

# Effector Phenotype of *Plasmodium falciparum*-Specific CD4<sup>+</sup> T Cells Is Influenced by Both Age and Transmission Intensity in Naturally Exposed Populations

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**Background.** Mechanisms mediating immunity to malaria remain unclear, but animal data and experimental human vaccination models suggest a critical role for CD4<sup>+</sup> T cells. Advances in multiparametric flow cytometry have revealed that the functional quality of pathogen-specific CD4<sup>+</sup> T cells determines immune protection in many infectious models. Little is known about the functional characteristics of *Plasmodium*-specific CD4<sup>+</sup> T-cell responses in immune and nonimmune individuals.

**Methods.** We compared T-cell responses to *Plasmodium falciparum* among household-matched children and adults residing in settings of high or low malaria transmission in Uganda. Peripheral blood mononuclear cells were stimulated with *P. falciparum* antigen, and interferon  $\gamma$  (IFN- $\gamma$ ), interleukin 2, interleukin 10, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production was analyzed via multiparametric flow cytometry.

**Results.** We found that the magnitude of the CD4<sup>+</sup> T-cell responses was greater in areas of high transmission but similar between children and adults in each setting type. In the high-transmission setting, most *P. falciparum*-specific CD4<sup>+</sup> T-cells in children produced interleukin 10, while responses in adults were dominated by IFN- $\gamma$  and TNF- $\alpha$ . In contrast, in the low-transmission setting, responses in both children and adults were dominated by IFN- $\gamma$  and TNF- $\alpha$ .

**Conclusions.** These findings highlight major differences in the CD4<sup>+</sup> T-cell response of immune adults and non-immune children that may be relevant for immune protection from malaria.

**Keywords.** CD4<sup>+</sup> T cells; malaria; *P. falciparum*; cellular immunity; IL-10; IFN- $\gamma$ .

Naturally acquired immunity to malaria emerges slowly in malaria-endemic populations. In areas of high transmission, adults experience relatively few clinical episodes, compared with children, despite similar exposure to parasites, indicating that the adult immune

system is able to control parasite burden and/or immunopathology associated with disease. Murine models have shown that cytokine-producing CD4<sup>+</sup> T cells are critical for protection from malaria [1, 2]. In naturally exposed humans, interferon  $\gamma$  (IFN- $\gamma$ ) production by CD4<sup>+</sup> T cells in response to merozoite [3, 4] and sporozoite [5–7] antigens has been associated with protection from disease. The importance of *Plasmodium falciparum*-specific CD4<sup>+</sup> T-cell responses was further demonstrated by a study of naive adults vaccinated with an ultra-low dose of blood-stage parasites, which found that the CD4<sup>+</sup> T-cell response was protective from malaria, even in the absence of antibody [8].

Advances in multiparametric flow cytometry have made it clear that assessment of pathogen-specific T cells by a single parameter is often inadequate for

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identifying correlates of protection. Antigen-specific T cells are functionally heterogeneous and can exhibit a complex variety of effector mechanisms. Mounting evidence from many infectious models suggests that the quality, rather than the quantity, of response is critical for immunity [9]. For instance, multifunctional CD4<sup>+</sup> T cells coproducing IFN- $\gamma$ , tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and interleukin 2 (IL-2) correlate with vaccine-mediated protection from *Leishmania major* [10] and are implicated in protection induced by vaccinia virus immunization [11]. Multifunctional CD4<sup>+</sup> T cells that coproduce TNF- $\alpha$  and/or IL-2 in conjunction with IFN- $\gamma$  are thought to have increased function [12] and are associated with nonprogression of human immunodeficiency virus (HIV) infection [13]. In *P. falciparum* malaria, sterilizing immunity induced by experimental vaccination with whole sporozoites is associated with the induction of CD4<sup>+</sup> T cells that coproduce IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$  [14–16]. CD4<sup>+</sup> T-cell responses of a similar phenotype have been implicated in protection from malaria conferred by the vaccine RTS,S [17–19].

Among naturally exposed children, several effector phenotypes of *P. falciparum*-specific CD4<sup>+</sup> T cells have been described, including distinct subsets producing inflammatory (TNF- $\alpha$  and IFN- $\gamma$ ) and regulatory (interleukin 10 [IL-10]) cytokines [20–22]. Recent studies suggest that the effector phenotype may be dependent on parasite exposure intensity, as recent symptomatic malaria is associated with increased frequencies of IFN- $\gamma$ /IL-10-coproducing cells and a reduced frequency of TNF- $\alpha$ -producing cells [20, 21]. Murine models of malaria and other parasitic infections indicate that IFN- $\gamma$ /IL-10-coproducing cells play an essential role in protecting the host from immunopathology, although this may come at the cost of reduced or delayed parasite clearance [23–25].

The identification of CD4<sup>+</sup> T-cell responses in children susceptible to symptomatic malaria that differ from those in immune adults may provide important information regarding the mechanisms mediating protection. In this study, we performed a detailed assessment of *P. falciparum*-specific CD4<sup>+</sup> T-cell responses among children and their adult caregivers in Uganda, in areas of high or low parasite transmission. We hypothesized that the frequency and functional characteristics of *P. falciparum*-specific CD4<sup>+</sup> T-cell responses among clinically immune adults may differ from those of children who remain vulnerable to symptomatic malaria.

## MATERIALS AND METHODS

### Ethics Approval

Written informed consent was obtained from the adult caregiver of all study participants. The study protocol was approved by Makerere University School of Medicine Research and Ethics Committee, the Uganda National Council of Science and Technology, and the University of California–San Francisco

Committee on Human Research (institutional review board number 11-05995; reference number 067647).

