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Cellular Tumor Necrosis Factor, Gamma Interferon, and Interleukin-6 Responses as Correlates of Immunity and Risk of Clinical *Plasmodium falciparum* Malaria in Children from Papua New Guinea▽

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The role of early to intermediate *Plasmodium falciparum*-induced cellular responses in the development of clinical immunity to malaria is not well understood, and such responses have been proposed to contribute to both immunity and risk of clinical malaria episodes. To investigate whether *P. falciparum*-induced cellular responses are able to function as predictive correlates of parasitological and clinical outcomes, we conducted a prospective cohort study of children (5 to 14 years of age) residing in a region of Papua New Guinea where malaria is endemic Live, intact *P. falciparum*-infected red blood cells were applied to isolated peripheral blood mononuclear cells obtained at baseline. Cellular cytokine production, including production of interleukin-2 (IL-2), IL-4, IL-6, IL-10, tumor necrosis factor (TNF) (formerly tumor necrosis factor alpha), and gamma interferon (IFN-γ), was measured, and the cellular source of key cytokines was investigated. Multicytokine models revealed that increasing *P. falciparum*-induced IL-6 production was associated with an increased incidence of *P. falciparum* clinical episodes (incidence rate ratio [IRR], 1.75; 95% confidence interval [CI], 1.20 to 2.53), while increasing *P. falciparum*-induced TNF and IFN-γ production was associated with a reduced incidence of clinical episodes (IRR for TNF, 0.55 [95% CI, 0.38 to 0.80]; IRR for IFN-γ, 0.71 [95% CI, 0.55 to 0.90]). Furthermore, we found that monocytes/macrophages and γδ-T cells are important for the *P. falciparum*-induced production of IL-6 and TNF. Early to intermediate cellular cytokine responses to *P. falciparum* may therefore be important correlates of immunity and risk of symptomatic malaria episodes and thus warrant detailed investigation in relation to the development and implementation of effective vaccines.

Individuals living in regions of moderate to high malaria endemicity slowly acquire clinical immunity to *Plasmodium falciparum* throughout their lives in an age- and exposure-dependent manner (3, 29). This immunity enables them to control parasite replication at densities below that which induces clinical symptoms (29, 46). Both antibody-dependent and T-cell-dependent acquired immune responses have been shown to play an important role in the development of clinical immunity (13, 29). The role of early to intermediate cellular responses, however, is less well understood, and such responses have been proposed to contribute to both immunity and risk of clinical malaria episodes (45, 49, 51).

Early cellular immune responses are rapidly initiated during malaria infection and are thought to play an important role both in limiting initial parasite replication and in directly shaping subsequent adaptive immune responses (45, 49, 51). However, the overproduction or inappropriate regulation of both proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, gamma interferon (IFN-γ), and tumor necrosis factor (TNF) (formerly tumor necrosis factor alpha), and anti-inflammatory cytokines, such as IL-10, IL-4, and transforming growth factor β (TGF-β), may also lead to localized and systemic inflammation and has been associated with symptomatic and severe malaria (7, 45). The clinical outcome of an infection may thus depend on the appropriate induction and counterregulation of both pro- and anti-inflammatory cytokine secretion. Understanding how this network of *P. falciparum*-induced cellular responses is associated with immunity and risk of clinical disease in malaria exposed-children could provide important insights for the development of effective vaccines.

Studies investigating the association between *P. falciparum*- or antigen-induced secretion of cytokines from peripheral blood mononuclear cells (PBMCs) and prospective risk of clinical episodes in malaria-exposed individuals provide the most powerful means by which to understand the relationship between these inducible cellular immune responses and risk of disease (8, 19, 29). Two such prospective cohort studies report that late-stage IFN-γ responses to liver-stage and merozoite surface antigens, as well as IL-10 responses to liver-stage antigens, are associated with resistance to reinfection (23, 26). However, stimulation of PBMCs with live, intact *P. falciparum*-infected red blood cells (iRBCs) at the late trophozoite-

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schizont stage is thought to more closely reflect the in vivo situation and in malaria-naïve adults has been shown to capture early cellular responses to diverse *P. falciparum* stimuli (1, 9, 11, 16). For malaria-exposed individuals, Dodoo et al. (8) reported an association between late-stage, live *P. falciparum*-induced IFN-γ production and reduced risk of fever and clinical malaria. More recently, we reported that early *P. falciparum*-induced IFN-γ production from γδ-T cells and αβ-T cells is associated with protection from clinical *P. falciparum* episodes in a cohort of 5- to 14-year-old children from a region of Papua New Guinea (PNG) where malaria is endemic (10).

The aims of the present study were to investigate the contribution of multiple *P. falciparum*-induced early cellular immune responses to immunity and risk of clinical malaria episodes and to evaluate these early cytokine responses together using a multivariate model rather than as isolated responses. A 6-month prospective cohort study was conducted in the Mugil region of PNG (31), and live, intact *P. falciparum*-iRBCs were applied to isolated PBMCs from 165 malaria-exposed children with ages of 5 to 14 years. Specifically, we sought to determine whether it is possible to define predictive correlates of immunity and risk by examining whether *P. falciparum*-induced production of cytokines (IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ) in defined cell populations is associated with age, infection status, and prospective risk of *P. falciparum* infection and clinical episodes.

