



## Modulation of immune responses by *Plasmodium falciparum* infection in asymptomatic children living in the endemic region of Mbita, western Kenya

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### ABSTRACT

Individuals living in malaria endemic areas become clinically immune after multiple re-infections over time and remain infected without apparent symptoms. However, it is unclear why a long period is required to gain clinical immunity to malaria, and how such immunity is maintained. Although malaria infection is reported to induce inhibition of immune responses, studies on asymptomatic individuals living in endemic regions of malaria are relatively scarce. We conducted a cross-sectional study of immune responses in asymptomatic school children aged 4–16 years living in an area where *Plasmodium falciparum* and *Schistosoma mansoni* infections are co-endemic in Kenya. Peripheral blood mononuclear cells were subjected to flow cytometric analysis and cultured to determine proliferative responses and cytokine production. The proportions of cellular subsets in children positive for *P. falciparum* infection at the level of microscopy were comparable to the negative children, except for a reduction in central memory-phenotype CD8<sup>+</sup> T cells and natural killer cells. In functional studies, the production of cytokines by peripheral blood mononuclear cells in response to *P. falciparum* crude antigens exhibited strong heterogeneity among children. In addition, production of IL-2 in response to anti-CD3 and anti-CD28 monoclonal antibodies was significantly reduced in *P. falciparum*-positive children as compared to -negative children, suggesting a state of unresponsiveness. These data suggest that the quality of T cell immune responses is heterogeneous among asymptomatic children living in the endemic region of *P. falciparum*, and that the responses are generally suppressed by active infection with *Plasmodium* parasites.

### 1. Introduction

It is estimated that > 40% (3.2 billion) of the world population is at risk of being infected with malaria, with the heaviest burden lying in sub-Saharan Africa [1]. Although recent control measures have reduced malaria-related morbidity and mortality in many African countries, malaria remains one of the leading causes of infection-related deaths [1,2]. Development of drug resistance among *Plasmodium* parasites to

anti-malaria drugs and among vector mosquitos against insecticides as well as the lack of an effective malaria vaccine underpin the need to improve existing strategies for malaria control [2,3]. Moreover, there is long-standing evidence that individuals living in malaria-endemic areas become clinically immune after multiple re-infections over time, and remain asymptotically infected as sterile immunity is rarely achieved by natural infection [4].

Both cellular and antibody-dependent immunity play indispensable

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roles for protection against blood-stage infection with *Plasmodium* parasites [5]. In addition, innate immunity such as provided by natural killer (NK) cells and macrophages has crucial function for the elimination of *Plasmodium* parasites in the host. These cellular responses are mediated by cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-12 that play key roles in the protective immunity [5–7]. However, excessive immune responses are sometimes harmful to the host and may lead to the severe symptoms of malaria. Although the mechanisms underlying severe malaria are not clearly understood, regulatory cytokines such as IL-10, IL-27, and TGF- $\beta$  are considered important for the modulation of severe manifestation of this disease [8,9]. In particular, *Plasmodium*-specific CD4<sup>+</sup> T cells co-producing IFN- $\gamma$  and IL-10 are predominant among children living in high endemic regions, and appear to have regulatory value for acute malaria inflammation [10,11]. Regulatory T cells that express the transcription factor Foxp3 are also induced and may modulate the immune responses as well [12]. Notably, a balance between pro- and anti-inflammatory responses may be important to attain clinical immunity.

In addition, immune responses are generally suppressed during acute infection with *Plasmodium* parasites [13]. Several mechanisms underlying such suppression have been reported, such as inhibition of T-cell production of IL-2 during *Plasmodium* infection [14], increased apoptosis of effector T cells, and down-regulated T-cell responses to unrelated antigens [15]. Moreover, Foxp3<sup>+</sup> regulatory T cells are induced and inhibit the expansion of T cells specific for unrelated antigens by producing inhibitory cytokines such as IL-27 [16,17], whereas acute malaria infection causes alterations in circulating levels of peripheral blood lymphocytes leading to a depletion of these populations, impacting the acquisition of immunity and control of infection [18–20]. These immunological findings were largely derived from studies on naturally infected patients with symptomatic malaria or animal models. However, residents in endemic regions of malaria acquire clinical immunity to malaria infection and remain asymptomatic despite maintaining low levels of infection. Studies on immunologic correlates in these individuals asymptomatic of malaria in the endemic region in Africa are relatively scarce, and there is little information available regarding the impact of infection on their immunological profiles [21–24].

Accordingly, to assess the impact of blood-stage *Plasmodium* infection on the immune responses in asymptomatic children, we conducted a cross-sectional study on the immune responses of peripheral blood mononuclear cells (PBMCs) from children in local schools of Mbita, Kenya, where malaria is holoendemic and a high prevalence of schistosomiasis has been reported [25]. We also assessed whether asymptomatic *Plasmodium* infection modifies the immune responses to *S. mansoni* antigens.

## 2. Materials and methods

### 2.1. Ethical statement

Study approval was granted by the Scientific Steering Committee and Ethical Review Committee of the Kenya Medical Research Institute, Kenya (KEMRI, SSC No. 2084) and the Ethical Review Board of the Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan (No. 140829127). Written informed consent was obtained from parents or guardians of the study participants and assent was sought orally from children.

### 2.2. Study location

The study was conducted in Mbita subcounty, on the Lakeside region of western Kenya, which lies in the malaria-endemic zone where there is intense malaria transmission throughout the year with some seasonal fluctuations. The estimated entomological inoculation rate of Kenya's malaria endemic zones is between 30 and 100 infectious bites

per person per year [26]. Mbita is also an endemic region of schistosomiasis, with a high prevalence of *S. mansoni* infection (approximately 60%) among children attending schools within a 5-km radius from the lakeshore [25].

### 2.3. Study participants

School children aged 4–16 years were recruited from 5 schools in the area to participate in this cross-sectional study. The children were involved in an ongoing longitudinal study of the epidemiology of schistosomiasis in the area, in which 160 children were initially enrolled, and followed for approximately one year to investigate the immune responses to infection with *S. mansoni* (unpublished). During the cohort study, some of the children were lost to follow-up due to migration or absence on the day of sample collection etc., and total 148 children participated in this study conducted in October 2015. Blood, stool, and urine samples were collected at inclusion. A positive infection status was defined by the presence of a single *P. falciparum* parasite of either asexual or gametocyte stages in the inspection of one thick blood smear slide per individual (*P. falciparum* positive). Slides were observed by four independent, whereby subjects were included for analysis when 3 or all 4 microscopists were in agreement regarding the slide readings, and subjects were excluded when only 2 slide readings coincided. Parasite densities were expressed relative to the white blood cell count and the final number of parasites per  $\mu$ l of blood was calculated based on the assumption that the number of white blood cells per  $\mu$ l of blood is 8000 as shown in the formula; parasites/ $\mu$ l blood = (number of parasite counts  $\times$  8000) / (number of white blood cell counts). The results of all readings for each slide were averaged. Rapid diagnostic testing (RDT) (CareStart™ Malaria Pf (HRP2) Ag RDT, Access Bio Inc., Somerset, NJ USA, and SD Bioline Malaria Ag Pf/Pan, Standard Diagnostics Inc., Yongin, Republic of Korea) for malaria was performed to advise treatment with antimalaria drugs on site. Stool samples were analyzed in duplicate using the Kato-Katz (KK) technique for detecting eggs of *S. mansoni* [27]. Urine testing for *S. mansoni* diagnosis was evaluated using a circulating cathodic antigen cassette test (CCA, Rapid Medical, Pretoria, South Africa) [28]. Infection status was defined by a positive outcome from either KK, CCA, or both. All children positive for malaria were treated with artemether/lumefantrine per the Kenya national guidelines for the treatment of uncomplicated malaria. Children infected with schistosomes as detected by KK were treated with 40 mg/kg praziquantel and those infected with soil-transmitted helminths were treated with 400 mg albendazole according to World Health Organization guidelines.