### Study Sites, Participants, and Follow-up Procedures

Samples were obtained from children and adults in households enrolled in a longitudinal cohort study of malaria in 2 districts, peri-urban Walukuba subcounty, Jinja, and rural Nagongera subcounty, Tororo, in eastern Uganda [26]. Both sites experience year-round malaria transmission, with 2 annual peaks during October–January and April–July. Children ages 6 months to 10 years and 1 adult caregiver from 100 randomly selected households were enrolled between August 2011 and August 2012. At time of sample collection, all participants had been followed for the entire previous year. All adult caregivers were >20 years old. Exclusion criteria included any known chronic medical condition (including HIV infection) requiring specialized care. All medical care was provided free of charge at dedicated study clinics open 7 days/week. If participants presented with documented fever (tympanic temperature,  $\geq 38^{\circ}\text{C}$ ) or a history of fever in the previous 24 hours, a thick blood smear was performed. If the smear was positive for any parasitemia, the patient received a diagnosis of malaria and was treated with artemether-lumefantrine. Quarterly routine evaluations, including thick blood smears, were performed for all study participants. Asymptomatic cases of *Plasmodium* infection identified during quarterly evaluations (defined as individuals with *Plasmodium*-positive blood smears and no fever) were not treated. All participants included in the current study were followed for at least 14 days following peripheral blood mononuclear cell (PBMC) collection for the development of symptomatic malaria. HIV serostatus was not assessed within the cohorts, but the Ugandan prevalence rates were estimated in 2011 as 7.1% in adults and 0.7% in children aged <5 years [27].

### Measurement of CD4<sup>+</sup> and CD8<sup>+</sup> T-Cell Responses to Infected Red Blood Cells (RBCs)

PBMCs were isolated by density gradient centrifugation from whole-blood specimens collected into tubes containing acid citrate dextrose and cryopreserved in liquid nitrogen. T-cell responses were measured as previously described [20]. PBMCs were thawed using standard methods and rested overnight in 10% fetal bovine serum. A total of  $10^6$  PBMCs were stimulated with intact purified trophozoite/schizont-stage *P. falciparum* (clone 3D7)-infected RBCs (iRBCs) or uninfected RBCs at an effector to target ratio of 1:2. Brefeldin A and monensin (BD Pharmingen) were added at 6 hours (10  $\mu\text{g}/\text{mL}$ ). At 24 hours, cells were washed, and surface and intracellular staining was performed with the following antibodies: from BD Pharmingen, anti-CD3-PerCP (SK7), anti-CD8-APC-H7 (SK1), anti-IFN- $\gamma$ -PE-Cy7 (B27), anti-IL-10-PE (JES3-19F1), and anti-TNF- $\alpha$ -FITC (6401.1111); from Biolegend, anti-CD4-BV650 (OKT4),

anti-CD45RA-Brilliant violet 605 (HI100), anti-CD27-Brilliant violet 711 (O323), anti-CD14-Alexa700 (M5E2), anti-CCR7-FITC (G043H7), anti-CD3-Brilliant violet 650 (OKT3), anti-CD4-PerCP (RPA-T4), anti-CD19-Alexa700 (HIB19), anti-IL-2-Brilliant violet 421 (MQ1-17H12), anti-TNF- $\alpha$ -Alexa700 (MAb11), anti-CD14-Brilliant violet 511 (M5E2), and anti-CD19-Brilliant violet 511 (HIB19); from Miltenyi Biotec, anti- $\gamma\delta$ 2-APC (123R3); and from Invitrogen, Live/Dead aqua amine. Samples were acquired on a BD LSR2 flow cytometer with FACSDiva. A mean of 100 000 CD4<sup>+</sup> T-cells (interquartile range [IQR], 85 000–130 000 cells) were collected, with a minimum of 10 000 cells collected. The proportion of CD45RA<sup>-</sup> cells was 40% (IQR, 31%–68%) and was higher in adults, compared with children (63% [IQR, 50%–71%] vs 35% [IQR, 28%–43%]).

### Data Analysis

Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carlos, California) and Pestre (version 1.7)/Spice (version 5.3; <http://exon.niaid.nih.gov>) [28]. Color compensation was performed using single-color cell controls or beads stained for each fluorochrome. Responding cells were gated as lymphocytes/singlets/CD14<sup>-</sup>CD19<sup>-</sup>Aqua<sup>-</sup>/CD3<sup>+</sup>  $\gamma\delta$  V $\delta$ 2<sup>-</sup> cells and then as CD4<sup>+</sup> or CD8<sup>+</sup> and CD45RA<sup>-</sup> cells. Cytokine production was gated as CD4<sup>+</sup>CD45RA<sup>-</sup> or CD8<sup>+</sup>CD45RA<sup>-</sup> cells producing IFN- $\gamma$ , IL-2, IL-10, or TNF- $\alpha$ , and Boolean gating was performed to categorize cells into 15 subsets. To calculate frequencies of *P. falciparum*-specific T cells, background responses to uninfected RBCs were subtracted from each subset. Responses were considered positive if the response was  $\geq 0.01\%$  of CD4<sup>+</sup>CD45RA<sup>-</sup> cells after background subtraction [28]. Based on this, total cytokine responses were considered positive if  $\geq 0.15\%$ , and total responses for individual cytokines were considered positive if  $\geq 0.08\%$ . To analyze the composition of the response, total individual cytokine responses were expressed as a fraction of the total response for each individual.