MATERIALS AND METHODS

Prospective cohort study. A detailed description of the study area, subjects, and design of this treatment-reinfection study has been published elsewhere (31). Briefly, 206 children with ages of 5 to 14 years from Madang Province, PNG, were enrolled at baseline and actively monitored for 6 months. At baseline, 10 ml of peripheral blood was collected using sterile sodium heparin Vacutainer tubes (BD), and PBMCs were separated and cryopreserved. All children then underwent a 7-day course of artesunate monotherapy to clear any existing *Plasmodium* infection. After treatment, children were actively monitored for reinfection and clinical episodes by performing fortnightly clinical examinations, during which a morbidity questionnaire was administered and finger-prick blood samples were collected to allow detection of parasitemia by light microscopy (LM) and a semiquantitative post-PCR ligase detection reaction-fluorescent microsphere assay that detects all four human malaria species (30, 31). Children found to be unwell were referred for treatment at the Mugil Health Centre, where they also had exclusive access during the study (passive case detection) (31). Clinical episodes of malaria were treated with chloroquine and sulfadoxine-pyrimethamine in accordance with PNG national treatment guidelines. Clinical malaria was defined as a measured fever (axillary temperature, ≥37.5°C) or history of febrile illness during the 48 h preceding clinical examination, in conjunction with concurrent *P. falciparum* parasitemia (any density) as determined by LM. O the 206 PBMC samples acquired at baseline, sufficient cells were available for 165 individuals to be included in this immunological study. Ethical approval was granted by the PNG Medical Research Advisory Council and The Walter and Eliza Hall Institute of Medical Research, and written informed consent was obtained from the parents or guardian of each child.

PBMC isolation. Blood was diluted 1:1 in phosphate-buffered saline, and PBMCs were isolated by density centrifugation over Ficoll-Paque PLUS (Amersham). PBMCs were washed twice and cryopreserved by resuspension at a concentration of 2×10^7 cells/ml in fetal bovine serum (FBS) and then dilution to 1×10^7 cells/ml by dropwise addition of 20% dimethyl sulfoxide–80% FBS. PBMCs were frozen to −80°C at a controlled rate of 1°C/min in Nalgene freezing containers and transferred to liquid nitrogen after 24 h.

***P. falciparum* culture.** *P. falciparum* strain 3D7 was maintained in continuous culture in human O-positive erythrocytes (kindly provided by the Australian Red Cross Blood Service) at 4% hematocrit in RPMI 1640 with 25 mM HEPES, 0.5% Albumax II (Gibco), 2 mg/ml glucose, 28 mM sodium bicarbonate, 25 mg/ml gentamicin, and 100 μg/ml hypoxanthine at 37°C in an atmosphere containing 1% O_2, 5% CO_2, and 94% N_2. Parasites were regularly sorbitol synchronized (25) and knob selected (14) and were routinely confirmed to be *Mycoplasma* free by PCR (Stratagene). Live and intact *P. falciparum* late-trophozoite- and schizont-iRBC were purified by magnetic separation over MACS CS columns (Miltenyi Biotec) using a VarioMACS magnet (Miltenyi Biotec).

PBMC stimulation assays. PBMCs were thawed at 37°C, diluted 1:10 by dropwise addition of complete culture medium (RPMI 1640, 5% heat-inactivated FBS, 2 mM L-glutamine, 25 mM HEPES, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate), and pelleted by centrifugation ($770 \times g$, 10 min). PBMCs were washed twice, and numbers of viable cells were determined by staining with Turks solution (Merck) and trypan blue (Sigma). Cells were resuspended in complete medium at a concentration of 2×10^6 cells/ml. PBMCs were then aliquoted in U-bottom 96-well plates at a density of 2×10^5 cells/well in 100 μl complete medium. Purified iRBCs and autologous uninfected red blood cells (uRBCs) were added to PBMCs at a ratio of three iRBCs or uRBCs to one mononuclear cell (6×10^5 RBCs/well). PBMC viability was assessed by stimulation with 4% phytohemagglutinin (PHA) (Gibco). Cultures were incubated at 37°C in 5% CO_2 for 72 h to capture early responses from cells of the innate, intermediate, and adaptive arms of the immune system. Supernatants were collected, and cytokine concentrations were determined by Cytometric Bead Array (CBA) (BD). uRBCs and PHA were used as negative and positive controls, respectively.

Cell depletion. To deplete the monocyte/macrophage population ($CD14^+$ cells), Dynabeads (Invitrogen) were used to positively select $CD14^+$ cells, leaving the remaining PBMCs untouched. γδ-T and αβ-T cells were depleted using anti-γδTCR-fluorescein isothiocyanate (FITC) and anti-αβTCR-FITC antibodies together with MACS anti-FITC microbeads, as previously described (9). Cell depletion was confirmed by flow cytometry. Depleted PBMCs were resuspended at a concentration of 2×10^6 cells/ml in complete medium for use in stimulation assays as described above. Nondepleted PBMC samples (2×10^6 cells/ml) were used as controls.