### 2.4. *P. falciparum* culture and schizont antigen preparation

The *P. falciparum* 3D7 strain used in this study was originally reported by Dr. David Walliker (Edinburgh Univ., Edinburgh, UK) [29] and was obtained from Dr. L. H. Miller (NIAID, National Institutes of Health, Bethesda, MD, USA). Red blood cells (RBCs) were obtained from the Japanese Red Cross Blood bank. Parasites were grown as previously reported [30]. Briefly, parasites were maintained in O<sup>+</sup> human RBCs cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with HEPES (25 mM, Sigma-Aldrich, St. Louis, MO, USA), hypoxanthine (200  $\mu$ M, Sigma-Aldrich), sodium bicarbonate (0.225%), Gentamicin (10 mg/ml, Invitrogen, Carlsbad, CA, USA), and AlbuMax I (0.5%, Invitrogen) under a gas environment of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> at 37 °C. Parasite development was monitored by examination of Giemsa-stained thin blood smears. After two rounds of synchronizations with 5% sorbitol (Sigma-Aldrich), schizont parasites were harvested at approximately 90% parasitemia by magnetic MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). Mature schizont-infected RBCs were lysed through five freeze-thaw cycles (–80 °C and 37 °C) to constitute *P. falciparum* schizont extract (iRBC). Freeze-thawed preparations of

uninfected RBCs (uRBC) were used as the control. Both preparations were aliquoted at  $1 \times 10^8$  cells/ml in phosphate buffered saline (PBS) without protease inhibitors and stored at  $-80^\circ\text{C}$  until use. Antigenicity of iRBCs was examined using PBMCs from malaria non-exposed Japanese donors.  $\text{CD4}^+$  T cells in PBMCs proliferated and PBMCs produced  $\text{IFN-}\gamma$  in response to iRBCs and not to uRBC, in line with the previous reports (data not shown) [31,32].

## 2.5. Schistosome antigen preparation

The life cycle of the *S. mansoni* Puerto Rican strain was maintained by passage through *Biomphalaria glabrata* snails and ICR mice (SLC, Shizuoka, Japan). The mice were percutaneously infected with 250 cercariae, sacrificed 7 weeks after infection, and the adult worms collected from the portal vein by intra-cardiac perfusion with PBS whereas eggs were isolated from the liver of infected mice. Mice were maintained in the Biomedical Research Center at Nagasaki University in specific pathogen free condition. The animal experiments represented here were approved by the Institutional Animal Care and Use Committee of Nagasaki University and were conducted according to the Guidelines for Animal Experimentation at Nagasaki University. *Schistosoma* antigens were prepared as described previously with modifications [33]. Briefly, the adult *S. mansoni* worms were washed and suspended in PBS containing of leupeptin ( $2\ \mu\text{g/ml}$ ) (Sigma-Aldrich) and phenylmethylsulfonyl fluoride ( $0.1\ \text{mM}$ ) (Wako, Osaka, Japan), homogenized on ice, subjected to 5 cycles of freeze and thaw, centrifuged at  $30,000 \times g$  for 30 min at  $4^\circ\text{C}$ , and the supernatant collected as soluble worm antigen (SWA). The livers were crushed through sieves and the eggs collected. Eggs were suspended in ice-cold PBS containing leupeptin ( $2\ \mu\text{g/ml}$ ), homogenized on ice, subjected to 5 cycles of freeze and thaw, and centrifuged at  $30,000 \times g$  for 30 min at  $4^\circ\text{C}$  to separate the soluble fractions as soluble egg antigens (SEA). Both SWA and SEA were dialyzed against PBS prior to sterile filtration through a  $0.22\text{-}\mu\text{m}$  filter. Protein amount was estimated by BCA Protein Assay (Thermo Fisher Scientific).

## 2.6. Culture and cytokine assay of PBMCs

Peripheral blood collected into heparinized vacutainer tubes (Terumo, Tokyo, Japan) was centrifuged for 20 min at  $800 \times g$  with no brake to isolate plasma, which was stored at  $-80^\circ\text{C}$  for further analysis. RPMI 1640 of equal volume to the plasma was added to the pellet. PBMCs were isolated by density centrifugation through a lymphoprep gradient (Axis-Shield, Oslo, Norway) and washed twice in RPMI 1640. Mononuclear cells were collected and resuspended in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine ( $4\ \text{mM}$ ), penicillin ( $100\ \text{U/ml}$ ), streptomycin sulfate ( $100\ \text{mg/ml}$ ), and  $\beta$ -mercaptoethanol ( $100\ \mu\text{M}$ ).

PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Thermo Fisher Scientific) at a final concentration of  $2\ \mu\text{M}$  for 10 min in  $37^\circ\text{C}$ , and washed in pre-warmed culture medium. Cells ( $1 \times 10^5$  cells/well) were cultured for 7 days in the presence of iRBC ( $1 \times 10^6$ /well), uRBC ( $1 \times 10^6$ /well), SEA ( $5\ \mu\text{g/ml}$ ), SWA ( $20\ \mu\text{g/ml}$ ), or culture medium alone. For polyclonal stimulation, cultures were maintained for 5 days in the presence of anti-CD3/28 Dynabeads ( $1 \times 10^5$  cells/well, Thermo Fisher Scientific), or left unstimulated for baseline reference. All samples were incubated at  $37^\circ\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ . After the culture period, the supernatant was harvested for cytokine detection assays and cells were assayed for lymphocyte proliferation. To determine *P. falciparum*-specific response, background proliferation and cytokine levels in uRBC culture were subtracted from those in iRBC culture, and the values in unstimulated culture were subtracted from those in culture in the presence of SEA, SWA, or anti-CD3/anti-CD28 monoclonal antibodies (mAbs).