### Statistical Methods

All statistical analyses were performed using Prism 4.0 (Graph-Pad); Stata, version 12 (College Station, Texas); or Spice, version 5.3 (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland). Responding cell frequencies were compared between children and adults and between low- and high-transmission sites, using the Wilcoxon rank sum test. Statistical comparisons of global cytokine profiles were performed by partial permutation tests, using Spice software, with a threshold of 0.01% [28].

## RESULTS

### Study Populations

Samples were obtained from 71 children and their 32 primary adult caregivers enrolled in a longitudinal cohort study at 2

study sites in Uganda, the peri-urban Walukuba subcounty, Jinja (low transmission; annual entomological inoculation rate, 2.8 infectious bites/person-year), and rural Nagongera subcounty, Tororo (high transmission; annual entomological inoculation rate, 310 infectious bites/person-year), between January and August 2013 [26]. Malaria incidence (based on all documented cases of malaria recorded at study clinics) was  $>20$ -fold lower in Walukuba than in Nagongera; among children from Walukuba, the malaria incidence in the year preceding collection of blood specimens was 0.1 episodes/person-year, while in Nagongera the incidence was 2.42 episodes/person-year (Table 1). The incidence was markedly lower among adult caregivers at both sites: no episodes of malaria in Walukuba adults were recorded, and 2 episodes of malaria in Nagongera adults were recorded (incidence, 0.09 episodes/person-year). Among Nagongera children, incidence declined with age (Spearman rho,  $-0.43$ ;  $P = .0018$ ), consistent with the acquisition of immunity. Nonetheless, 81% of Nagongera children aged  $>7$  years experienced at least 1 episode of malaria in the prior year, indicating that even these older children were only partially immune. At the time of blood sample collection, no participants had symptomatic malaria. All participants from Walukuba were uninfected, whereas 17 of 51 children (33%) and 1 of 22 adults (4.6%) in Nagongera had asymptomatic *P. falciparum* infection detected by microscopy. Of these participants, 1 child (age, 2.5 years) developed symptomatic malaria within 14 days following blood sample collection. Exclusion of this participant did not change the results. Therefore, this participant was included in all analyses.

### Frequency of *P. falciparum*-Specific CD4<sup>+</sup> T Cells Does not Differ Between Children and Adults but Is Greater in Settings of High Transmission

To investigate the frequency and function of T-cell responses, PBMCs were stimulated with *P. falciparum* iRBCs, and production of IFN- $\gamma$ , IL-2, IL-10, and TNF- $\alpha$  by CD45RA<sup>-</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells was analyzed (Figure 1A). Overall, higher frequencies of cytokine-producing CD4<sup>+</sup>CD45RA<sup>-</sup> cells were observed among both children and adults residing in Nagongera, compared with Walukuba. However, the magnitude of responses did not differ significantly between children and adults within each study site (Figure 1B). Similarly, the proportions of individuals with a detectable *P. falciparum*-specific CD4<sup>+</sup> T-cell response were significantly higher among Nagongera children and adults (88% and 96%, respectively) than among Walukuba children and adults (55% and 60%, respectively), again with no significant difference between children and adults at each study site. Thus, both the frequency and prevalence of *P. falciparum*-specific CD4<sup>+</sup> T-cell responses appear to be heavily influenced by parasite exposure. There was no difference in the overall frequency of cytokine-producing cells between uninfected children and asymptomatic infected children (as determined by microscopy) in Nagongera ( $P = .45$ ). *P. falciparum*-specific CD8<sup>+</sup> T-cell

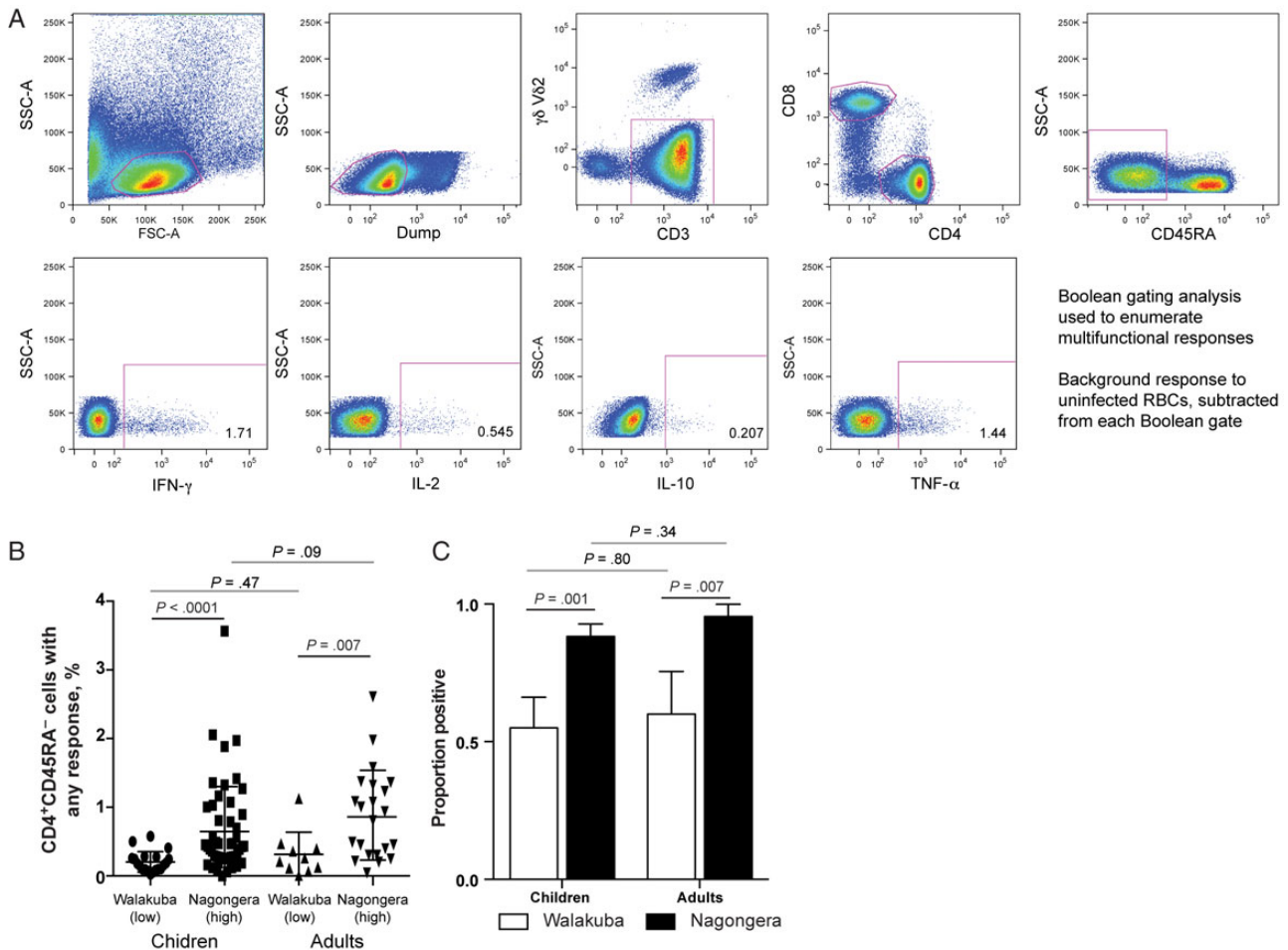
**Table 1. Characteristics of Children and Adults From Settings of Low (Walukuba) or High (Nagongera) Malaria Transmission**

