This immune remodelling has consequences for subsequent pathogenesis of a broad range of diseases and induction of antibody following infection with other pathogens.[26] The global seroprevalence of CMV is 83%, which ranges from less than 50% in high income countries to up to 100% in LMIC,[27] where the majority of infants are infected in the first year of life.[28] These geographical differences in CMV seroprevalence have implications for vaccine efficacy for pathogens where CMV can modify the immune response.[29] Whether CMV is associated with changes to host immune response to malaria infection or vaccination is unknown.

Here we aimed to investigate the associations between CMV serostatus and antibody induction during malaria infection and after vaccination. To do so we investigated antibody induction in individuals who were CMV seronegative and seropositive enrolled in CHMI studies[17] and in a phase 1a clinical trial of a full-length MSP1 vaccine candidate.[30,31] We found that CMV seropositivity was associated with reduced antibody induction of specific antibody subclasses and functions following both CHMI and vaccination, and that these reductions were correlated with CMV associated changes within of the CD4+ T cell compartment. These findings have implications for the development of malaria immunity, either induced by infection or vaccination, in children in endemic areas where CMV infection occurs in infancy, and CMV seropositivity reaches 100%.[28,32]

Methods

Ethics

Written informed consent was obtained from all participants. Ethics approval for the use of human samples in the relevant studies was obtained from the Alfred Human Research and Ethics Committee for the Burnet Institute (#225/19), the Human Research and Ethics Committee of the QIMR-Berghofer Medical Research Institute (P1479, P3444 and P3445) and the Ethics Committee of the Medical Faculty of Heidelberg (AFmo-538/2016) and the relevant regulatory authority (Paul Ehrlich Institute, Langen, Germany). Study complied with all ethics approval conditions.

Study populations

Controlled human malaria infection (CHMI) studies were performed as previously described using the induced blood stage malaria model.[33] Individuals who were malaria naïve were inoculated by intravenous injection of 2800 *P. falciparum* infected red blood cells and monitored for parasite growth with qPCR.[34] Here, blood samples were collected at baseline (day 0), day of treatment (day 8) and at 14 or 15 days and at end of study (EOS), 27–36 days after inoculation (4 studies, across 6 independent infection cohorts). Clinical trials were registered at ClinicalTrials.gov NCT02867059,[35] NCT02783833,[36] NCT02431637,[37] NCT02431650.[37] Trials were completed between May 2015 and February 2017. Whole blood was stained and analysed immediately by flow cytometry and plasma was collected from lithium heparin collection tubes. Cumulative parasite burden was calculated as previously described with a trapezoidal method on serial log10 transformed parasites/mL data from 4 days after inoculation to end of study timepoint, which ranged from 27 to 36 days post inoculation.[17]

Full-length MSP1 vaccination was performed as previously described in a Phase 1a clinical trial of full-length MSP1 adjuvanted with GLA-SE[30,31] registered with EudraCT (No. 2016-002463-33). Trial was completed between April 2017 and December 2018. Volunteers received three vaccinations at intervals of 29 ± 3 days (days 0, 29 and 57). Vaccinations were of either 25, 50 or 150 mg of MSP1 adjuvanted with 5 mg of GLA-SE. For the current study, antibody responses at day 85 following first vaccination, and cellular responses at day 0 and day 85 were considered. At these time points, induced antibody titres and functions were comparable across vaccine antigen dose,[30,31] as such vaccine dosage groups were collapsed into a single analysis. Antibody responses were measured from sera collected in S-Monovetter serum-gel, and PMBCs were collected from sodium-citrate tubes.

Within some CHMI trials, females of child baring age were excluded due to use of experimental drugs. For MSP1 vaccine study, both sexes were included, with 19/32 females in the parent study.[30] In both studies sex was self-reported. All available data from both CHMI and vaccine cohorts were included in this study.

CMV and EBV serostatus

For CHMI cohorts CMV and EBV seroprevalence was assessed using baseline plasma samples by commercially available ELISA kits (ab108724 and ab108730), according to manufacturer's instructions. CMV ELISA kit has a reported sensitivity and specificity of 98% and 97.5% respectively. For the vaccination cohort, CMV seroprevalence was assessed using day 0 plasma samples by commercially available CMV IgG ELISA (EUROIMMUN EI 2570-9601 G). One individual fell into a CMV intermediate status in the ELISA. For this sample, an immunoblot (MIKROGEN DIANOSTIK

*recom*Line CMV IgG 5572) was performed which was positive. The EBV seroprevalence was established using the Anti-EBV CA_ELISA (IgG) Assay (EUROIMMUN EI 2791-9601 G).

Induced antibody responses

For CHMI studies, antibody response to intact merozoites and merozoite surface antigen MSP2 were quantified as previously described.[17] To isolate merozoites, *P. falciparum* 3D7 parasites were maintained in continuous culture in RPMI-HEPES medium supplemented with hypoxanthine (370 mM), gentamicin (30 mg/mL), 25 mM sodium bicarbonate and 0.25% AlbuMAX II (GIBCO) or 5% heat-inactivated human sera in O + RBCs from malaria-naive donors (Australian Red Cross blood bank). Cultures were incubated at 37 °C in 1% O_2, 5% CO_2, 94% N_2 and schizont stage parasites were purified by MACS separation (Miltenyl Biotec). To isolate merozoites, magnet purified schizonts were incubated with the protease inhibitor E64 (10 mg/mL), and following complete development, merozoites were isolated by membrane filtration (1.2 mm). 50 mL of *P. falciparum* 3D7 merozoites (2.5×10^5 merozoites/mL) or 50 mL of 0.5 mg/mL MSP2 recombinant antigen[38] in PBS were coated to 96-well flat bottom MaxiSorb plates (Nunc) overnight at 4 °C. Plates were blocked with 10% skim milk for merozoites, or 1% casein (Sigma–Aldrich) for MSP2 for 2 h at 37 °C. Plasma was diluted in 0.1% casein in PBS1/100 for IgG, 1/250 for IgG subclasses and IgM, 1/100 for C1q, 1/100 for FcgR targeting merozoites or 1/50 for FcgR targeting MSP2) and incubated for 2 h at room temperature. Plasma dilutions were optimised to maximise signal to noise ratio based on malaria-naïve (day 0) responses. For total antigen specific IgG detection, plates were incubated with goat polyclonal anti-human IgG HRP- conjugate (1/1000; Thermo Fisher Scientific) for 1 h at room temperature. For detection of IgG subclasses and IgM, plates were incubated with a mouse anti-human IgG1 (clone HP6069), mouse anti-human IgG3 (HP6050, Cat# MH1031, RRID AB_2539709) or mouse anti-human IgM (clone HP6083, Cat# 05-4900, RRID AB_2532927) at 1/1000 (Thermo Fisher Scientific) for 1 h at room temperature. This was followed by detection with a goat polyclonal anti-mouse IgG HRP-conjugate (1/1000; Cat# 12-349, RRID AB_390192, Millipore). For all ELISAs, plates were washed three times with PBS (for merozoite ELISAs) or PBS-Tween 0.05% (for MSP2) between antibody incubation steps. For detection of complement fixing antibodies, following incubation with human sera, plates were incubated with purified C1q (10 mg/mL; Millipore) as a complement source, for 30 min at room temperature. C1q fixation was detected with rabbit anti-C1q antibodies (1/2000; in-house) and a goat anti-rabbit-HRP (1/2500; Millipore). For FcγR assays, 100 µL of biotin-conjugated rsFcγRIIa H131 or rsFcγRIIIa V158 ectodomain dimer (0.2 µg/mL) was incubated at 37 °C for 1 h followed by 3 washes with PBS-Tween. The binding was detected with horseradish peroxidase (HRP)-conjugated streptavidin antibody (1:10,000) in PBS-BSA at 37 °C for 1 h.