Cytokine levels in the culture supernatant were determined by sandwich enzyme-linked immunosorbent assay (ELISA). Immunoplates

**Table 1**  
Demographic and clinical characteristics of the study subjects.

|                                | <i>P. falciparum</i> -positive | <i>P. falciparum</i> -negative | <i>p</i> -Value <sup>a</sup> |
|--------------------------------|--------------------------------|--------------------------------|------------------------------|
| Number of subjects             | 45                             | 91                             |                              |
| Gender (male/female)           | 20/25                          | 39/52                          | $> 0.9999$                   |
| Age (years) <sup>b</sup>       | 11 (5.5)                       | 11 (6)                         | 0.7257                       |
| Hemoglobin (g/dl) <sup>b</sup> | 12.5 (2.1)                     | 13.1 (2.2)                     | 0.0519                       |
| Body mass index <sup>b</sup>   | 16.0 (3.6)                     | 15.8 (3.1)                     | 0.7012                       |
| <i>S. mansoni</i> prevalence   | 72%                            | 76%                            | 0.6675                       |

<sup>a</sup> Comparison between *P. falciparum*-positive and -negative children by Mann-Whitney test.

<sup>b</sup> Clinical and laboratory data from the 136 school children examined. Data are expressed as the median (interquartile range).

(Thermo Fisher Scientific) were coated with anti-human  $\text{IFN}\gamma$ , IL-2, IL-10, or IL-13 mAbs (BioLegend, San Diego, CA, USA) in coating buffer overnight at  $4^\circ\text{C}$ . Plates were washed and blocked with 10% fetal calf serum in PBS for 1 h at room temperature. Supernatants ( $50\ \mu\text{l}$ , diluted  $3 \times$ ) or serially diluted standard cytokines in sample diluent (0.05% Tween-20 in blocking buffer) were added to each well. Plates were incubated for 4 h at room temperature, washed, and then incubated for 1 h with  $50\ \mu\text{l}$  of  $1\ \mu\text{g/ml}$  biotinylated anti-human  $\text{IFN}\gamma$ , IL-2, IL-10, or IL-13 mAbs (BioLegend). Samples were then reacted with Streptavidin-Alkaline Phosphate (Jackson ImmunoResearch, West Grove, PA, USA) and 4-nitrophenyl phosphate disodium salt hexahydrate substrate (Sigma-Aldrich). The absorbance at 415 nm was determined using a plate reader (BioRad, Hercules, CA, USA). Sample protein values were calculated by interpolation from the standard curves of recombinant cytokines, which were included on every plate.

## 2.7. Flow cytometry

To assess the frequency of various circulating cell subsets, freshly isolated PBMCs were stained with various combinations of fluorochrome-conjugated mAbs at  $4^\circ\text{C}$  in the dark for 30 min. The following antibodies were used: APCCy7-anti-CD3, PECy7-anti-CD8, PE-anti-CD56, PECy5-anti-CD4, FITC-anti-CD62L, PE-anti-CD45RO, APC-anti-279 (PD-1), APCCy7-anti-CD19, and APC-anti-CD23 mAbs. For the lymphocyte proliferative assays, cells were harvested after the appropriate culture period and surface-stained with APCCy7-anti-CD3, PECy7-anti-CD8, and APC-anti-CD4 mAbs. Antibodies were purchased from BioLegend, BD Biosciences, or TONBO Biosciences (San Diego, CA, USA). Dead cells were excluded using 7-AAD. Relevant isotype control antibodies were used. Samples were assessed on the Guava<sup>®</sup> easyCyte™ 8HT Flow Cytometer (Merck Millipore, Billerica, MA, USA), and analyzed using FlowJo software (Tree Star, San Carlos SA).

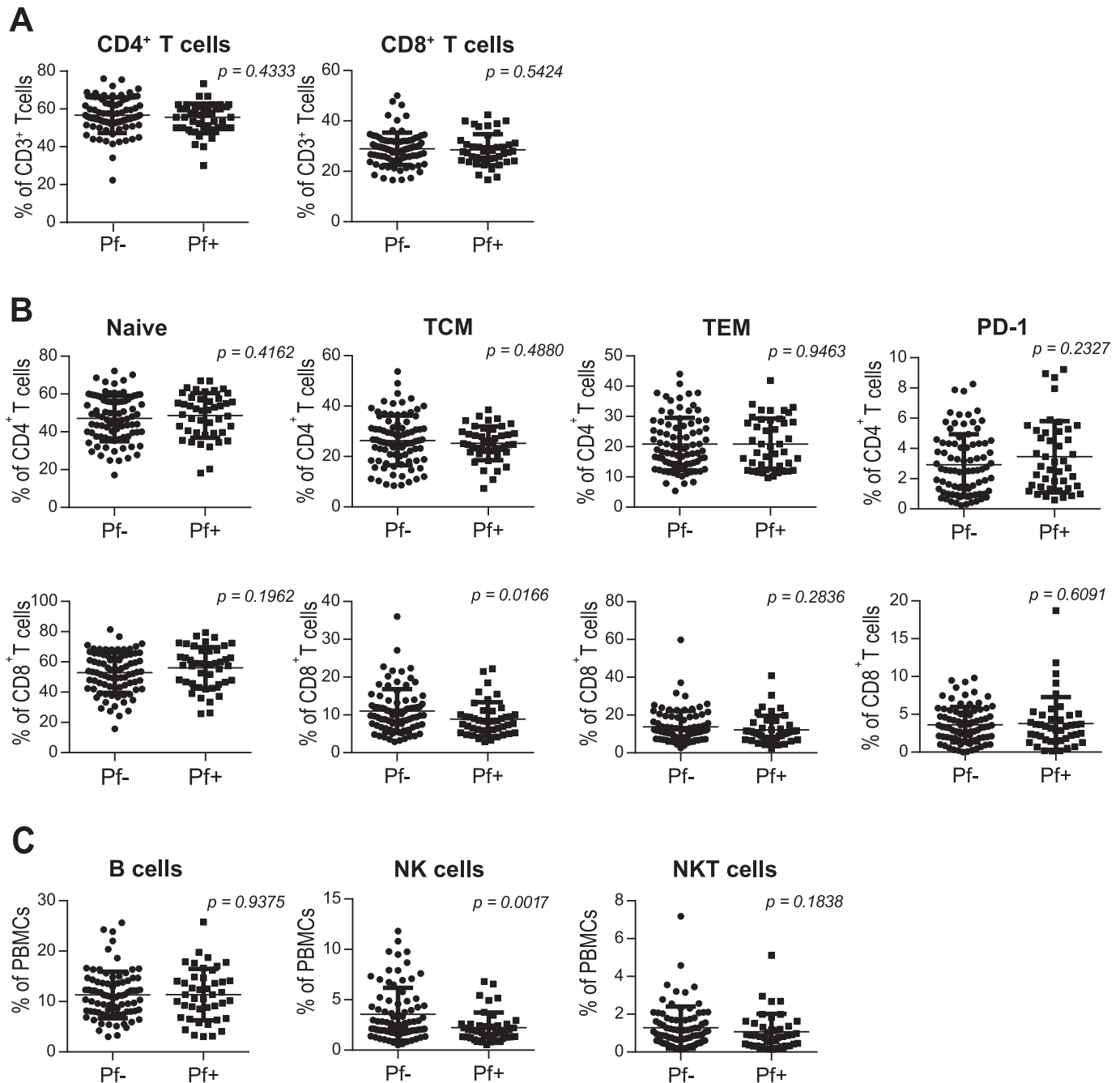
## 2.8. Statistical methods

Statistical analyses were performed using GraphPad Prism software version 6.0 (San Diego, CA, USA). The Mann-Whitney non-parametric test was used for pairwise comparisons. Correlations were analyzed using Spearman's rank correlation coefficient. Comparisons with *p* values  $< 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Characteristics of study participants