Characteristic	Walukuba		Nagongera	
	Children (n = 20)	Adults (n = 10)	Children (n = 51)	Adults (n = 22)
Age, y, median (IQR)	5.1 (3.7–6.5)	28 (25–30)	7.1 (4.6–8.7)	54 (31–63)
Female sex, subjects, %	55	100	47	77
Malaria incidence in past year, cases/person-year, mean ± SD	0.10 ± 0.31	0	2.42 ± 2.35	0.09 ± 0.29
Asymptomatic with <i>P. falciparum</i> -positive blood smear, subjects, no. (%)	0	0	17 (33)	1 (4)

Abbreviations: IQR, interquartile range; *P. falciparum*, *Plasmodium falciparum*.

responses were infrequently observed in all participants; we observed 3 CD8<sup>+</sup> T-cell IL-10 responses, 4 CD8<sup>+</sup> T-cell IFN-γ

responses, a single CD8<sup>+</sup> T-cell TNF-α response, and no CD8<sup>+</sup> T-cell IL-2 responses.



**Figure 1.** Frequency of *Plasmodium falciparum*-specific CD4<sup>+</sup> T-cell responses is dependent on exposure intensity. *A*, Gating strategy to identify CD4<sup>+</sup>CD45RA<sup>-</sup> cytokine cells producing interferon γ (IFN-γ), interleukin 2 (IL-2), interleukin 10 (IL-10), and tumor necrosis factor α (TNF-α) following stimulation with *P. falciparum*-infected red blood cells (RBCs). Background responses (to uninfected RBCs) were subtracted from each Boolean gate. *B*, Frequencies of CD4<sup>+</sup> T cells producing any cytokine in response to *P. falciparum*-infected RBC stimulation are shown separately for children and adults from Walukuba (low-transmission setting) and Nagongera (high-transmission setting). *C*, The proportions of children and adults with a detectable CD4<sup>+</sup> T-cell response to *P. falciparum* were higher in Nagongera, compared with Walukuba. Abbreviations: FSC, forward scatter; SSC, side scatter.

### ***P. falciparum*-Specific CD4<sup>+</sup> T Cells in Children and Adults Differ Markedly in Their Functional Phenotype in a High-Transmission Setting**

The functional quality of pathogen-specific CD4<sup>+</sup> T cells, reflected in part by their effector cytokine production, is believed to be critical for protective immunity [9]. We compared the effector phenotype of *P. falciparum*-specific CD4<sup>+</sup> T-cell responses between children and their adult caregivers who resided in the same household and who were thus similarly exposed to *P. falciparum* but with differing levels of immunity. In Nagongera, the overall frequencies of *P. falciparum*-specific IFN- $\gamma$  producing CD4<sup>+</sup> T cells were not statistically significantly different between children and adults. However, there were striking differences in other cytokines (Figure 2A–C). Among adults, higher frequencies of TNF- $\alpha$ -, TNF- $\alpha$ /IFN- $\gamma$ -, TNF- $\alpha$ /IL-2-, IFN- $\gamma$ /IL-2-, and TNF- $\alpha$ /IFN- $\gamma$ /IL-2-producing CD4<sup>+</sup> T cells were observed. In contrast, children had higher frequencies of IL-10- and IL-10/IFN- $\gamma$ -producing cells. We also analyzed the composition of the response by calculating the proportional contribution of each cytokine to the total response. As with the frequencies of responding cells, in Nagongera the proportional contribution of IL-10-producing cells was dramatically higher in children ( $P < .0001$ ), and the contributions of TNF- $\alpha$ - and IL-2-producing cells were greater in adults ( $P < .0001$  and  $P = .0002$ , respectively; Figure 3A and 3B). Of interest, these age-related differences were not observed in Walukuba, where the proportion of cells producing IL-10 was somewhat higher in children ( $P = .053$ ), but the proportions of cells producing IFN- $\gamma$ , IL-2, and TNF- $\alpha$  were similar (Supplementary Figure 1).