Detection of multiple cytokines by CBA. CBA human Th1/Th2 cytokine II kits (BD) were used to simultaneously measure IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ levels in supernatants. Samples were analyzed using a FACSCalibur flow cytometer, and data were analyzed using BD FCAPArray software.

Measure of blood-stage immunity. Levels of immunoglobulin G (IgG) to *P. falciparum* schizont extract were determined by an enzyme-linked immunosorbent assay, using established methods (37).

Statistics. Statistical analyses were performed using STATA software (Stata Corp.). Univariate analysis of baseline and follow-up variable data was performed using the χ^2 test for proportions and unpaired two-tailed Student's *t* tests for continuous variables. Cytokine responses were transformed by using $\log_e$(pg/ml cytokine + 1) to normalize the distribution of cytokine values, and the geometric means were compared using unpaired two-tailed Student's *t* tests with unequal variance. For analysis of cytokine ratios, the variance of $\log_e$-transformed data was standardized prior to calculation of the cytokine ratios, such that the units represented the number of standard deviations from the mean. Univariate analysis of cytokine responses with baseline categorical variables (age and *P. falciparum* infection status) was performed using the $\log_e$-transformed data and unpaired two-tailed Student's *t* tests with unequal variance. Age-adjusted analysis of cytokine responses with baseline *P. falciparum* infection status was performed using the $\log_e$-transformed data and linear regression. In all models age was analyzed as a categorical variable with the cutoff set at a median age of 9 years (interquartile range, 8.1 to 10.3 years).

Poisson regression was used to investigate the association of cytokine production as a continuous variable with the incidence of *P. falciparum* clinical episodes. For the purposes of analysis, three definitions of clinical malaria were used. Clinical malaria of any density was defined as a measured fever (axillary temperature, ≥37.5°C) or a history of febrile illness during the 48 h preceding clinical examination, in conjunction with concurrent *P. falciparum* parasitemia of any density as determined by LM. Moderate-density clinical episodes were defined as fever plus ≥500 *P. falciparum* parasites/μl of blood, and high-density clinical episodes were defined as fever plus ≥5,000 *P. falciparum* parasites/μl of blood as determined by LM (48). All *P. falciparum* episodes observed during active follow-up and passive morbidity surveillance were considered. Adjustments for *Plasmodium vivax* were not considered necessary since there was no association between the presence of *P. vivax* at baseline and the risk of a *P. falciparum* episode during follow-up and the incidence of *P. vivax* febrile episodes during follow-up was very low (0.06/year) (31). Furthermore, we observed only three febrile episodes with concurrent *P. falciparum* and *P. vivax* parasitemia (any density). The association of cytokine production with time to first reinfection above specific density thresholds was assessed by Cox regression as described elsewhere (31). Children were considered to be at risk until they did not provide

TABLE 1. Malariometric indices of a subset of 165 children from the Mugil cohort according to time point and age group[a]

Ages (yr)	Baseline				Follow-up		
	No. of children	No. (%) of children *P. falciparum* positive by LM	No. (%) of children *P. falciparum* positive by PCR	PBMC concn (10^6 cells/ml) (mean ± SD)	No. (%) of children reinfected with *P. falciparum* by PCR	No. (%) of children with ≥1 febrile *P. falciparum* episode	No. (%) of children with ≥1 high-density *P. falciparum* clinical episode[b]
All	165	53 (32)	113 (68)	2.88 ± 0.13	159 (96)	82 (50)	65 (39)
≤9	72	19 (26)	45 (63)	2.99 ± 0.17	70 (97)	45 (63)	38 (53)
>9	93	34 (37)[c]	68 (73)[c]	2.79 ± 0.18[c]	89 (96)[c]	37 (40)[d]	27 (29)[e]

[a] χ^2 and *t* tests were used as described in the text to compare the two age groups.
[b] A high-density *P. falciparum* clinical episode was defined as fever in the presence of ≥5,000 parasites/μl as determined by LM.
[c] The *P* value for comparison of the two age groups was >0.1.
[d] The *P* value for comparison of the two age groups was <0.001.
[e] The *P* value for comparison of the two age groups was 0.002.

two consecutive biweekly samples, were retreated with antimalarials, or withdrew from the study (31). Children were censored for 4 weeks after treatment with chloroquine and sulfadoxine-pyrimethamine and for 2 weeks after treatment with Coartem. Episodes of the same species within 2 weeks were considered the same episode. In all multivariate analyses, backward selection and likelihood ratio (LR) tests were used to identify the best-fitting models. All estimates of the effects on incidence of clinical episodes or risk of reinfection were adjusted for previously identified confounders (31). For further details concerning modeling strategies, see reference 31.