TMB liquid substrate (Life Technologies) was added for 1 h at room temperature and the reaction was stopped using 1M sulfuric acid. The optical density (OD) was read at 450 nm.

For opsonic phagocytosis, THP1 cells were incubated with intact merozoites (stained with Ethidium Bromide and opsonised with plasma diluted 1/100) or latex beads coated with MSP2 (opsonised with plasma diluted 1/10) for 20 min at 37 °C and cells washed with FACS buffer. The proportion of THP-1 cells containing fluorescent-positive beads was evaluated by flow cytometry (FACS CantoII, BD Biosciences), analysed using FlowJo software and presented as phagocytosis index (the percentage of THP-1 monocytes with ingested merozoites or beads).

To calculate antibody score antibody responses below positive cut-off threshold were set as negative, and remaining positive responses were used to calculate median and used to categorise responses into low (below median) and high (above median) responses. Antibody score was calculated by giving categories zero/low/high a numerical score of 0/1/2 and then summing across all antibody responses.

For full-length MSP1 vaccination studies, antibodies were measured as previously reported.[30,31] Total IgG and IgM antibody levels were determined by ELISA using MaxiSorp plates (Thermo Fisher Scientific) coated with 100 nM recombinant full-length MSP1 (Glycotope Biotechnology GmbH, Heidelberg). Sera were titrated in two-fold dilutions and incubated for 2 h. A secondary antibody goat anti-human IgG and IgM alkaline phosphatase conjugate (Sigma–Aldrich, Cat#A154 RRID AB_257935 and Cat#A3437 RRID AB_258080 respectively) was used at a dilution of 1:20,000 for 1 h. The substrate p-nitrophenyl-phosphate was added and incubated for 1 h in the dark, and stopped with 1M NaOH. The absorbance at 405 nm was determined using the plate reader Cytation 3 (BioTek). For the IgG subtype ELISA, the same protocol was applied, with the exception that subclass-specific peroxidase-conjugated secondary antibodies were used (The Binding Site GmbH). The substrate 1-step turbo TMB (Thermo Fisher) was added for 20 min in the dark, then stopped by adding 1 M HCl. Optical density was recorded at 450 nm. For the C1q fixation ELISA assay, full-length MSP1 coated plates were also incubated overnight at 4 °C. The next day, the plates were washed four times with 1× PBS containing 0.05% Tween 20 and blocked with 1% casein/PBS at 37 °C for 2 h. After washing, the plates were incubated with 50 µl of purified IgG at 1 mg/mL blocking buffer, respectively at 37 °C for 1 h. After incubation, the plates were washed and recombinant C1q

(Abcam) at 10 μg/mL in blocking buffer was added for 30 min at 37 °C. To detect C1q binding, anti-C1q horse radish peroxidase (HRP)-conjugated secondary antibodies (Abcam Cat#ab46191 RRID AB_726908) was added at a 1:100 dilution in blocking buffer for 1 h at 37 °C. Then SigmaFAST OPD (Sigma–Aldrich) was added for 30 min to 1 h in the dark at room temperature for development. The reaction was stopped with 1M HCL and the signal intensity was measured at 492 nm using the Biotek Cytation 3 plate reader. For the Antibody dependent respiratory burst (ADRB) assay full length MSP1 protein was coated at 100 nM in PBS in opaque 96-well Lumitrac microplates (Greiner Bio-One), blocked with 1% casein/PBS and incubated for 1 h at 37 °C with 50 μl/well of IgG at 1 mg/mL. The plates were washed with PBS and 50 μl/well of luminol at 0.04 mg/mL were added to each well. Next, freshly isolated neutrophils were added at 10^7 cells/mL and absorption was immediately read at 450 nm using the Biotek Cytation 3 reader for every 2 min over 1 h. For the Opsonic phagocytosis activity assay, full-length MSP1 coupled to fluorescent beads were performed as previously reported[30,31] using THP1 cells (ATCC TIB-202), and neutrophils). Briefly, an antigen coupled bead suspension was added to 96-well to U-bottomed plates and opsonised for 1h at 37 °C with 50 μl/well of purified IgG. The plates were centrifuged at 2000×g for 7 min and washed with PBS. Opsonised beads were resuspended in culture medium (RPMI 1640 media with 2 mM L-glutamine, 10% FCS and 1% penicillin-streptomycin) before incubation with 5×10^4 phagocytes at 37 °C for 30 min. Phagocytosis was arrested by centrifugation at 1200 rpm for 7 min at 4 °C and washing with ice-cold FACS buffer (0.5% BSA and 2 mM EDTA in PBS). Cells were resuspended in 2% formaldehyde/PBS and the proportion of phagocytes containing PE fluorescent beads was determined on a FACS Canto II (BD Biosciences). NK activity assays, full-length MSP1-coated plates were washed with PBS and blocked with 1% casein/PBS for 4 h at 37 °C. The plates were washed and incubated for 2 h at 37 °C with IgG at 1 mg/mL. Then 5×10^4 freshly isolated human NK cells together with anti-human CD107a PE (H4A3, Cat#560948, RRID AB_396135, BD Biosciences, 1:70), brefeldin A (Sigma–Aldrich, 1:200) and monensin (Sigma, 1:200) was added for 18 h at 37 °C. Afterwards, the cells were transferred to 96-well V-bottomed plates, centrifuged at 1500 rpm for min at 4 °C and washed with ice-cold FACS buffer. Cell viability was determined by adding dye eFluor™520 (Thermo Fischer). NK cell surface markers were labelled with an antibody cocktail of anti-CD56 APC (B169, Cat#555518, RRID AB_398601, BD Biosciences, 1:17) and anti-CD3 PE-Cy5 (UCHT1, Cat# 555334, RRID AB_395741, BD Biosciences, 1:33) for 30 min at 4 °C in the dark. After washing, NK cells were fixed with CellFIX (BD Biosciences) at 4 °C and permeabilised with permwash (BD Biosciences) for 10 min at 4 °C. Intracellular IFN-γ was measured by adding anti-IFN-γ PE-Cy7 (B27, Cat#560924, RRID, AB_396760, BD Biosciences, 1:33) for 1 h at 4 °C. NK cells were washed with permwash, resuspended in FACS buffer and Ab-NK activity (proportion of NK cells with CD107a and/or IFN-γ staining was assessed by a FACS CantoII (BD biosciences).