A total of 148 school children were enrolled in the study for whom infection status of *P. falciparum* and *S. mansoni* was examined. Among these, one *P. malariae* single infection and 11 samples whose diagnosis disagreed among microscopists were excluded from the analyses. None of the children exhibited fever ( $> 37.5^\circ\text{C}$ ). The prevalence of

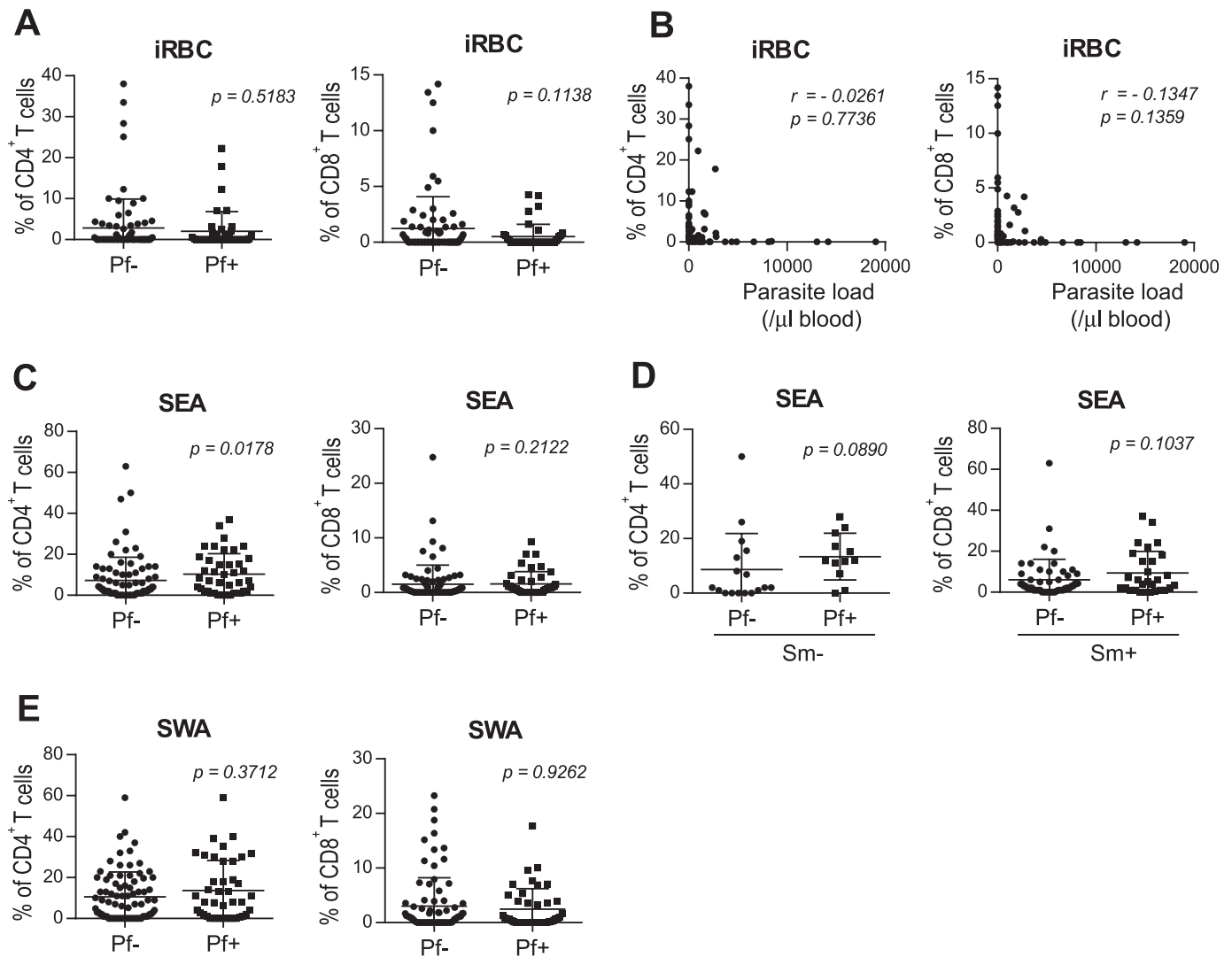


**Fig. 1.** Subpopulations of peripheral lymphocytes. PBMCs from *P. falciparum*-positive (Pf+) and -negative (Pf-) children were stained with specific mAbs and were analyzed by flow cytometry. A. Proportions of CD4<sup>+</sup> CD3<sup>+</sup> (CD4<sup>+</sup> T cells) and CD8<sup>+</sup> CD3<sup>+</sup> (CD8<sup>+</sup> T cells) in *P. falciparum*-positive and -negative children are plotted. B. Proportions of CD62L<sup>+</sup> CD45RO<sup>-</sup> (naive), CD62L<sup>+</sup> CD45RO<sup>+</sup> (central memory), CD62L<sup>-</sup> CD45RO<sup>+</sup> (effector memory), and PD-1<sup>+</sup> (PD-1) CD4<sup>+</sup> T cells (upper panel) and CD8<sup>+</sup> T cells (lower panel) in the *P. falciparum*-positive and -negative children are plotted. C. Proportions of CD3<sup>-</sup> CD19<sup>+</sup> (B cells), CD56<sup>+</sup> CD3<sup>-</sup> (NK cells), and CD56<sup>+</sup> CD3<sup>+</sup> (NK T cells) in *P. falciparum*-positive and -negative children. Pf-; n = 83, Pf+; n = 43. Mann-Whitney test *p*-values are indicated.

microscopy-positive *P. falciparum* infection was 33% (45/136) in this study with a median infection intensity of 1,237 parasites/ $\mu$ l blood and a range of 39–19,006 parasites/ $\mu$ l blood. There were no significant differences in age, hemoglobin level, body mass index, or prevalence of schistosomiasis between children that were *P. falciparum*-positive and those that were *P. falciparum*-negative (Table 1). Children positive for *P. falciparum* tended toward having lower hemoglobin levels when compared to those negative, although the difference was not statistically significant. The levels of parasitemia of *P. falciparum* showed no correlation with age (Fig. S1).