### ***P. falciparum*-Specific CD4<sup>+</sup> T Cells From Children in the High-Transmission Setting Produce More IL-10 Than Those From Children in the Low-Transmission Setting**

To assess the influence of parasite exposure on cytokine production by *P. falciparum*-specific CD4<sup>+</sup> T cells, we compared responses of children living in a low-transmission setting with those of children living in a high-transmission setting. We found markedly higher frequencies of IFN- $\gamma$ -, IL-10-, and IFN- $\gamma$ /IL-10-producing CD4<sup>+</sup> T cells among children from Nagongera ( $P < .0001$  for all comparisons; Figure 4A). When analyzed as a fraction of the total response, the difference in cytokine production was more striking. Among Nagongera children, a greater proportion of *P. falciparum*-specific CD4<sup>+</sup> T cells produced IFN- $\gamma$  and IL-10 ( $P = .002$  and  $P = .0001$ , respectively), whereas among Walukuba children a greater proportion of CD4<sup>+</sup> T cells produced IL-2 and TNF- $\alpha$  ( $P = .0003$  and  $P = .0001$ , respectively; Figure 4B and 4C). Among Nagongera children, we observed no differences in the composition of the cytokine response between asymptotically infected and uninfected children, as defined by microscopy (Supplementary Figure 2). Together these data indicate that the IL-10-dominant

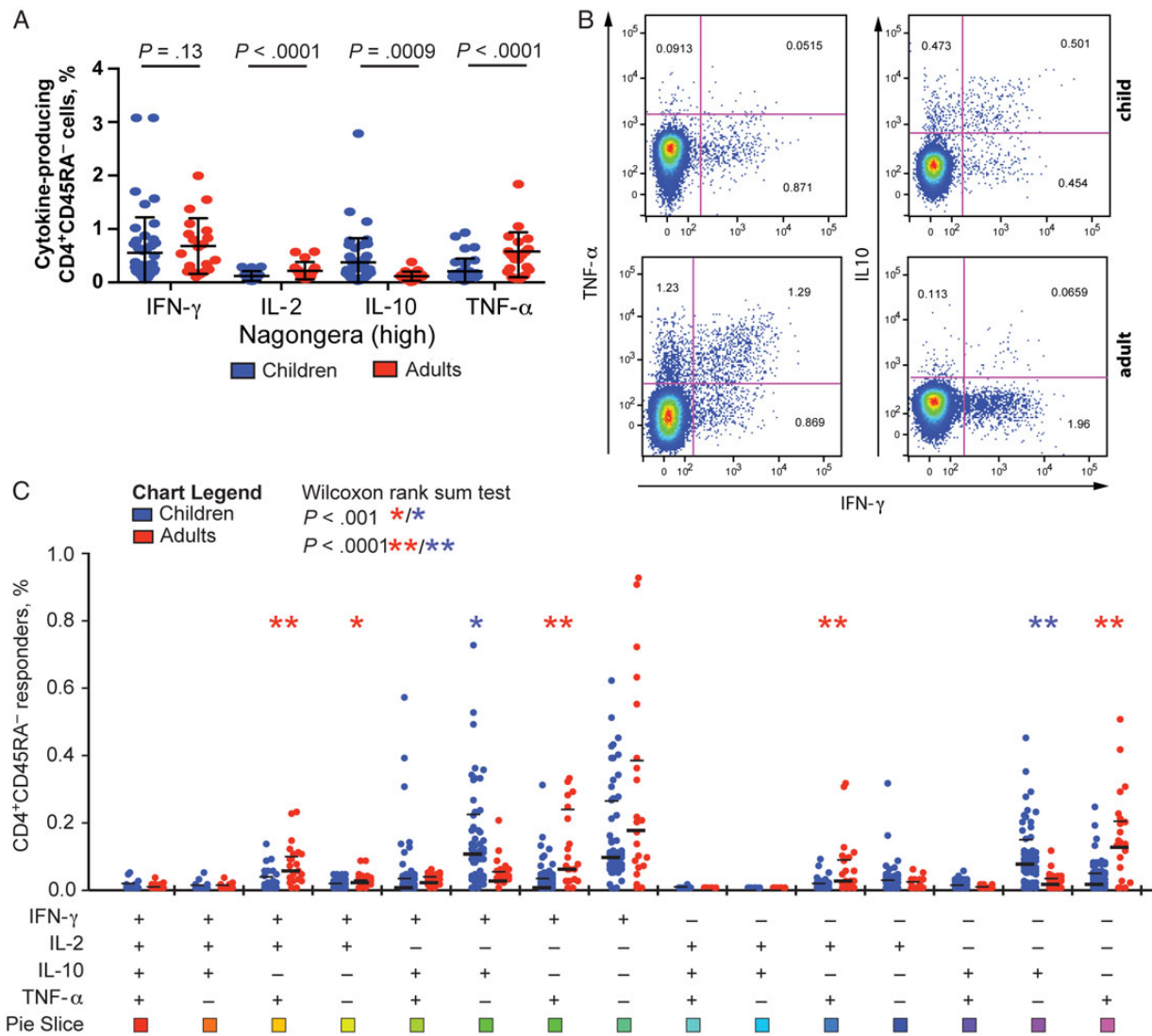
phenotype of *P. falciparum*-specific CD4<sup>+</sup> T cells observed among Nagongera children is related to residence in a high-transmission setting, because children from the low-transmission setting mount a CD4<sup>+</sup> T-cell response that is more typical of that for adults, with a greater proportion of inflammatory cytokines. In contrast, there was little difference in the composition of responses observed among adults at these 2 sites. While the frequencies of CD4<sup>+</sup> T cells producing IFN- $\gamma$ , IL-2, IL-10, and TNF- $\alpha$  and the proportion of the response dominated by IFN- $\gamma$  were all higher among adults from high-transmission as compared to low-transmission settings, the proportion of cells producing IL-2, IL-10, and TNF- $\alpha$  was similar (Supplementary Figure 3).