RESULTS

Characteristics of the cohort. Malariometric indices of the 165 children in this immunological study at baseline and follow-up are shown in Table 1. The data are consistent with rates of infection and illness observed for the entire cohort (31), confirming that there was no selection bias. At baseline, 68% of the children were positive for *P. falciparum* as determined by PCR, 32% were positive for *P. falciparum* as determined by LM, and 7% had a moderate-density infection (parasitemia, ≥500 parasites/μl [Table 1]). During the 6-month follow-up period, 96% of the children became reinfected with *P. falciparum* (as diagnosed by PCR), and 39% experienced at least one high-density (≥5,000 parasites/μl) *P. falciparum* clinical episode (Table 1). The overall incidence of *P. falciparum* clinical episodes in the study cohort was estimated to be 1.68 episodes per child per year (95% confidence interval [CI], 1.41 to 2.04 episodes per child per year) for all *P. falciparum* febrile episodes and 1.23 episodes per child per year (95% CI, 0.98 to 1.52 episodes per child per year) for high-density *P. falciparum* clinical episodes. Children older than 9 years of age had a significantly reduced risk of experiencing a *P. falciparum* episode (incidence rate ratio [IRR] for febrile episodes, 0.45 [95% CI, 0.29 to 0.69; $P < 0.001$]; IRR for high-density febrile episodes, 0.49 [95% CI, 0.34 to 0.71; $P < 0.001$]), indicating that older children were more immune to symptomatic *P. falciparum* episodes and suggesting that the children in this cohort are in the process of acquiring clinical immunity. The concentrations of PBMCs in samples collected from children in this study were not observed to be associated with age (Table 1) or any other clinical outcome variable.

In vitro PBMC cytokine responses. In the cohort of malaria-exposed PNG children examined, the level of background or spontaneous cytokine production in response to uRBCs was low, and the geometric mean levels of iRBC- and PHA-stimulated cytokine production were significantly greater than the background (uRBC) levels for all cytokines tested ($P < 0.001$). In order to specifically assess iRBC-induced and PHA-induced cytokine production, cytokine responses to uRBCs were subtracted from responses to iRBCs and PHA. Cellular responses to iRBCs were all positively correlated with each other ($P < 0.001$) (Table 2), and several PHA-induced cytokine responses were also correlated (Table 2). These observations support previous findings that cytokine secretion is highly coregulated (7, 8).

Association between age at enrollment and cellular cytokine responses. To investigate the effect of age on in vitro cellular responses, we compared the geometric mean levels of iRBC- and PHA-induced cytokine production in children who were ≤9 years of age to the geometric mean levels in children who were >9 years of age. *P. falciparum*-induced PBMC production of IL-10, TNF, and IFN-γ was significantly elevated in children who were >9 years of age compared to children who were ≤9 years of age (Fig. 1A). However, this was not a malaria-specific phenomenon, as there was also an age-dependent increase in the PHA-induced production of all cytokines except IL-6 (Fig. 1B). Age was therefore investigated as an important variable in the subsequent multivariate analysis of cellular cytokine responses and risk of clinical malaria. Since many cytokines have overlapping and counterregulatory functions, we also investigated whether there was any association between cytokine ratios and age. However, neither iRBC- nor PHA-induced cytokine ratios were significantly different for the two age groups ($P > 0.1$).

Association between *P. falciparum* infection status at enrollment and cellular cytokine responses. We investigated whether parasite status at baseline was associated with cellular cytokine responses. No significant associations were observed between *P. falciparum* infection status at baseline (as determined by PCR or LM) and either individual cytokine levels (iRBC or PHA stimulated) (Fig. 2) or cytokine ratios, both unadjusted and age adjusted. Due to the limited number of children who had *P. falciparum* moderate-density infections (≥500 parasites/μl) at baseline ($n = 12$) or fever in the presence of parasites at baseline ($n = 5$), a meaningful statistical analysis of cellular cytokine responses in relation to these variables was not possible.

Association between individual cellular cytokine responses, risk of *P. falciparum* reinfection, and incidence of clinical episodes. The relationship between individual cytokine responses

TABLE 2. Correlation of *P. falciparum*-induced cytokine production and PHA-induced cytokine production

Production conditions	Cytokine	Pairwise correlation[a]					
		IL-2	IL-4	IL-6	IL-10	TNF	IFN-γ
P. falciparum induced	IL-2	1.0					
	IL-4	0.47***	1.0				
	IL-6	0.23***	0.71***	1.0			
	IL-10	0.35***	0.61***	0.81***	1.0		
	TNF	0.42***	0.80***	0.65***	0.45***	1.0	
	IFN-γ	0.45***	0.59***	0.38***	0.50***	0.88***	1.0
PHA-induced	IL-2	1.0					
	IL-4	0.18	1.0				
	IL-6	0.04	−0.14	1.0			
	IL-10	0.26***	0.15*	0.50***	1.0		
	TNF	0.87***	0.18*	0.13*	0.32***	1.0	
	IFN-γ	0.50***	0.01	0.26***	0.30***	0.62***	1.0

[a] Asterisks indicate *P* values, as follows: ***, $P < 0.001$; **, $P < 0.05$; *, $P < 0.1$.

and time to reinfection with *P. falciparum* was investigated, revealing that neither *P. falciparum*- nor PHA-induced production of IL-2, IL-4, IL-6, IL-10, TNF, or IFN-γ was associated with risk of *P. falciparum* reinfection ($P > 0.1$). Similarly there were no significant associations between PHA-induced production of IL-2, IL-4, IL-6, IL-10, TNF, or IFN-γ and incidence of clinical episodes of any density or high density (parasitemia, ≥5,000 parasites/μl) (Table 3). However, *P. falciparum*-induced production of IL-2, TNF, and IFN-γ showed some evidence of an association with a reduced incidence of high-density *P. falciparum* clinical episodes (IRR, < 1) (Table 3), but the effects were not statistically significant.