### 3.2. Cellular subsets in PBMCs

Cellular composition in PBMCs was examined by flow cytometry, and the proportions of each cell type were compared between *P. falciparum*-positive and -negative groups (Fig. 1, Fig. S2). There were no significant differences in the proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and their naïve subsets (Fig. 1A, B). The frequency of CD8<sup>+</sup> T cells with central memory phenotype (CD62L<sup>+</sup> CD45RO<sup>+</sup>) was lower in *P. falciparum*-positive children relative to *P. falciparum*-negative subjects, whereas that of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells with effector memory phenotype (CD62L<sup>-</sup> CD45RO<sup>+</sup>) were similar (Fig. 1B). In addition,



**Fig. 2.** Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in responses to antigen stimulation. PBMCs were labeled with CFSE and cultured for 6–7 days in the presence and absence of uRBC, iRBC (A, B), SEA (C, D), and SWA (E). Cells were stained for CD3, CD4, and CD8, and CFSE profiles of CD4<sup>+</sup> CD3<sup>+</sup> (CD4<sup>+</sup> T) and CD8<sup>+</sup> CD3<sup>+</sup> (CD8<sup>+</sup> T) cells were analyzed. Background responses to uRBC and unstimulated controls were subtracted from those to iRBC and SEA/SWA stimulations, respectively. Proportions of cells that specifically divided more than once were plotted in *P. falciparum*-positive (Pf+) and -negative (Pf-) groups (A, C–E). The correlations between the proportion of dividing cells and parasitemia are plotted (B). Children were divided into those uninfected (Sm-) and infected (Sm+) with *S. mansoni*, and then the proportions of dividing cells were plotted in each group. Pf-; n = 82, Pf+; n = 42. *p* values by Mann-Whitney and Spearman Rank tests are indicated on the scatter plots.

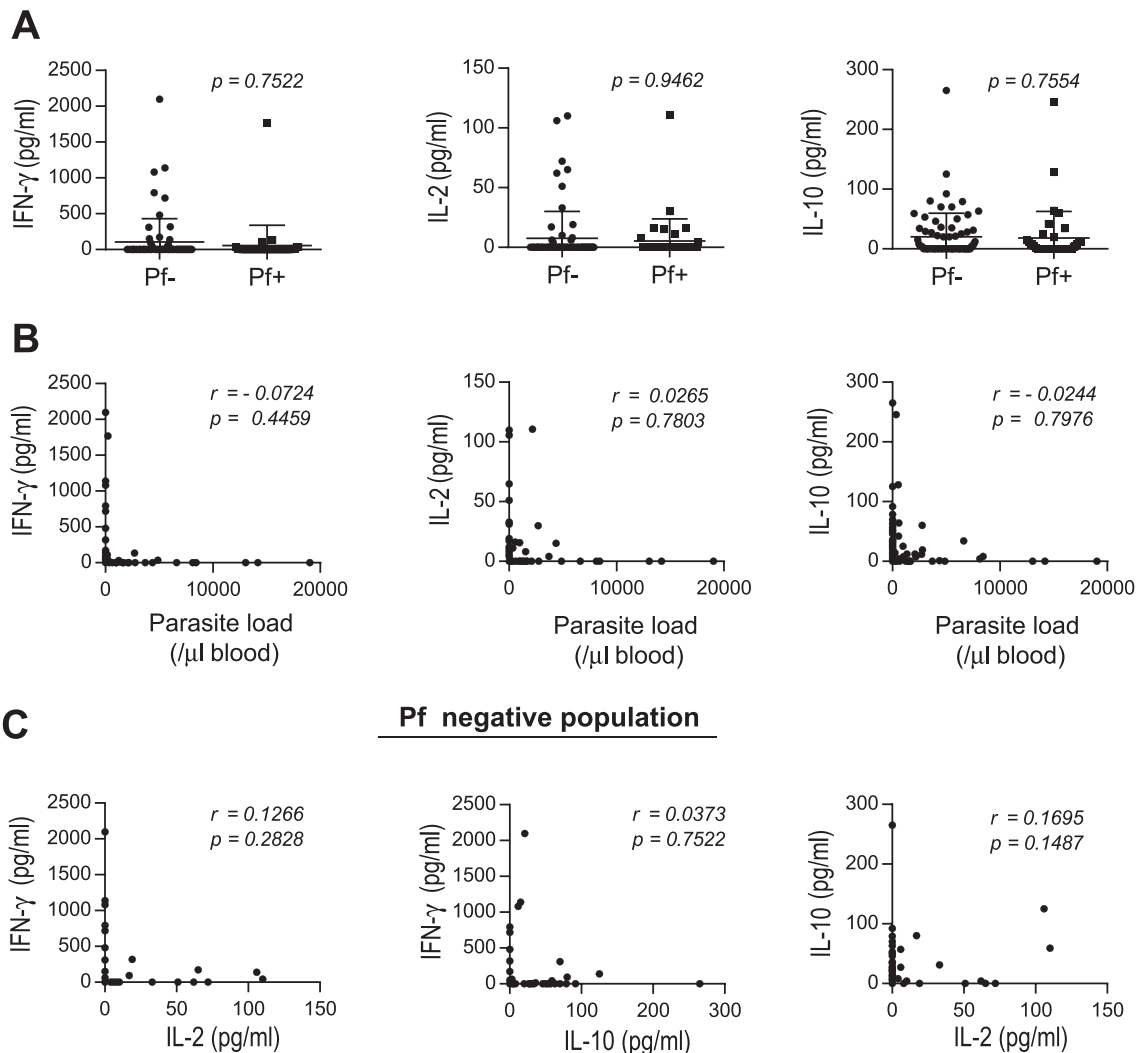
there was no difference in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that expressed PD-1. However, the proportion of NK cells was significantly lower in *P. falciparum*-positive children when compared to -negative ones, whereas that of B cells and NKT cells was comparable (Fig. 1C).

### 3.3. Proliferative response of T cells to antigen stimulation

Antigen-specific proliferative responses of PBMCs to stimulation with malarial antigens (iRBC) and *Schistosoma* antigens (SEA and SWA) were assessed (Fig. 2). Schistosomal antigens were selected as control antigens unrelated to malaria, as the Mbita region is highly endemic to *S. mansoni* and the majority of children were expected to have a history of exposure [26]. PBMCs were labeled with CFSE prior to culture, and proliferation was determined by the diminution of CFSE signal in each cell type after culture (Fig. S3). Background proliferative responses to uRBC lysates were subtracted from those to iRBC to determine specific responses to *P. falciparum* antigens. There were large variations within each group with a majority of cells failing to proliferate in response to iRBC among both *P. falciparum*-positive and -negative children. The

proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that proliferated in response to iRBC did not significantly differ between *P. falciparum*-positive and -negative groups (Fig. 2A). In addition, we did not find correlation between the proportions of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T cells and the levels of parasitemia, although it was clear that children with high levels of parasitemia did not exhibit proliferation (Fig. 2B). Notably, in *P. falciparum*-positive children, the proportions of CD4<sup>+</sup> T cells that proliferated in response to SEA were significantly higher than those in *P. falciparum*-negative children, although this difference was not observed in CD8<sup>+</sup> T cells and CD4<sup>+</sup> T-cell response to SWA (Fig. 2B, C, E). To evaluate the effects of *S. mansoni* infection on T-cell response to SEA in more detail, we divided the subjects into *S. mansoni*-infected and uninfected cohorts and performed comparisons between *P. falciparum*-positive and -negative children within each cohort (Fig. 2D). However, we did not find a significant difference between children positive or negative for *P. falciparum* infection in either cohort, perhaps owing to the reduction in the number of subjects.