Quantification of T cells

For CHMI studies, whole blood was analysed as described previously.[17] 200 mL of whole blood were stained with the following conjugated antibodies, all from BD Biosciences; anti-CD20-BUV395 (2H7, 1:150, Cat# 563782, RRID AB_2744325), anti-CXCR5-BV421 (RF8B2, 1:50, Cat#562747, RRID AB_2737766), anti-CD4 (V500, 1:30, Cat# 560768, RRID AB_1937323), anti-CCR6-BV650 (11A9, 1:200, Cat#563922, RRID AB_2738488), anti-CD38-BV786 (HIT2, 1:400, Cat#563964, RRID AB_2738515), anti-CXCR3-APC (1C6, 1:25, Cat#550967, RRID AB_398481), anti-CD27-APC-R700 (M-T271, 1:100, Cat#565116, AB_2739074), anti-CD8-APC-Cy7 (SK1, 1:150, Cat#557834, RRID AB_396892), anti-CD19-FITC (HIB19, 1:20, Cat#560994, RRID AB_395812), anti-CD45-PerCP-Cy5.5 (2D1, 1:50, Cat#340953, RRID AB_400194), anti-ICOS-PE (DX29, 1:10, Cat#557802, RRID AB_396878), anti-CD3-PE-CF594 (UCHT1, 1:600, Cat#562280, RRID AB_11153674), anti-PD1-PE-Cy7 (EH12.1, 1:100, Cat#561272, AB_10611585). RBCs were lysed with FACS lysing solution (BD) and resuspended in 2% FBS/PBS. Samples were acquired on the BD LSR Fortessa TM 5 laser cytometer (BD Biosciences). These data were analysed using FlowJo version 10.8 software (Tree Star, San Carlos, CA, USA).

For full-length MSP1 vaccine cohorts, cellular responses were analysed as previously reported.[31] The phenotyping of T- and B-lymphocytes was conducted in whole blood samples within 24 h. To monitor the dynamics of lymphocyte subpopulations, comprehensive T-cell and B-cell panels were employed, based on modified recommendations from the "Human Immunophenotyping Consortium".[39]

For all commercial antibodies and cell lines, validation were performed by the suppliers per quality assurance documents provided. All validation data are available on the manufacture's websites.

Statistical analysis

Non-parametric testing was preformed except for data which was normally or near-normally distributed. Normality was assessed visually using histogram frequency distributions. Non-normalised continuous data of antibody score, or individual antibody titres and functions, and cellular responses was compared between CMV seronegative and seropositive groups using Mann–Whitney U test. Correlations between non-normalised continuous data antibody score and cell responses were measured by Spearman's correlations.

Centred and z-scored transformed antibodies induced by MSP1 vaccination were compared between CMV seronegative and seropositive groups by MANOVA and t-tests. Statistical comparisons were not adjusted for multiple comparisons. All analyses were performed in R (version 4.4.4). Graphical outputs were made in ggplot2 (version 3.5.1) and ggpubr (version 0.6.0). No sample size calculation was performed, instead all available participant data was included. No subgroup analysis was performed. Data generation was performed with blinding to participant demographic data (including CMV status, sex, age etc).

Role of funders

Funders had no role in data generation, analysis, interpretation, writing of the manuscript or the decision to submit for publication.

Results

Latent CMV infection is associated with reduced antibody induction following controlled human malaria infection in adults

To investigate the impact of latent CMV infection (quantified as CMV seropositive) on the immune response to *P. falciparum* infection, we analysed antibody and Tfh responses in 40 malaria-naïve adults during blood stage CHMI (median age 25.5, range 18–52 years).[17] Within the cohort, 21/40 (52%) individuals were seropositive for CMV. Sex, Epstein–Barr virus (EBV) seropositivity and age, were comparable between the individuals who were CMV seronegative and seropositive (Table 1).

As previously reported, the induced antibody response was measured as an antibody score, that captured the breadth, magnitude and functionality of antibodies to the merozoite parasite stage, and major merozoite antigen, merozoite surface protein 2 (MSP2).[17] Antibody score was significantly lower in individuals who were CMV seropositive (Fig. 1a). There was no difference in antibody score with EBV status, Sex, and antibody score was not associated with age (Supplementary Fig. S1a). CMV associated differences in antibody score where not driven by differences in cumulative parasitaemia, which was not different between individuals who were CMV seronegative and seropositive (Fig. 1b). We have previously reported that antibody score is not associated with cumulative parasitaemia.[17]

	CMV serostatus	
	Negative	Positive
Total, n (%)	19 (48%)	21 (52%)
Sex, male, n (%)	18 (95%)	18 (86%)
EBV, positive, n (%)	18 (95%)	17 (81%)
Age years, median [IQR]	26 [20.25–31.75]	25 [21–29]

Table 1: **Study populations demographics.**

CMV seropositive adults have reduced induction of antibodies following controlled human malaria infection

To assess the antibodies that were contributing to reduced antibody score in individuals who were CMV seropositive, we first divided the antibody score into responses targeting the merozoite, and responses targeting MSP2 antigen. Antibody score for the merozoite was significantly higher in individuals who were CMV seronegative ($p = 0.025$, Mann–Whitney U test), but antibody score for MSP2 was not different ($p = 0.24$, Mann–Whitney U test) (Supplementary Fig. S1b). To further analyse the differences in induced antibodies targeting the merozoite and the function of these antibodies, IgM, IgG (total and subclasses) were analysed along with C1q fixation, Fcγ binding and opsonic phagocytosis. In individuals who were CMV seropositive, IgG to the merozoite was lower at end of study (EOS, Day 27–36), but there were no differences in the magnitude of induced IgM (Fig. 2a). The reduced IgG in individuals who were CMV seropositive was driven by a reduced IgG1 response. The magnitude of IgG2 and IgG3 were also lower, however differences were not statistically significant (Fig. 2b). IgG1 is a cytophilic antibody which has important functional capacity to fix complement and interact with Fc receptors on phagocytes. These functional antibody responses have essential roles in immunity to malaria and can target the merozoite to block invasion of the red blood cell and mediate protection.[11,40,41] Consistent with reduced IgG1 induction in individuals who were CMV seropositive, these individuals also had reduced induction of antibodies that could fix complement (measured by C1q fixation, the first step in the classical complement cascade[40]), and reduced binding of FcγRII and FcγIII, which are involved in phagocytosis of parasites by neutrophils and other immune cells[42] (Fig. 2c and d). However, antibodies that could mediate opsonic phagocytosis by the THP-1 pro-monocytic cell line, which primarily involves FcγRI,[42] did not differ between the two groups (Fig. 2e). This finding is consistent with the correlation between levels to IgG1 recognising the merozoite and C1q and FcγIII fixation, but not opsonic phagocytosis reported previously.[17] There was no difference in the antibody response to MSP2 (Supplementary Fig. S2). Together these data suggest that CMV is an important modulator of the primary immune response to malaria infection.