Association between multiple cellular cytokine responses, risk of *P. falciparum* reinfection, and incidence of clinical episodes. Cytokines are known to act concomitantly rather than individually (12); therefore, a statistical model including all cytokine responses as continuous variables was constructed to investigate the association of multiple cytokine responses with incidence of *P. falciparum* clinical episodes. The best-fitting multicytokine model included IL-6, TNF, and previously iden-

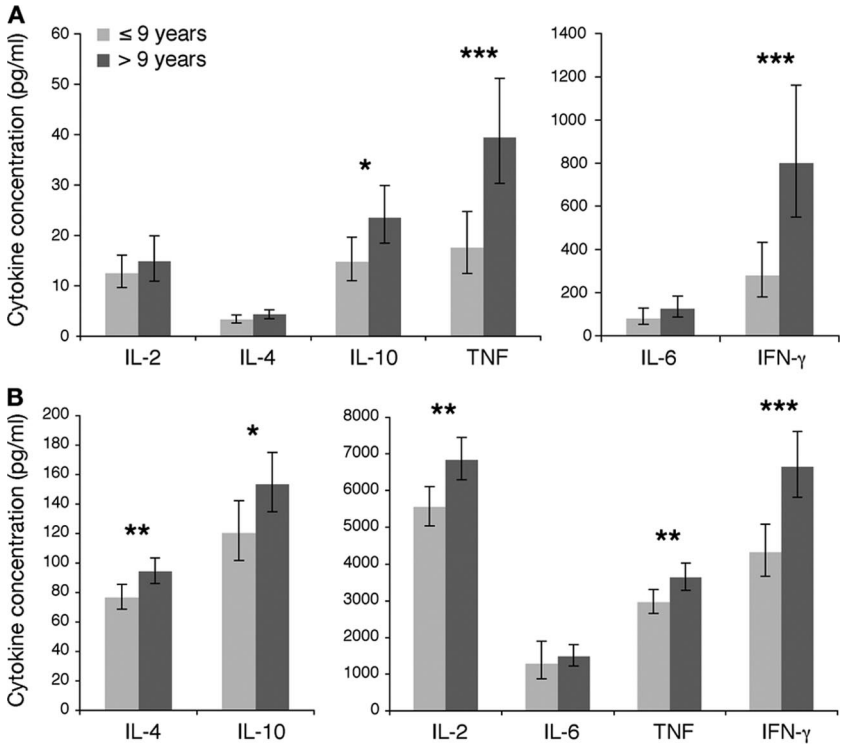

FIG. 1. Cellular responses of (A) iRBC-stimulated and (B) PHA-stimulated PBMCs from children ≤9 years of age compared to those from children >9 years of age. The graphs show the geometric means and 95% CI for cytokine production. Note the different scales for the *y* axis. Asterisks indicate *P* values estimated using two-tailed Student's *t* tests with unequal variance, as follows: ***, $P < 0.01$; **, $P < 0.05$; *, $P < 0.1$.

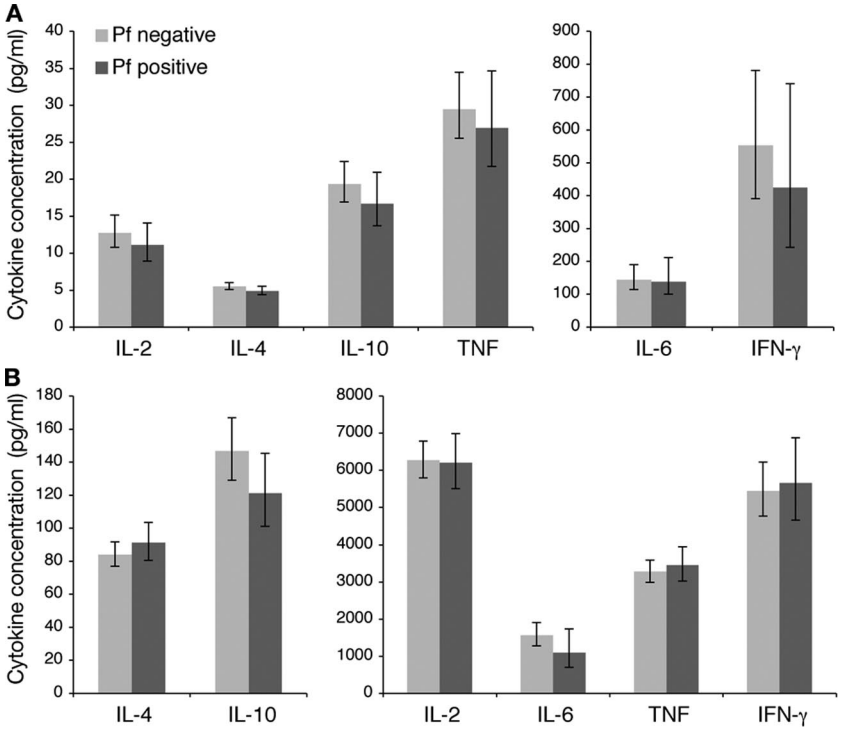

FIG. 2. Cellular responses of (A) iRBC-stimulated and (B) PHA-stimulated PBMCs from children who were *P. falciparum* negative and positive at baseline. The graphs show the geometric means and 95% CI for cytokine production. Note the different scales for the *y* axis. *P* values were estimated for linear regression models, adjusting for age. All *P* values were >0.1.