## **DISCUSSION**

Here we show that the effector phenotype of the *P. falciparum*-specific CD4<sup>+</sup> T-cell response is critically dependent on both age and *P. falciparum* exposure intensity. In high-transmission settings, CD4<sup>+</sup> T cells from adults produced predominantly inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and/or IL-2), while the response in children was of a more regulatory phenotype, dominated by IL-10- and IFN- $\gamma$ /IL-10-producing CD4<sup>+</sup> T cells. These findings suggest that induction of *P. falciparum*-specific CD4<sup>+</sup> T cells producing the regulatory cytokine IL-10 is age dependent and likely requires recent or persistent exposure to a high *P. falciparum* antigen burden.

Production of IL-10 (with or without IFN- $\gamma$ ) by *P. falciparum*-specific CD4<sup>+</sup> T cells in naturally exposed children has been described in several recent studies [20–22]. However, the frequency of IL-10-producing CD4<sup>+</sup> T cells does not appear to be associated with prospective protection from symptomatic malaria [20, 22]. A similar population of IL-10-producing T-helper type 1 (Th1) cells has been shown to play an essential role in preventing immunopathology and tissue inflammation in murine models of *Plasmodium* and in other parasitic infections, although this appears to come at the cost of reduced or delayed parasite clearance [23, 24, 29–32]. Thus, parasite-specific IL-10-producing CD4<sup>+</sup> T cells likely represent a peripheral tolerance mechanism that limits pathological inflammation but may interfere with the development of immunity. Interestingly, Th1 IL-10-producing cells suppress dendritic cell maturation [33] and production of interleukin 12 by antigen-presenting cells [23], preventing further Th1 differentiation. It is possible that, in *P. falciparum*-infected children, IL-10 provides local feedback to prevent the further development of CD4<sup>+</sup> T cells with the inflammatory cytokine profile typical of adults.

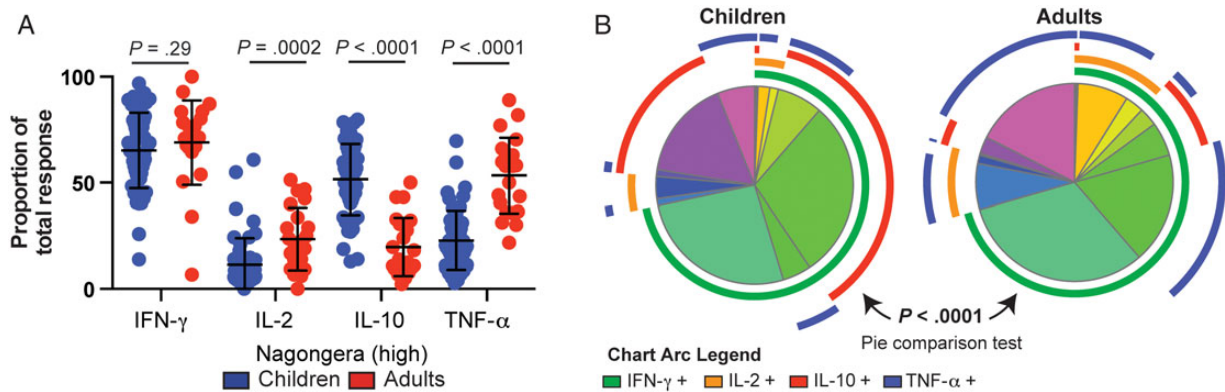
In contrast to highly exposed children, highly exposed adults, and adults and children with low exposure exhibited a response that was dominated by coproduction of IFN- $\gamma$  and TNF- $\alpha$ . In mouse models, IFN- $\gamma$  and TNF- $\alpha$  production by CD4<sup>+</sup> T cells is important for protection from *Plasmodium* [1, 2, 34]. Furthermore,



**Figure 2.** In a high-transmission setting, children have increased frequencies of regulatory CD4<sup>+</sup> T-cell responses, and adults have increased frequencies of inflammatory CD4<sup>+</sup> T-cell responses. *A*, The frequency of *Plasmodium falciparum*-specific CD4<sup>+</sup>CD45RA<sup>-</sup> cells producing interferon  $\gamma$  (IFN- $\gamma$ ), interleukin 2 (IL-2), interleukin 10 (IL-10), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) among children (blue) and adults (red) from Nagongera. *B*, Representative plots of CD4<sup>+</sup>CD45RA<sup>-</sup> cell responses from a child and an adult following stimulation with *P. falciparum*-infected red blood cells, showing typical coproduction of IL-10 and IFN- $\gamma$  (for the child) and coproduction of TNF- $\alpha$  and IFN- $\gamma$  (for the adult). *C*, Frequencies of *P. falciparum*-specific CD4<sup>+</sup>CD45RA<sup>-</sup> cells producing all possible cytokine combinations are shown separately for children and adults from Nagongera.