**Fig. 3.** Cytokine production of PBMCs in response to *P. falciparum* antigens. A. Levels of IFN- $\gamma$ , IL-2, and IL-10 in the culture supernatant of PBMCs stimulated with iRBC were determined by ELISA. Values of cytokines produced in the culture stimulated with uRBC were subtracted from those of culture stimulated with iRBC. Pf-; n = 74, Pf+; n = 39. A Mann-Whitney test was performed for the pairwise comparisons. B. Relationship between the production of cytokines (IFN- $\gamma$ , IL-2, and IL-10) and infection intensity (parasite load) was evaluated. p-values by Spearman Rank tests are indicated on the scatter plots. C. Correlations between the indicated cytokine production levels are plotted.

### 3.4. Cytokine production of PBMCs in response to antigens

PBMCs were cultured in the presence of uRBC or iRBC, and their production of IFN- $\gamma$ , IL-2, and IL-10 in response to *P. falciparum* antigens was determined. A significant proportion of children did not show any detectable production of IFN- $\gamma$  (75%), IL-2 (81%), or IL-10 (53%) (Fig. 3A). The levels of these three cytokines were not significantly different between *P. falciparum*-positive and -negative children. To investigate whether the production of these cytokines correlated with infection intensity, cytokine responses to iRBC were plotted against the parasite load (Fig. 3B). Although statistically significant correlations were not observed between the levels of cytokine production and parasitemia, children with high parasitemia levels produced little cytokine in response to iRBC. We further examined the pattern of cytokine production following iRBC stimulation in *Plasmodium*-negative children to examine correlation in the production of the three cytokines (IFN- $\gamma$  vs IL-2; IFN- $\gamma$  vs IL-10; and IL-10 vs IL-2) (Fig. 3C). *Plasmodium*-positive children were excluded from the analysis because CD4<sup>+</sup> T cells from the majority of these did not produce cytokines. Notably, we did not find any positive correlation between producers of these cytokines; rather, responders for each of the three cytokines appeared to be independent of each other and thus individuals that produced one

cytokine likely did not produce the other two. This was unexpected, in particular for IFN- $\gamma$  vs. IL-2, as the production of each cytokine is generally associated with the other. We also determined the immune responses to malaria-unrelated antigens, SEA and SWA, and evaluated the production of IFN- $\gamma$ , IL-2, IL-10, and IL-13 (Fig. 4). Similarly, a large proportion (45%–76%) of children did not show a positive response to these antigens, although 75% of children were actively infected with *S. mansoni*. We did not observe significant differences in the levels of cytokines produced by cells from *P. falciparum*-positive and -negative groups.

### 3.5. Cytokine production of PBMCs in response to polyclonal stimulation

To examine the general responsiveness of T cells to T-cell receptor-mediated stimulation, PBMCs were cultured in the presence or absence of anti-CD3 and anti-CD28 mAbs, and the production of IFN- $\gamma$ , IL-2, and IL-10 was determined (Fig. 5). The level of IFN- $\gamma$  production was not significantly different between *P. falciparum*-positive and -negative children. However, the level of IL-2 production was significantly lower in *P. falciparum*-positive compared with -negative children. Production of IL-10 also showed a lower tendency in *P. falciparum*-positive children, although this difference was not statistically significant. To

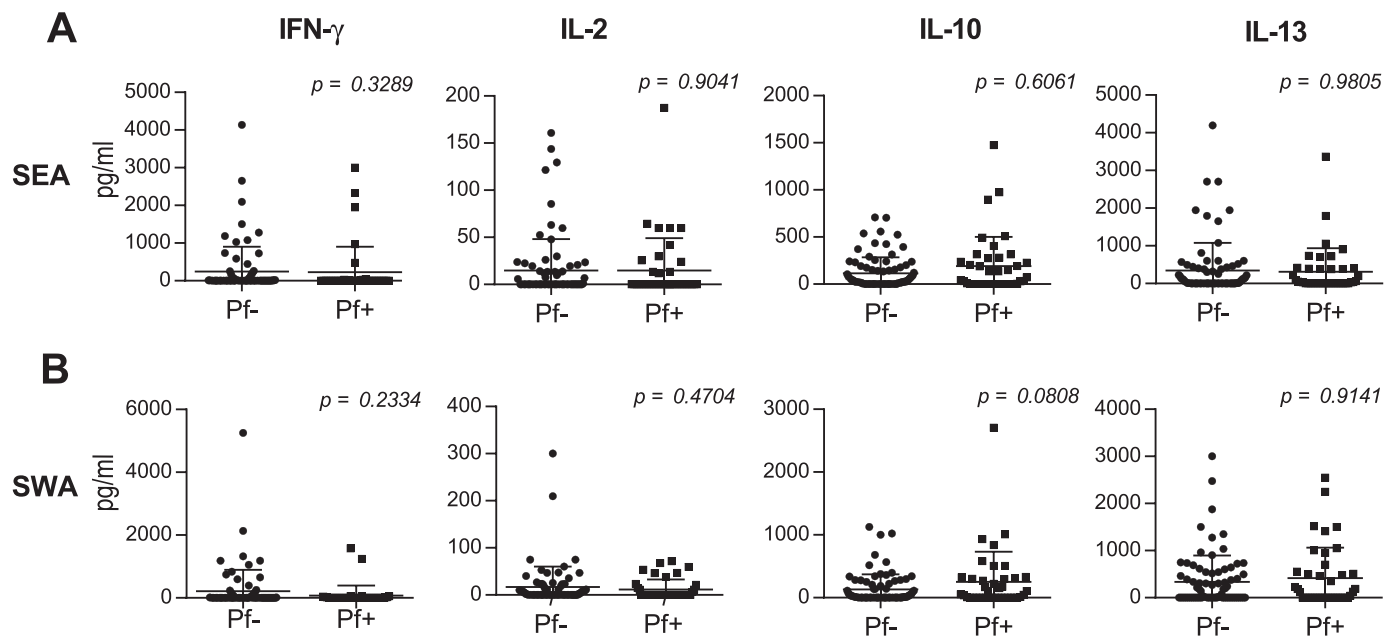


Fig. 4. Cytokine production of PBMCs in response to *Schistosoma* antigens. Levels of IFN- $\gamma$ , IL-2, IL-10, and IL-13 in the culture supernatant of PBMCs stimulated with SEA or SWA were determined by ELISA. Values of cytokines produced in the culture without antigens were subtracted from those of cultures stimulated with *Schistosoma* antigens. Pf+, n = 39; Pf-, n = 74. p values by Mann-Whitney test are indicated on the scatter plots.