CMV seropositive adults have expansion of Tfh1 cells, and reduced proportions of activated Tfh2 cells during infection

We have previously shown that in this cohort of CHMI participants, early activation of Tfh2 cells was associated

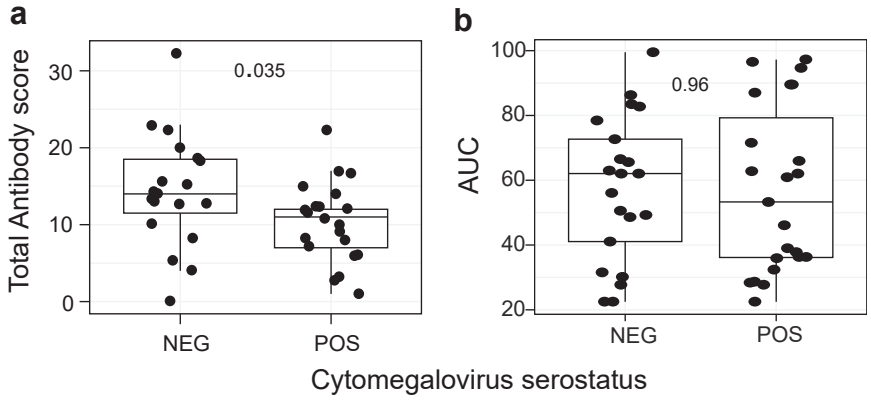

Fig. 1: **Antibodies induced against merozoite by controlled human malaria infection are reduced in adults with latent CMV infection a)** Individuals were inoculated with blood stage malaria. IgM, IgG and functional antibodies to merozoite and major merozoite antigen MSP2 were quantified at end of study and categorised as negative, low or high responses with a score 0, 1, 2 respectively. Scores were combined to calculate an antibody score used to capture the total magnitude, breadth and functionality of the responses. Antibody score was stratified CMV serostatus (CMV seronegative n = 19 [NEG], CMV seropositive n = 21 [POS]), **(b)** In the same individuals, during blood stage malaria, cumulative parasitemia was measured by Area Under Curve (AUC) from day 4 post inoculation to end of study. Data is stratified by CMV serostatus. Data is Tukey boxplots with the median, 25th and 75th percentiles. The upper and lower hinges extend to the largest and smallest values, respectively but no further then 1.5XIQR from the hinge. P are Mann–Whitney U test. See also Supplementary Fig. S1.

with anti-parasitic antibody induction.[17] In contrast, activation of Tfh1 cells was not associated with antibody levels, but was associated with increased antibody secreting cell development which may impair germinal centres by acting as a nutrient sink during infection.[18] To assess if CMV seropositivity impacted Tfh cell differentiation either before or during CHMI, we analysed the same data set, and stratified by CMV serostatus. Tfh cells were defined as all CXCR5$^+$ cells, subsets identified based on CXCR3 and CCR6 expression, and activation measured by expression of PD1, CD38 and ICOS. Non-Tfh effector CD4$^+$ cells (CXCR5$^-$ CD4$^+$ T cells) were also analysed (Supplementary Fig. S3a and b). Within the Tfh cell population, individuals who were CMV seropositive had a significantly higher proportion of Tfh1 cells, and a significantly decreased proportion of Tfh2 cells, before and during CHMI (Fig. 3b). This expansion towards Th1-like cells was not seen in non-Tfh effectors, suggesting that the expansion of Tfh1 cells in individuals who were CMV seropositive was not due to a systemic inflammatory phenotype (Supplementary Fig. S3d). Importantly, amongst activated Tfh cells (analysed as either PD1$^+$, ICOS$^+$ or CD38$^+$ Tfh cells), there was a significantly increased proportion of Tfh1 cells (PD1$^+$) and a reduced proportion of Tfh2 cells (ICOS$^+$ and CD38$^+$) at day 14/15 in individuals who were CMV seropositive (Fig. 3c). There was also a significantly reduced proportion of Tfh2 cells within PD1+ Tfh, and a trend towards reduced proportions of Tfh2 cells in ICOS+ and CD38+ Tfh at day 0, prior to inoculation (Supplementary Fig. S3c), however it should be noted that the magnitude of activation prior to infection is low.[17] Within the CD4$^+$ T cell population, there was no difference in the proportion of Tfh cells, nor consistent major differences in CD4$^+$ effector (CXCR5-) Th1-, Th2- and Th17- like populations between individuals who were CMV infected and uninfected, with only a higher proportion of Th2- cells detected in individuals who were CMV seronegative at EOS (Supplementary Fig. S3d and e). Further, there was no difference in magnitude of activation in Tfh cells, nor non-Tfh effector cells during CHMI, aside from an increase in Th2 cell activation at day 14 post-inoculation (Fig. 3a, Supplementary Fig. S3d and e). While previous studies have shown that CMV antigen specific Tfh cells are dominated by Tfh1 cell subsets,[43] these findings show that latent CMV infection is associated with changes within the entire Tfh cell compartment.

The relationship between Tfh cell subsets and antibody induction is associated with CMV serostatus

Different subsets of Tfh cells have specific functions within the germinal centre to drive B cell activation.[15] In the cohort studied here, we have previously reported that activation of Tfh2 cells at day 8 was associated with induction of functional antibodies at end of study.[17] To assess if the CMV associated bias of the Tfh cell compartment towards Tfh1 cells may impact antibody development in malaria, we assessed the correlations between Tfh cell subsets and antibody score in our cohort, stratified by CMV serostatus. The activation of Tfh2 cells at day 8,[17] was only associated with antibody score in individuals who were CMV seronegative (Fig. 4a). Further, amongst individuals who were CMV seropositive, the proportion of Tfh1 cells in the Tfh cell