TABLE 3. Association of cellular cytokine production with incidence of all clinical episodes and high-density clinical episodes (≥5,000 parasites/μl)

Cytokine production	All *P. falciparum* episodes		High-density *P. falciparum* episodes	
	IRR[a]	95% CI	IRR[a]	95% CI
IL-2	0.85	0.67–1.06	0.82	0.64–1.07
P. falciparum specific				
PHA specific	0.97	0.61–1.56	1.21	0.73–2.03
IL-4				
P. falciparum specific	1.08	0.82–1.43	1.01	0.73–1.38
PHA specific	0.87	0.57–1.30	0.74	0.46–1.19
IL-6				
P. falciparum specific	1.07	0.96–1.20	1.04	0.91–1.18
PHA specific	1.16	0.95–1.42	1.04	0.83–1.29
IL-10				
P. falciparum specific	0.99	0.82–1.21	0.93	0.74–1.15
PHA specific	1.04	0.78–1.39	0.90	0.64–1.25
TNF				
P. falciparum specific	0.94	0.78–1.13	0.87	0.71–1.07
PHA specific	1.01	0.68–1.50	1.04	0.67–1.60
IFN-γ				
P. falciparum specific	0.93	0.54–1.08	0.84[b]	0.69–1.02
PHA specific	0.99	0.76–1.30	0.89	0.66–1.19

[a] IRR were estimated from multivariate Poisson regressions of continuous $\log_e$-transformed cytokine levels, with adjustment for age, school, distance from the coast, and hemoglobin (known confounders) (31).

[b] $P = 0.079$. *P* values that are >0.1 are not shown.

tified confounders (school, distance from the coast, and hemoglobin level) (LR, 10.3; $P = 0.006$). Increased levels of IL-6 were associated with increased incidence of high-density *P. falciparum* febrile episodes (IRR, 1.75; 95% CI, 1.20 to 2.53) (Fig. 3A, model 1), whereas increased levels of TNF were associated with reduced incidence (IRR, 0.55; 95% CI, 0.38 to 0.80) (Fig. 3A, model 1). The same relationships were also predictive of risk of any febrile *P. falciparum* episode, and the effect sizes and significance levels were almost identical (Fig. 3B, model 1). As *P. falciparum*-induced production of TNF and IFN-γ are strongly correlated (Table 2), we also constructed a model in which IFN-γ was retained and TNF was excluded (LR, 7.6; $P = 0.02$) and observed similarly significant associations. In this model, increased levels of IL-6 relative to IFN-γ levels were associated with increased risk of high-density *P. falciparum* febrile episodes (IRR, 1.35; 95% CI, 1.03 to 1.78), while increased levels of IFN-γ relative to IL-6 levels were associated with protection from clinical episodes (IRR, 0.71; 95% CI, 0.55 to 0.90). An investigation of IL-6, TNF, and IFN-γ using cytokine ratio analysis revealed that increased ratios of IL-6 to TNF (IRR, 2.13; 95% CI, 1.18 to 3.85; $P = 0.01$) and of IL-6 to IFN-γ (IRR, 1.89; 95% CI, 1.11 to 3.20; $P = 0.02$) were associated with increased risk of high-density *P. falciparum* febrile episodes. There was no association between PHA-induced cytokine responses and risk of clinical disease ($P > 0.2$), indicating that these associations are malaria specific. These associations suggest that there is an important relationship between TNF, IFN-γ, IL-6, and risk of clinical episodes.

As age and cumulative exposure are associated with reduced risk of clinical episodes in the cohort examined, we