CD4<sup>+</sup> T-cell production of TNF- $\alpha$ , with or without IFN- $\gamma$  and/or IL-2, induced by vaccination with RTS,S has been associated with protection in naturally exposed children and experimentally challenged adults [17–19] and in studies using whole-parasite vaccination strategies [8, 14, 15, 35]. Together, these data suggest that inflammatory CD4<sup>+</sup> T-cell responses may contribute to protection from clinical disease. The dominance of this CD4<sup>+</sup> T-cell phenotype among high exposed adults, who exhibit a high degree of clinical immunity to malaria, is consistent with a protective role. Longitudinal clinical follow-up studies will be required to determine the relationship between these Th1 cells and prospective protection from symptomatic malaria.

Our data suggest that the intensity of exposure to *P. falciparum* parasites, independent of age, influences the magnitude of the CD4<sup>+</sup> T-cell response, while the functional phenotype of *P. falciparum*-specific CD4<sup>+</sup> T cells is influenced by both age and exposure. In naturally exposed populations, protection from symptomatic malaria is strongly associated with age [36]. However, the degree to which this age-dependent acquisition of immunity is due to the accumulation of exposure to multiple parasite variants, as opposed to inherent differences in child and adult immune responses, remains unclear. Studies of Indonesian transmigrants suggest that the acquisition of immunity is age dependent regardless of exposure; in these studies,



**Figure 3.** In a high-transmission setting, the proportion of the *Plasmodium falciparum*-specific CD4<sup>+</sup> T-cell response is dominated by regulatory responses in children and inflammatory responses in adults. *A*, Proportional contribution of interferon  $\gamma$  (IFN- $\gamma$ )-, interleukin 2 (IL-2)-, interleukin 10 (IL-10)-, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-producing cells to the total *P. falciparum*-specific CD4<sup>+</sup> T-cell response differed between children and adults in Nagongera. *B*, The overall composition of responding cells differed significantly between Nagongera children and adults ( $P < .0001$ , by the partial permutations test).

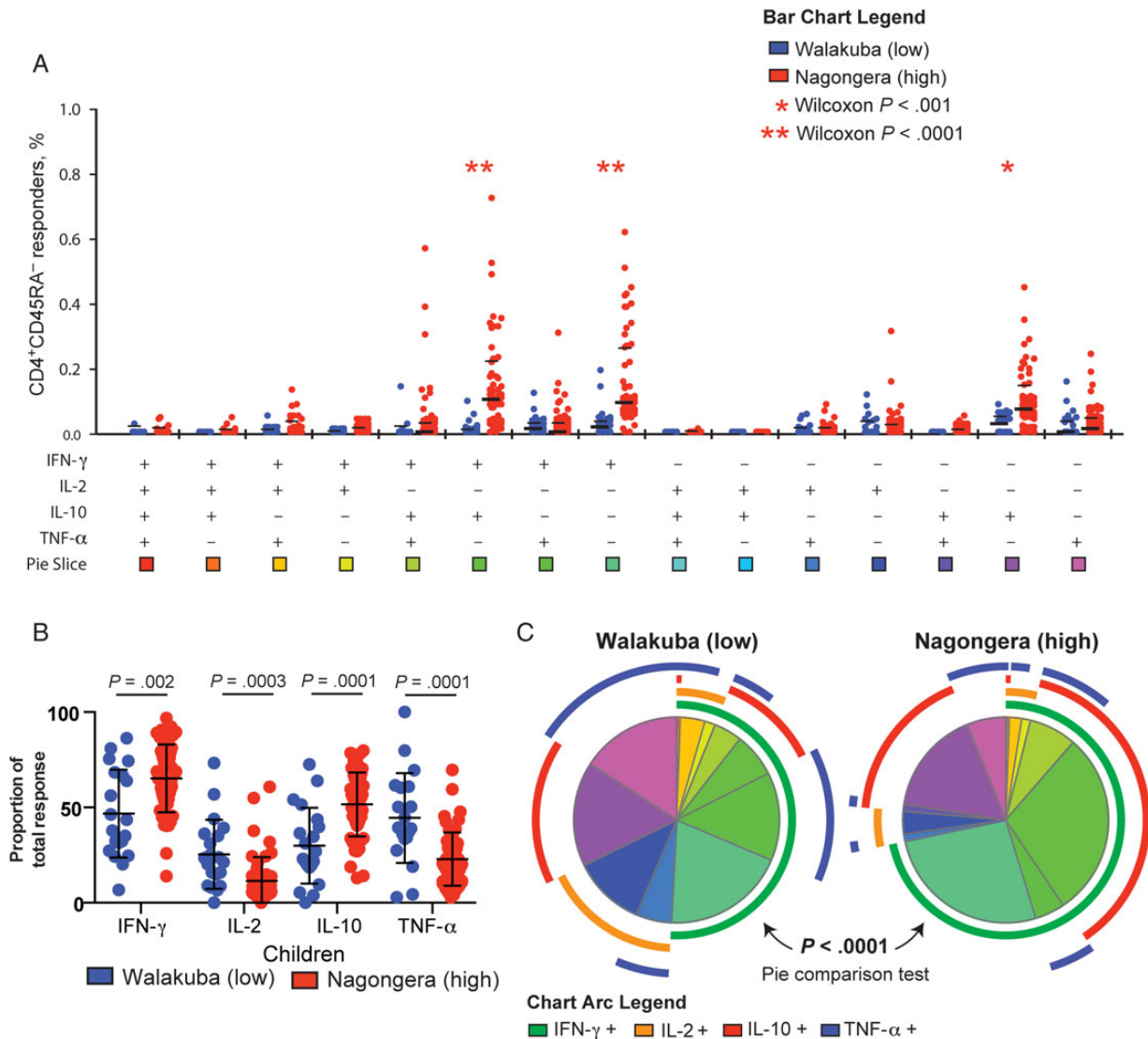
although adults were initially more susceptible to severe disease, they rapidly acquired protective immunity, while children remained susceptible to repeated episodes of symptomatic malaria [37, 38]. Age-dependent differences in the immune system itself [39] may result in different T-cell responses in children and adults with similar exposure levels. This possibility is supported by our data demonstrating that, although the composition of responses was remarkably different between children and adults from high-transmission settings, responses of adults from the low-transmission setting were similar to those of adults from high-transmission setting.