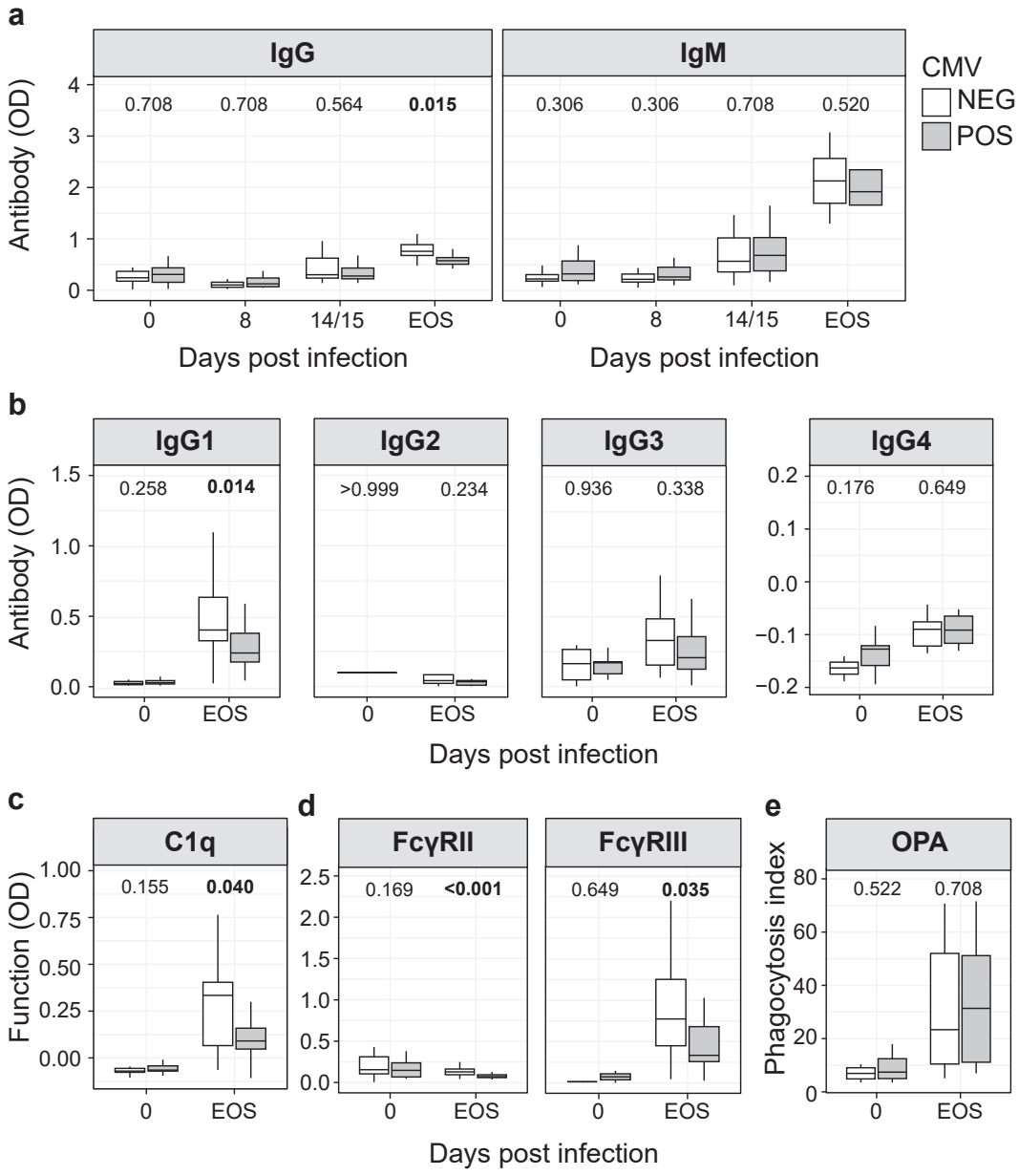

Fig. 2: **IgG1 and functional antibodies to the merozoite induced by controlled human malaria infection are reduced in CMV infected adults (a)** IgG and IgM antibodies, **(b)** IgG subclass antibodies and levels of functional antibodies that can **(c)** fix C1q complement component, **(d)** bind FcγRII and FcγRIII, and **(e)** drive opsonic phagocytosis by THP1 cells, targeting 3D7 strain whole merozoite parasites, were quantified and stratified by CMV serostatus. For IgG and IgM antibodies were measured at day 0, 8, 14/15 and end of study (EOS) timepoints after controlled human malaria infection. For IgG subclasses and functional antibodies, responses were measured at day 0 and end of study timepoints. CMV seronegative white bars (n = 19), CMV seropositive grey bars (n = 21), data is Tukey boxplots with the median, 25th and 75th percentiles. The upper and lower hinges extend to the largest and smallest values, respectively but no further then 1.5 XIQR from the hinge. P are Mann–Whitney U test. See also Supplementary Fig. S2.

compartment both prior to infection at day 0 and at day 8, negatively correlated with antibody score (Fig. 4b). These data expands on our previous findings, by highlighting that the previously reported association between Tfh2 cells and antibody induction[17] is only detected in individuals who were CMV seronegative. Further, the association between latent CMV infection and increased proportions of Tfh1 cells may negatively impact induction and function of antibodies during malaria.