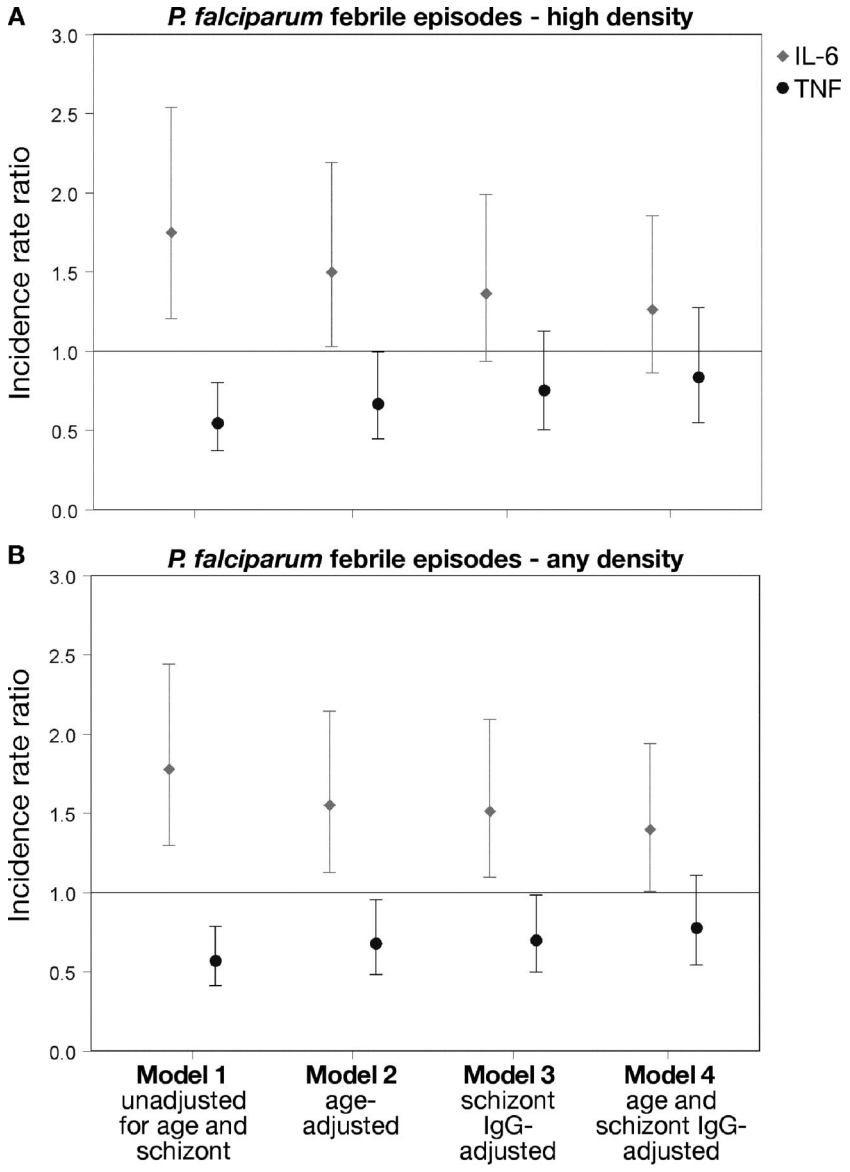

FIG. 3. Association of cellular cytokine responses with incidence of high-density *P. falciparum* febrile episodes (≥5,000 parasites/μl) (A) and any *P. falciparum* febrile episodes (B), adjusted for markers of age and exposure. Increased levels of IL-6 were significantly associated with an increased risk of *P. falciparum* febrile episodes (IRR, >1), while increased levels of TNF were significantly associated with a reduced risk of *P. falciparum* febrile episodes (IRR, <1). Model 1 adjusts for previously identified confounders of school, distance from the sea, and hemoglobin (31). In order to investigate the effect of age and cumulative exposure to *P. falciparum*, models 2 to 4 were constructed. Model 2 is model 1 with adjustment for age at enrollment; model 3 is model 1 with adjustment for the levels of antischizont IgG (a measure of blood-stage exposure); and model 4 is model 1 with adjustment for both age and exposure. The IRR were estimated from multivariate Poisson regressions of standardized, $\log_e$-transformed cytokine levels. The effects correspond to a change in risk with a one-standard deviation increase in cytokine output. The error bars indicate 95% CI. Adjusting for age and exposure (as measured using antischizont IgG) progressively reduced the strength and significance of the associations. The greater significance of associations in panel B is probably due to the greater number of clinical episodes of any density compared to the number of high-density clinical episodes (Table 1).

also incorporated these variables into the multivariate models (Fig. 3, models 2, 3, and 4). The IgG response to blood-stage *P. falciparum* schizont extract was used as a proxy for cumulative blood-stage exposure (34) and is a significant correlate of immunity in the cohort (J. S. Richards, unpublished). Adjustments for age, exposure, and combined age and exposure progressively reduced the strength and statistical significance of the associations between IL-6, TNF, and the incidence of clinical episodes (Fig. 3, models 3 and 4), indicating that the responses are at least partially dependent on the child's age and exposure. Importantly, the effects did not disappear and were still of moderate strength when the data were adjusted for both age and exposure together. This suggests that early to intermediate cellular responses might have components that are also independent of age and exposure.

There was also an association between IL-6, TNF, and risk of moderate-density *P. falciparum* reinfection (≥500 parasites/μl) as detected by LM (LR, 6.6; $P = 0.04$). Increased *P. falciparum*-induced IL-6 production was associated with an increased risk of moderate-density *P. falciparum* reinfection (hazard ratio, 1.30; 95% CI, 1.05 to 1.63), while increased *P. falciparum*-induced TNF production was associated with a reduced risk of moderate-density *P. falciparum* reinfection (hazard ratio, 0.64; 95% CI, 0.45 to 0.90). However, this association was not significant when the data were adjusted for age. There was no association with risk of reinfection as determined by either PCR or LM. Lastly, there were no significant associations between PHA-induced cellular responses and risk of acquiring a *P. falciparum* infection (as determined by LM or PCR).