evaluate the correlation between the cytokine production and parasite load, cytokine responses to polyclonal T-cell stimulation were plotted against parasitemia levels (Fig. 5B). Whereas no significant correlation was found between IFN- $\gamma$  production and parasitemia levels, production of IL-2 in response to polyclonal T cell stimulation negatively correlated with the levels of parasitemia. Moreover, there was a trend toward a negative association between the IL-10 producers and their parasitemia levels, although the correlation failed to reach statistical significance (Fig. 5B). From the *P. falciparum*-positive and -negative groups we separately analyzed the relationship among children whose CD4<sup>+</sup> T cells produced different cytokines within each group (Fig. 5C). In both *P. falciparum*-positive and -negative groups, there was a positive correlation among children whose CD4<sup>+</sup> T cells produced IFN- $\gamma$  vs. IL-2, or among those whose CD4<sup>+</sup> T cells produced IFN- $\gamma$  vs. IL-10 in response to polyclonal stimulation, although this relationship was not observed between IL-2 vs. IL-10 production (Fig. 5C).

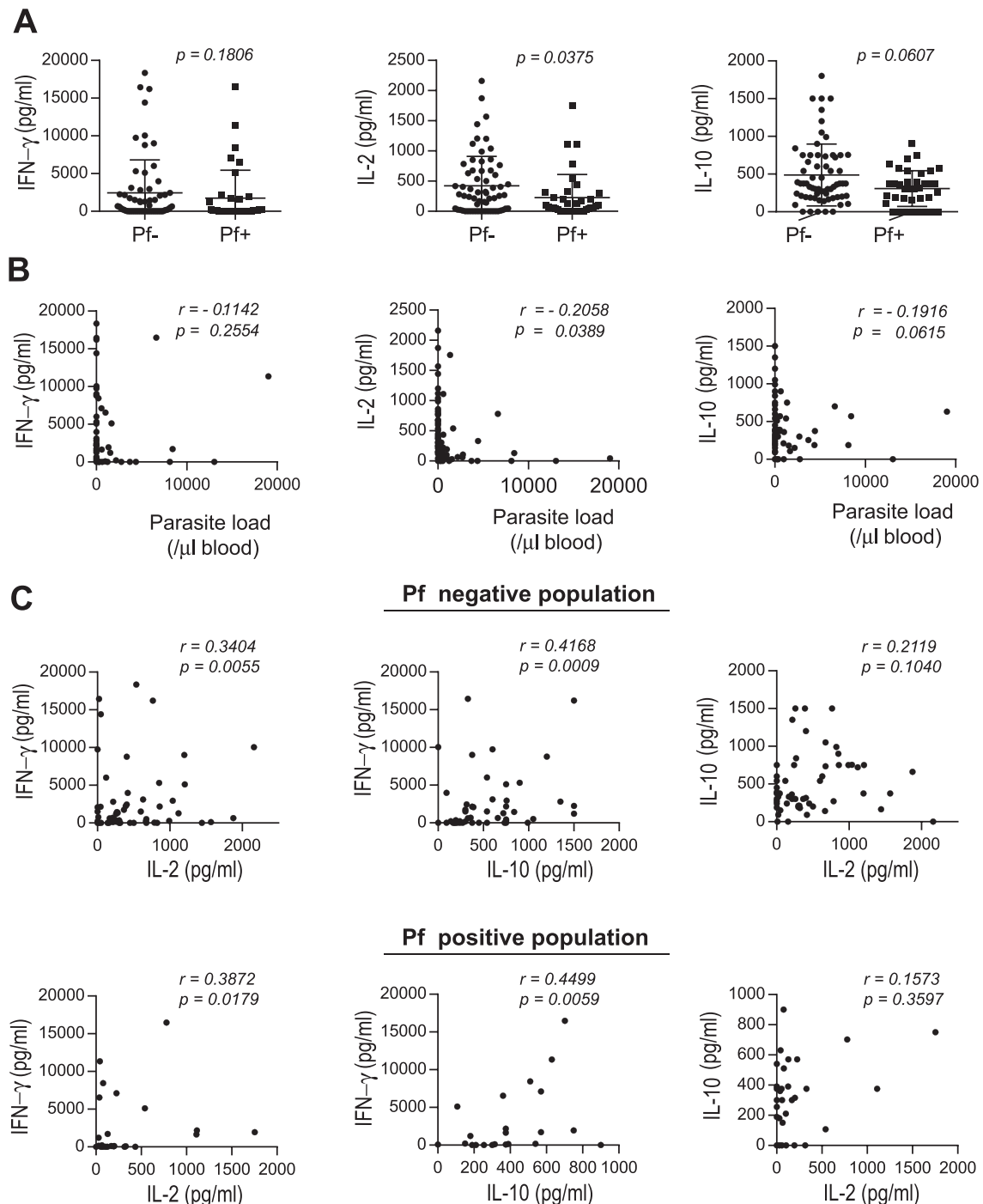
#### 4. Discussion

We performed a cross-sectional study of school children living in a *P. falciparum* holoendemic region, Mbita, Kenya, to evaluate the influence of *Plasmodium* infection on the immune status in asymptomatic children. Among 136 school children examined, parasitemia was microscopically detected in 45 (33%); we therefore compared the immune status of children who were positive and negative for parasitemia at the microscopy-level. We determined the infection status of children by microscopic examination of *P. falciparum* infection, because we considered that the level of parasitemia was likely to affect the host adaptive immune responses. The prevalence of *P. falciparum* infection among children living in Ungoye, the coastal area of Lake Victoria located near Mbita region was reported to be nearly 60–80% by PCR and approximately 30–60% by microscopy, indicating that a significant proportion of children are infected at submicroscopic levels [24]. Consistent with this study, within 91 microscopy-negative children in our study, 26 were positive by RDT, suggesting that a significant proportion of microscopy-negative children was infected with *P. falciparum* at submicroscopic levels. However, we did not detect significant differences between RDT-positive and -negative children in the

microscopy-negative group in the proportions of NK cells, central memory phenotype CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells that proliferate in response to SEA, and the production of IL-2 by PBMCs in response to anti-CD3 and CD28 mAbs (data not shown). Thus, we believe that a certain level of parasitemia is required to affect the observed immune status of the *P. falciparum*-infected children, although submicroscopic infection might have additional effects. It is likely that both the duration and the levels of parasitemia affect the immune status of individuals infected with *P. falciparum*.