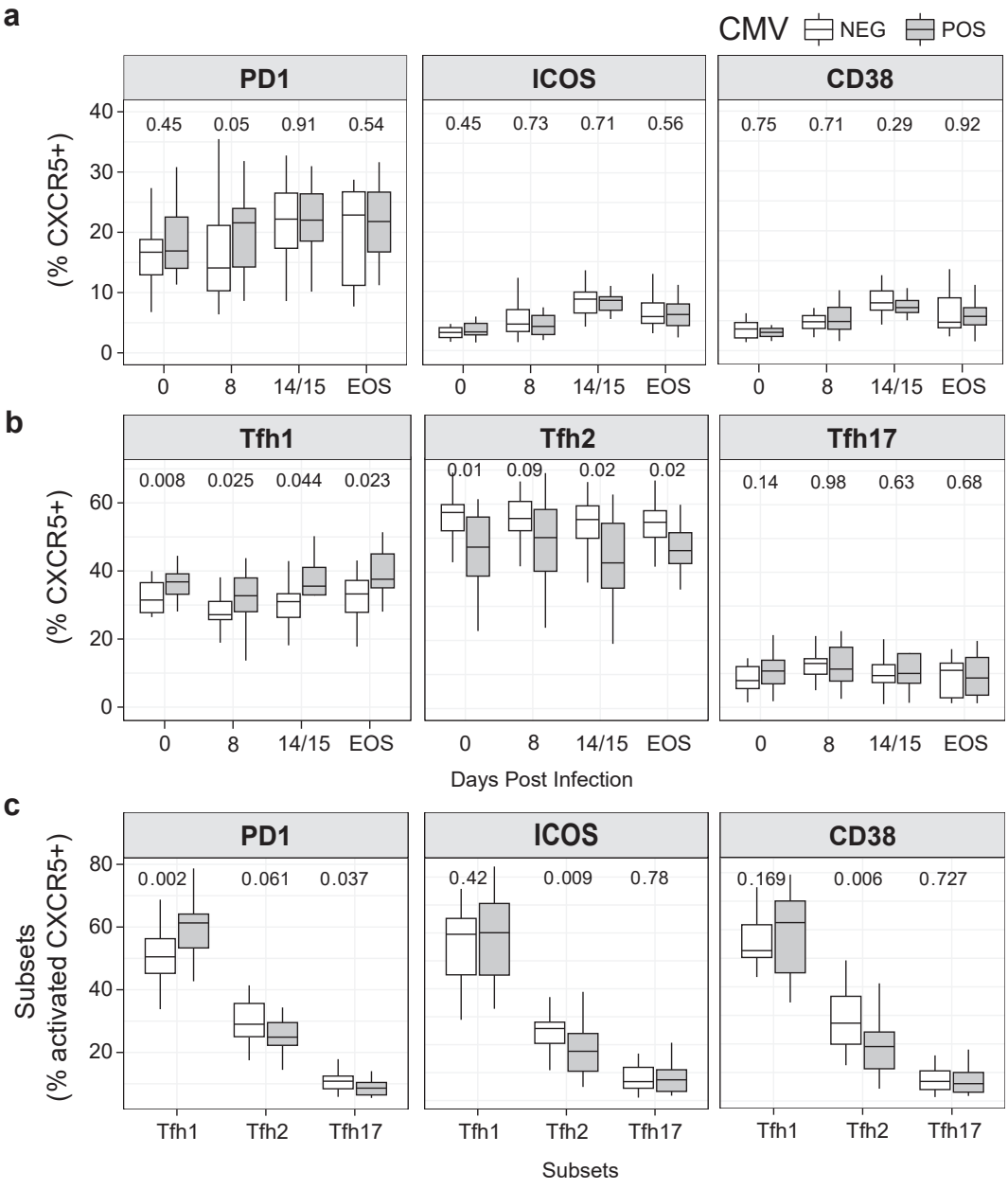

Fig. 3: **Tfh cell compartment is skewed to Tfh1 subsets in CMV seropositive adults (a)** Activation of Tfh cells as measured by quantifying PD1+, ICOS+, and CD38+ cells (% of Tfh CD4 T cells) on whole blood in individuals during CHMI at day 0, 8, 14/15 and end of study (EOS) timepoints. Data is stratified by CMV serostatus. **(b)** Tfh cell subsets (% of Tfh CXCR5+ CD4 T cells), stratified by CMV status. **(c)** Tfh cell subsets as a proportion of activated Tfh cells, stratified by CMV serostatus at day 14/15. CMV seronegative white bars (n = 19 for day 0, 8, 15, n = 17 for day EOS), CMV seropositive grey bars (n = 21 for day 0, 8, 15 and n = 20 for EOS), data is Tukey boxplots with the median, 25th and 75th percentiles. The upper and lower hinges extend to the largest and smallest values, respectively but no further then 1.5XIQR from the hinge. P are Mann–Whitney U test. See also Supplementary Fig. S3.

Latent CMV infection is associated with a reduced antibody response to malaria vaccination

To assess if latent CMV infection is also negatively associated with malaria vaccine responses, we analysed antibody levels in 24 individuals vaccinated with full-length MSP1 formulated with GLA-SE in a Phase 1a clinical trial.[30,31] Within this study 12 individuals were CMV seronegative and 12 were CMV seropositive. Sex, age and EBV status were comparable between individuals who were CMV seropositive and seronegative

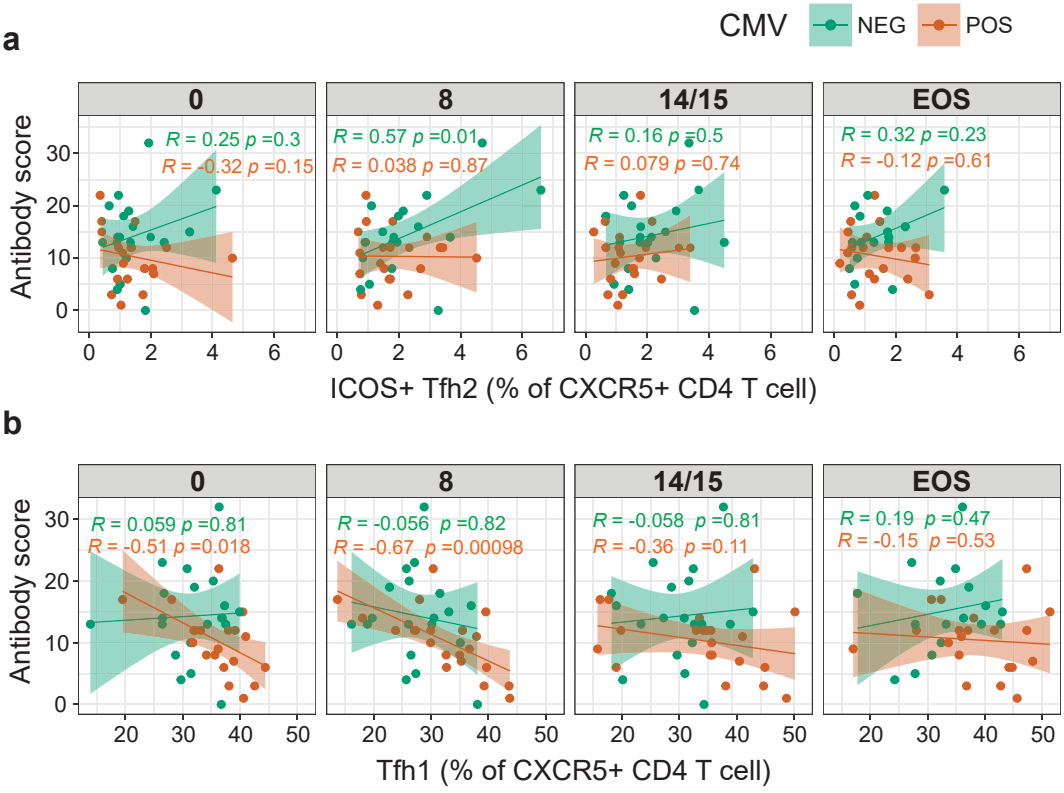

Fig. 4: **Correlation between Tfh cell subsets and antibody development based on CMV serostatus (a)** Correlation between activated Tfh2 cells (ICOS + Tfh2 (% of Tfh CXCR5+ CD4 T cells) and **(b)** the proportion of Tfh1 (% of Tfh CXCR5+ CD4 T cells) at day 0, 8, 14/15, end of study (EOS) and Antibody score measured at EOS. CMV seronegative green (n = 19 for day 0, 8, 15, n = 17 for day EOS), CMV seropositive orange (n = 21 for day 0, 8, 15 and n = 20 for EOS). Spearman's Rho and p are indicated.

(Supplementary Table S1). We analysed induced MSP1 antibodies at day 85 following vaccination, which has been shown to be when antibody response peak.[30,31] Induced IgG and IgM to MSP1, along with antibody functions of C1q fixation, opsonic phagocytosis by THP1 and neutrophils, and neutrophil antibody dependent respiratory burst (ADRB) responses were assessed. Additionally, IgG subclasses and NK IFNγ production and degranulation (measured by CD107a expression), were available for 11–13 participants. Unbiased clustering of all antibody responses aggregated the cohort into two clusters, low responders who were 8/12 (66%) CMV seropositive, and high responders who were 4/12 (33%) CMV seropositive. While the difference in proportions were not statistically different likely due to limitations in cohort size (Fisher Exact test p = 0.22) findings are consistent with a negative association between latent CMV infection and vaccine induced antibodies (Fig. 5a). To evaluate whether there was a difference in the global and specific antibody response between individuals who were CMV seropositive and seronegative, we used a MANOVA test which includes all antibody data in a single analysis. This found that there was a significant difference between CMV serostatus groups based on antibody parameters (MANOVA p = 0.027, Fig. 5b). MSP1 targeted IgG, IgM, C1q and neutrophil ADRB were all reduced in individuals who were CMV seropositive. Differences were most pronounced for C1q fixation which reached statistical significance (t test p = 0.034, Fig. 5b). For antibody responses where only partial data was available, there was reduced IgG1, IgG3, IgG4, along with reduced NK IFNγ and degranulation in individuals who were CMV seropositive (Fig. 5c).