Alternatively, it is possible that the development of adaptive immunity to malaria in adults, mediated by antibody and/or cellular immune responses, prevents many infections and limits parasite antigen load, which in turn influences the evolution of the CD4<sup>+</sup> T-cell response. In both viral and bacterial infections, the effector phenotype of pathogen-specific CD4<sup>+</sup> T cells, particularly IL-10 production by Th1 cells, is influenced by antigen load and persistence [40, 41]. Experimental induction of IFN- $\gamma$ /IL-10-producing CD4<sup>+</sup> T cells specific for myelin basic protein requires repeated antigen dosing; the first dose results in IFN- $\gamma$  and IL-2 effector responses, while repeated doses induce tolerance mediated via IFN- $\gamma$ /IL-10-producing CD4<sup>+</sup> T cells [42]. In murine models of *Toxoplasma gondii*, IFN- $\gamma$ /IL-10 responses are short lived and dependent on continued antigen exposure [23].

Consistent with these models, our prior study of highly exposed children demonstrated that the frequency of IFN- $\gamma$ /IL-10-producing CD4<sup>+</sup> T cells declines markedly within months of a child's most recent symptomatic malaria episode [20]. Further, in another study in an area of seasonal malaria transmission, continued *P. falciparum* infection throughout the dry season was required for the maintenance of CD4<sup>+</sup> T-cell IL-10

responses [21]. Together, these data suggest that these cells either have a relatively short half-life or are capable of functional plasticity and turn off IL-10 production over time. The latter possibility is supported by an elegant study of beekeepers exposed to repeated high doses of bee venom, which resulted in switching of cellular responses from an IFN- $\gamma$ -dominant response to an IL-10-dominant response, even within clonal populations [43]. Maintenance of antigen-specific IL-10-producing cells required continuous antigen exposure, with a reversion of cells to IFN- $\gamma$  dominance within 2–3 months following antigen withdrawal. In regions of exceptionally high transmission of parasites, such as Nagongera, where children regularly incur new high-burden blood-stage infections, exposure to parasite antigens may be nearly constant. In contrast, although adults presumably have similar environmental exposure, they do not experience comparable levels of antigenemia: their parasite burden is often only detectable via sensitive polymerase chain reaction (PCR) methods [44]. As a result, highly exposed children may develop IL-10 CD4<sup>+</sup> T-cell responses, while in adults low-burden infection may maintain IFN- $\gamma$ /TNF- $\alpha$ /IL-2 polyfunctional T-cell responses.

Our study has several limitations. Only T cells in peripheral blood specimens were studied; because the majority of memory T cells reside in tissues such as the spleen, our study may underestimate the total *P. falciparum*-specific T-cell response or provide biased assessments of function. Further, although we measured 4 different cytokine responses, other responses and/or cell types, such as regulatory T cells [45] or T follicular helper cells [46], may have critical importance in the development of immunity. Indeed, interleukin 4 responses to the *P. falciparum* antigen PfEMP1 have been identified in children and were associated with protection [22, 47]. With regard to clinical data, our study only measured current parasite infection via microscopy.



**Figure 4.** Composition of *Plasmodium falciparum*-specific CD4<sup>+</sup> T-cell response differs between children in a low-transmission setting and those in a high-transmission setting. *A*, Frequencies of *P. falciparum*-specific CD4<sup>+</sup>CD45RA<sup>-</sup> cells producing all possible cytokine combinations are shown separately for children from Walakuba and those from Nagongera. *B*, Proportional contribution of interferon  $\gamma$  (IFN- $\gamma$ ), interleukin 2 (IL-2), interleukin 10 (IL-10), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-producing cells to the total *P. falciparum*-specific CD4<sup>+</sup> T-cell response differed between Nagongera children and Walakuba children. *C*, The overall composition of responding cells differed significantly between Nagongera children and Walakuba children ( $P < .0001$ , by the partial permutations test).

It is possible that some microscopy-negative children may have had subpatent infection and that the identification of parasite-positive individuals via PCR may reveal differences not seen here. Our study also has potential to be biased by differences in other demographic and clinical characteristics, such as genetic composition, prevalence of coinfections, and/or socioeconomic factors, between adults and children and the populations at each study site. In particular, HIV serostatus was not measured in our study participants, but the seroprevalence is known to be significantly higher in adults than in children in Uganda [27].

In conclusion, among children and adults in malaria-endemic settings, CD4<sup>+</sup> T-cell responses to *P. falciparum* are dependent

on both transmission intensity and age. Adults in a high-transmission setting exhibited higher frequencies of CD4<sup>+</sup> T cells producing combinations of IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$ , compared with children, whose responses were dominated by IL-10- and IL-10/IFN- $\gamma$ -producing cells. In children, the increased frequency of IL-10-producing cells was dependent on transmission intensity. These differences may arise from intrinsic differences in the host immune responsiveness of children and adults, or they may be a result of changing parasite burden. The induction of regulatory CD4<sup>+</sup> T-cell responses in heavily exposed children has important implications for vaccine development, as these responses may interfere with the induction of



robust effector mechanisms. These results contribute to our understanding of the acquisition of immunity in children and adults, and they provide important insights into cellular immune responses in naturally exposed populations.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

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