We found that the proportion of central memory phenotype CD62L<sup>+</sup> CD45RO<sup>+</sup> CD8<sup>+</sup> T cells was significantly lower in *P. falciparum*-positive children. This reduction may be due to the differentiation of these cells to effector cells in response to active *P. falciparum* infection, although clarification of the precise mechanisms requires further study. In cellular subset analysis of PBMCs, we observed a striking reduction in the proportion of NK cells in *P. falciparum*-positive children. NK cells have been implicated in the protection against erythrocytic *Plasmodium* infection and pathogenesis of malaria through the production of cytokines including IFN- $\gamma$  and their cytotoxic activity in some cases [34]. They are reported to be severely reduced among PBMCs in patients with acute *P. falciparum* malaria, albeit not in asymptomatic individuals [35]. In contrast, our study showed that the numbers of NK cells in PBMCs were severely reduced in asymptomatic children that were *P. falciparum*-positive when compared with negative children. The reduction in PBMCs may be due to the sequestration or redistribution of NK cells in organs such as the spleen. Alternatively, NK cell reduction may prevent an excessive proinflammatory response and contribute toward maintaining asymptomatic infections in the face of the active infection with *P. falciparum*. Further studies are required to determine mechanisms underlying the reduction of these cells from the peripheral circulation in asymptotically infected children.

We evaluated the specific response of PBMCs by stimulation with antigens from *P. falciparum* or *S. mansoni* as well as polyclonal responses after stimulation with anti-CD3 and anti-CD28 mAbs, and found that the variations in the response in both *P. falciparum*-positive and -negative children were quite large with the majority of CD4<sup>+</sup> T cells failing to respond. Strikingly, CD4<sup>+</sup> T cells from 64% (27/42) and 70% (57/82) of *P. falciparum*-positive and -negative children, respectively,



**Fig. 5.** Cytokine production of PBMCs in response to polyclonal stimulation with anti-CD3 and anti-CD28 mAbs. **A.** Levels of IFN- $\gamma$ , IL-2, and IL-10 in the culture supernatant of PBMCs stimulated with beads coated with anti-CD3 and anti-CD28 mAbs were determined by ELISA. Values of cytokines produced in the culture of unstimulated control groups were subtracted from those of stimulated culture. Pf+,  $n = 37$ ; Pf-,  $n = 65$ . A Mann-Whitney test was performed for the pairwise comparisons. **B.** Relationship between the production of cytokines (IFN- $\gamma$ , IL-2, and IL-10) and infection intensity was evaluated. **C.** Relationships between the levels of IFN- $\gamma$ /IL-2, IFN- $\gamma$ /IL-10, and IL-10/IL-2 were evaluated separately in *P. falciparum*-positive and -negative groups.  $p$  values by Spearman Rank tests are indicated on the scatter plots.

failed to proliferate in response to iRBC stimulation, suggesting a state of cellular unresponsiveness. There were also trends that PBMCs from children with high parasite loads produced few cytokines (Fig. 3B). When PBMCs were stimulated with anti-CD3 and CD28 mAbs, production of IL-2 was significantly reduced in *P. falciparum*-positive children, in a manner dependent on the levels of parasite load (Fig. 5B). Notably, reduction of T cell responses was previously reported in patients with malaria. During acute infection with *P. falciparum*, IL-2 production of PBMCs in response to malaria antigen as well as polyclonal stimulation

was down-regulated in hospital studies in Thailand [14]. PBMCs from people living in a highly endemic area of *P. falciparum* in Ghana were also reported to produce minimum amounts of IFN- $\gamma$  in response to *P. falciparum* antigens, although no difference was detected in IL-10 production [36]. The immune response in individuals with asymptomatic malaria is associated with the reduction in proinflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , and increased levels of IL-10 [37]. Our study showed a possibility that the T-cell immune responses are reduced in *P. falciparum*-positive asymptomatic children. This reduction is likely to be



independent from IL-10 as the production of IL-10 also tended to be reduced. It is conceivable that owing to the holoendemic nature of the study area, children may have had frequent malaria exposure and consequently have undergone multiple mechanisms to reduce T-cell immunity to cope with the infection pressure with minimum immune-mediated host damage.

We analyzed the relationship among children whose PBMCs produced IFN- $\gamma$ , IL-2, and IL-10, and found that cells from each child did not produce both IFN- $\gamma$  and IL-2/IL-10 in response to *P. falciparum* antigens: in particular, PBMCs from children producing high levels of IL-2 or IL-10 did not produce high levels of IFN- $\gamma$  and vice versa. Although IL-10 might inhibit production of IFN- $\gamma$ , the reciprocal production pattern of IFN- $\gamma$  and IL-2 is likely to be mediated by distinct mechanisms. These data suggest that the quality of immune responses to *P. falciparum* antigen is heterogeneous among children living in this region, with IFN- $\gamma$  dominantly produced by PBMCs from some children and IL-2 or IL-10 from others. As this study was cross-sectional, it is unclear whether the status of T-cell responses is maintained for a prolonged period in each individual living in the endemic region of *P. falciparum* or whether their immune status alters with time. However, it may be informative to evaluate the effect of such differential cytokine production by T cells on the protection and pathogenesis of *P. falciparum* infection. Further multifaceted, large-scale cohort investigation for a prolonged period is required to better understand cellular and molecular mechanisms underlying the heterogeneity of the immune responses in asymptomatic children living in the malaria holoendemic regions.

In contrast to the general reduction of the immune responses in *P. falciparum*-positive children, when PBMCs were stimulated with SEA, an antigen unrelated to *P. falciparum*, the proportions of proliferating CD4<sup>+</sup> T cells were significantly higher in *P. falciparum*-positive children when compared with their -negative counterparts. The data suggest that reduction of T cell responses during *P. falciparum* infection may not apply to all immune responses, with immune responses to unrelated antigens potentially being relatively unaffected by the inhibition during *P. falciparum*-infection. In addition, we observed a positive correlation in the production level of IFN- $\gamma$  and IL-2 as well as IFN- $\gamma$  and IL-10 by PBMCs in response to anti-CD3 and anti-CD28 mAbs, suggesting that cytokine production in response to T cell stimulation was maintained.

In this study, we reported the reduction of immune responses and the heterogeneity in the quality of immune responses to *P. falciparum* antigen in asymptomatic children living in a high transmission area of malaria. Altogether, our study has several limitations. First, this study is cross-sectional and we measured current parasite infection status only. It is possible that some of the infected asymptomatic individuals may be in the pre-symptomatic period and present with clinical manifestations at a subsequent date. Second, we cannot exclude a possibility that these children might have received anti-malaria treatment, so the natural course of the infection may have been altered. Third, we may have missed the effect of subpatent infections that could be detected only by polymerase chain reaction analysis as we discussed previously. Nevertheless, this study revealed the modulation of T cell function and the reduction of NK cells in asymptomatic school children living in the holoendemic region of *P. falciparum* in Mbita, Kenya. The inhibition of IL-2 production might underlie the resistance to the disease outcome in asymptomatic children. In addition, the impairment, as well as heterogeneity of the immune response in asymptomatic children, may modify susceptibility of children to upcoming infection with *P. falciparum*, and the immune responses to future vaccine interventions when administered to these children.

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## Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.parint.2018.01.001>.

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