To assess if CMV associated cellular changes contributed to the reduced MSP1 antibody induction, we analysed the cellular compartment in individuals who were vaccinated at time of vaccination (day 0) and peak antibody response (day 85) (Supplementary Fig. S4a). Unlike in the CHMI cohort, we did not detect any significant differences in subset distribution based on CMV infection status within Tfh and non-Tfh $CD4^+$ T cells, nor were there differences in subset distribution of activated Tfh cells (Supplementary Fig. S4b–e). Previous studies have reported CMV to be associated with down regulation of co-stimulatory marker CD28, and upregulation of terminally differentiation marker CD57,[25] both of which were included in cellular analysis.

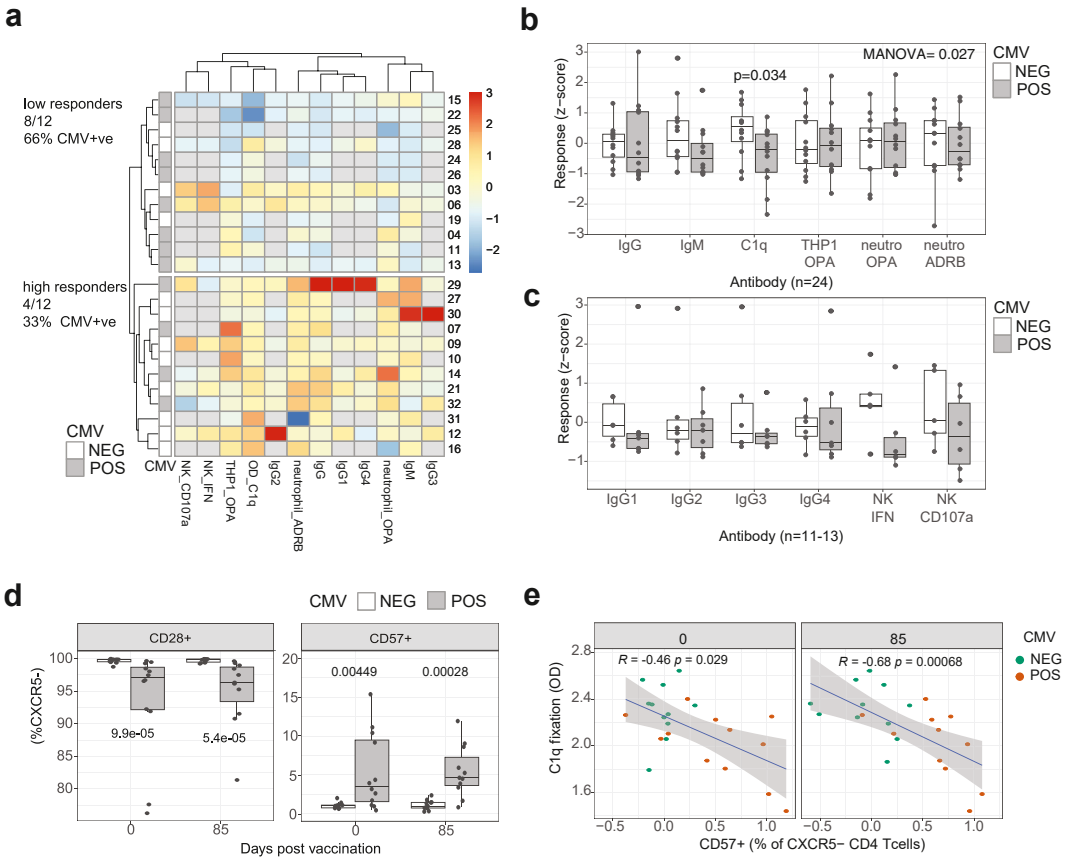

***Fig. 5:* Impact of CMV serostatus on antibody responses to MSP1 vaccination.** Antibodies induced by MSP1 vaccination at day 85 were measured to quantify MSP1 targeted IgM, IgG subclasses (IgG1, IgG2, IgG3, IgG4) and functional antibodies that could mediated C1q complement fixation, opsonic phagocytosis (OPA) from THP1 cells and neutrophils, neutrophil antibody dependent respiratory burst (ADRB), and NK production of IFNγ and degranulation (CD107a). Antibody responses were normalised and centred and represented as z-scores. **(a)** Unbiased clustering of all z-score response in individuals within vaccination cohort Individuals aggregated into low and high responders. CMV status is shown (n = 24). **(b)** Impact of CMV serostatus on antibody responses for IgG, IgM, C1q, THP1 OPA, neutrophil OPA and neutrophil ADRB (n = 24). Manova p is shown, ttest for C1q is shown. **(c)** IgG subclasses, and NK responses between individuals who were CMV negative and positive (n = 11–13). **(d)** Proportion of CD28+ and CD57+ non-Tfh CD4 T cells (CXCR5-) at day 0 and day 85 stratified by CMV infection status. **(e)** Correlation between frequency of CD57+ non-Tfh CD4 T cells at day 0 and day 85, and C1q antibodies at day 85 following vaccination. p values is Mann Whitney U test for D and for E Spearman's Rho and p are indicated. See also Supplementary Fig. S4.

Consistent with previous studies, individuals who were CMV seropositive had decreased frequencies of $CD28^+$ $CD4^+$ T cells, and increased frequencies of $CD57^+$ $CD4^+$ T cells within our cohort, both at day 0 and day 85 (Fig. 5d). These differences were significant within $CXCR5^-$ $CD4^+$ T cells, but not within the Tfh cell compartment (Supplementary Fig. 4c). The frequency of $CD57^+$ CD4 T cells at both day 0 and day 85 was negatively associated with the levels of C1q fixation, consistent with a role of CMV mediated terminally differentiation in reduced antibody responses to vaccination. Taken together, the data shows that latent CMV infection is associated with both the Tfh and non-Tfh $CD4^+$ T cell compartments and that these changes are negatively associated with antibody production during malaria or following vaccination.

Discussion

Here, we show that CMV positive serostatus is associated with a substantial negative impact on antibody development during malaria infection and following vaccination. Within our CHMI cohort, individuals who were CMV seropositive had a bias in Tfh cell compartment towards Tfh1 cells, with the proportion of Tfh1 cells being negatively associated with antibody development. In our vaccination cohort, individuals who were CMV seropositive had decreased frequencies of $CD28^+$ and increased frequencies of $CD57^+$ $CD4^+$ T cells, which was negatively associated with antibody development. These findings have significant implications for immune development during malaria via infection or vaccination in endemic areas, where there is typically a very high CMV seroprevalence.[28,32]

CHMI studies are a powerful platform to evaluate mechanisms of immunity to malaria. In previous studies using this model, we have identified that expression of specific miRNAs prior to CHMI can predict individual control of parasite growth and antibody development following drug treatment,[44] suggesting that preexisting host factors have major roles in the immune response to malaria. Here, we show that the protective role of Tfh2 cells in antibody induction[17] is associated with CMV serostatus, thus identifying a host factor underpinning immune heterogeneity. The impact of CMV on antibody induction was associated with a bias towards Tfh1 cells within the Tfh cell compartment. This link between CMV and modified Tfh cell compartment may explain previous reports that indicate that the impact of CMV on immune development in response to vaccination and infection is highly pathogen and/or context dependent. For example, while the impact of CMV serostatus on antibody induction to influenza vaccination is well studied, the findings are highly variable. Some studies have reported a negative association with CMV seropositivity,[45–52] others no impact,[48,53–55] and others reporting a positive association of CMV on antibody induction.[56–58] The impact of CMV on immune induction in the context of infection is also mixed, with antibodies induced by H1N1 influenza infection higher in individuals who were CMV seropositive,[59] but no CMV mediated differences in antibodies induced following SARS-CoV-2 infection.[60] The role of Tfh cells in antibody induction is pathogen and context dependent.[15] In malaria, where Tfh2 cells have been associated with antibody development in response to infection,[17] including in children in endemic areas,[20] or after vaccination with both the licenced RTS,S[61] and experimental blood stage malaria vaccines,[62,63] latent CMV infection may inhibit antibody induction. In contrast, following influenza vaccination, where Tfh1 cells are consistently positively associated with antibody induction,[64,65] CMV seropositive individual may have enhance antibody development, as seen in some studies.[56–58] Our data also suggest that the specific subclass and function of induced and the individual antibody target may also be important. These factors may contribute to previously reported differences in the impact of CMV depending on the context. For both CHMI and vaccination cohorts, the negative association between induced antibodies and CMV seropositivity was strongest for IgG1 and associated functions, specifically C1q fixation. This data may suggest that the impact of CMV is subclass specific. Further, within the CHMI, while the total antibody response to the merozoite was lower in individuals who were CMV seropositive, there was no differences seen in MSP2 responses. Identifying specific merozoite antigens where antibody responses are associated with CMV status is the focus of ongoing work.

Highlighting the important role of our findings with CHMI to vaccine development for malaria, we also show that antibodies induced by full-length MSP1 vaccination are reduced in individuals who were CMV seropositive within a Phase 1 a clinical study. Despite the limited cohort size within this study, significantly reduced induction of complement fixing antibodies was shown in individuals who were CMV seropositive. Within the vaccine cohort we did not detect differences in the phenotype of Tfh cells, instead reduced vaccine induced antibodies were associated with increased frequencies of $CD57^{+}$ $CD4^{+}$ T cells, a cell subset thought to be terminally differentiated. The absence of detectable CMV mediated differences in the Tfh compartment may be due to the small cohort size, technical differences in Tfh subset staining, or may reflect genetic, environmental and past infection histories distinct between the CHMI and vaccine cohorts. Nevertheless, our findings are consistent with a recent study of immune responses induced by Ebola vaccination in UK and Senegalese participants.[29] In that study latent CMV infection was also associated with the reduced induction of antibodies following vaccination, and similarly changes to CD28 and CD57 expression within the T cell compartment were reported. Differences in CMV seroprevalence between recipients of this Ebola vaccine in the UK (50% CMV positive) and Senegal (100% CMV positive) accounted for the previously reported reduced vaccine responsiveness in the Senegalese cohort, with participants who were CMV infected and from the UK having comparable responses to individuals who were Senegalese.[66] These differences in vaccine responsiveness in UK compared to participants from Senegal mimic differences in responsiveness to malaria vaccines seen between cohorts in the US and malaria endemic countries.[6–10] As such, studies to investigate the role of latent CMV infection on malaria vaccine responsiveness are required, particularly in target populations were CMV infection occurs early in life.

Limitations of our study include the relatively small cohort sizes of both CHMI and vaccine studies, which limits statistical power to detect differences. Of importance, cohorts used here in Australia and Germany may not be demographically and characteristically representative of the wider population, particularly those in malaria endemic countries. Studies in additional larger cohorts are required to assess the generalisability of our findings, particularly in children in malaria endemic areas. Additionally, all findings reported here are of associations, and future studies are required to investigate causality between CMV seropositivity and reduced antibody induction to malaria. CMV is only one factor of many that may influence antibody responses,[5] and studies in larger cohorts are required to assess the impact of CMV in the context of other social determinants of health. Of note, due to limited sample sizes, particularly when considering subgroup analysis such as sex and EBV, we have not included multivariant analysis or propensity score modelling. As such, these

factors and other sources of confounding such as race/ethnicity, BMI, socio-economic factors and lifestyles are possibility within our study. Additionally, within the CHMI study, only small numbers of females were included in the parent studies, some of which excluded women of childbearing age due to testing experimental drugs.[35–37] As such, we were unable to investigate sex associated differences in antibody induction within CMV sero-negative and sero-positive groups. Sex has been shown in previous studies to influence naturally acquired antibodies to malaria in children,[20,67] and in other infection and vaccination settings.[68,69] Similarly, the majority of individuals were EBV seropositive, and we were thus unable to assess the impact of EBV. Additionally, while previous reported changes to CD4 T cell expression of CD28 and CD57,[24] were associated with reduced antibody induction following MSP1 vaccination, expression of CD27 was not available, nor were these exhaustion markers assessed in the CHMI cohort. Further studies are required to comprehensively investigate associations between CMV mediated changes to CD4 T cells and antibody induction in malaria. Additionally, all associations with CD4 T cells assessed here were at population level, and further studies are required to analyse malaria-specific CD4 T cells.[19]

Concluding remarks and interpretation

Taken together, these data suggest that latent CMV infection is associated with heterogeneity in the host immune response to malaria, with a negative association on the development of malaria-specific antibodies induced by infection or vaccination. During malaria infection, this reduced antibody induction was linked to the skewing of Tfh cells to the Tfh1 cell subsets, and away from Tfh2 cells, which have previously been linked to antibody induction in CHMI.[17] In vaccination, reduced antibodies were associated with increased frequencies of $CD57^+$ $CD4^+$ T cells, highlighting a potential role of CMV mediated immune senescence in reduced antibody induction. Further studies with larger cohorts are required to assess generalisability of findings and to confirm if latent CMV infection contributes to the slow acquisition of protective functional antibodies in children in malaria endemic areas induced by infection or vaccination. Nevertheless, these findings have potential important implications for understanding heterogeneity in immune responses to malaria infection and vaccination, particularly as CMV is typically highly prevalent and acquired in early life in areas of malaria transmission.[28,32]

Contributors

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Investigation—RM, WAF, JAC, FdLR, DA, RTL.
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Visualisation—RM, JRL, MJB.
Writing—original draft—RM, JRL, MJB.
Writing—review and editing—all authors.
All authors read and approved the final version of the manuscript.

Data sharing statement

The data underlying all figures are available in the published article and its online supplemental material. Data is uploaded as a supplementary data file (Supplementary Table S2).

Declaration of interests

RTL is employee from Sumaya GmbH & Co. KG who provide contracts/grants and travel support. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105419.

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