Cellular source of IFN-γ, IL-6, and TNF. Resting frequencies of NK cells, γδ-T cells, and αβ-T cells were determined by flow cytometry for a subset of children from the cohort ($n = 28$). On average, NK cells comprised 8.0% of the total cells, γδ-T cells comprised 5.8% of the total cells, αβ-T cells comprised 64.9% of the total cells, and 21.4% of the cells were other cells. γδ-T cells and αβ-T cells were previously identified by flow cytometric intracellular IFN-γ staining as the predominant source of *P. falciparum*-induced IFN-γ in the malaria-exposed children examined, with a minor contribution by NK cells (10). Similar to IFN-γ production, several cell types are capable of producing IL-6 and TNF, and the role of these cytokines during malaria infection may vary depending on their cellular origin. Flow cytometric intracellular staining for IL-6 and TNF proved to be insufficiently sensitive to detect the levels of TNF and IL-6 produced by PBMCs from these children using this *P. falciparum* cellular elicitation assay. Studies of malaria-naïve adults have shown that monocytes/macrophages and γδ-T cells are the main sources of early TNF (16, 47), and IL-6 appears to be produced predominantly by monocytes/macrophages and some T cells (17, 38, 42). We therefore sought to specifically assess the contribution of these cells to *P. falciparum*-induced early TNF and IL-6 production using selective cell depletion assays with a limited number of PBMC samples ($n = 9$). In the malaria-exposed children examined, depletion of 96.4% of the $CD14^+$ monocytes/macrophages (Fig. 4A and B) almost completely abrogated IL-6 production (Fig. 4C). The TNF levels were also reduced (Fig. 4D). γδ-T-cell depletion (95.1%) (Fig. 4A and B) also reduced the levels of TNF (Fig. 4D) and to a lesser extent the levels of IL-6 (Fig. 4C). These findings are consistent with results obtained using PBMCs from malaria-naïve adult donors (data not shown) and suggest that monocytes/macrophages and γδ-T cells are important for the *P. falciparum*-induced production of IL-6 and TNF in this assay. Conversely, increased levels of IL-6 and TNF were observed following αβ-T-cell depletion (98.4%) (Fig. 4A and B). While it is possible that αβ-T cells negatively regulate the production of these cytokines (43), the increase most likely resulted from the fact that all samples (depleted and whole) were resuspended at a concentration of 2×10^6 cells/ml, which resulted in proportionally greater representation of responding cells in the αβ-T-cell-depleted population than in the whole-cell population.

DISCUSSION

Cytokines are key mediators in the cellular program of innate and adaptive immune responses to *P. falciparum*, controlling both the induction and the regulation of important effector immune responses. A critical balance between early pro- and anti-inflammatory cellular responses is crucial both for effectively controlling parasitemia and for preventing pathology (54). Therefore, we sought to determine which of these tightly regulated *P. falciparum*-induced cellular responses are associated with protection and which are associated with risk in malaria-exposed children. The high rates of reinfection and clinical episodes, together with the demonstrated age-dependent acquisition of clinical immunity, made this study well suited for investigating immunological correlates of risk and immunity to *P. falciparum* reinfection and symptomatic malaria.

This study differed methodologically from many previous studies in that we did not measure circulating levels of cytokines in the plasma but instead applied live, intact *P. falciparum* iRBCs to isolated PBMCs and measured cellular cytokine production. This live parasite-PBMC elicitation assay has previously been used for characterization of early cellular responses (e.g., NK cell and γδ-T-cell IFN-γ responses) in naïve individuals (1, 9, 11, 16), but it has not been widely used with PBMCs of malaria-exposed individuals. This assay differs from measurement of recall T-cell responses to synthetic peptides (26) and avoids the problem of uninfected erythrocytes inhibiting *P. falciparum*-specific cellular immune responses (50) by using purified PBMCs rather than whole blood (8). Furthermore, the prospective study design (31) allowed us to link *P. falciparum*-induced cellular cytokine responses to risk of subsequent *P. falciparum* infection and clinical episodes, overcoming the limitations involved in interpretation of cause and effect in case-control and cross-sectional studies.

In this study, the only individual cytokine that tended toward a significant association with incidence of clinical malaria was *P. falciparum*-induced IFN-γ. We previously reported an association between *P. falciparum*-induced IFN-γ production and reduced incidence of clinical malaria in the full Mugil cohort of 206 children, which was the focus of a separate study (10). Compared to children with low *P. falciparum*-induced IFN-γ responses, children with high *P. falciparum*-induced IFN-γ responses showed a reduced incidence of moderate- and high-density *P. falciparum* infections, and γδ-T cells and αβ-T cells were shown to be the predominant sources of the early, *P. falciparum*-induced production of IFN-γ in these malaria-exposed children (10).

The main focus of this study, however, was to investigate associations between multiple *P. falciparum*-induced cytokines and the incidence of clinical malaria episodes. The results of such analyses point toward an important relationship between TNF, IFN-γ, and IL-6 secretion by early to intermediate immune cells and the incidence of clinical malaria episodes. Increased levels of *P. falciparum*-induced TNF and IFN-γ relative to that of IL-6 were associated with reduced incidence of *P. falciparum* clinical episodes, while an increased *P. falciparum*-induced IL-6 level relative to TNF and IFN-γ levels was associated with increased incidence of *P. falciparum* febrile episodes. This suggests that IL-6 may be an important febrile

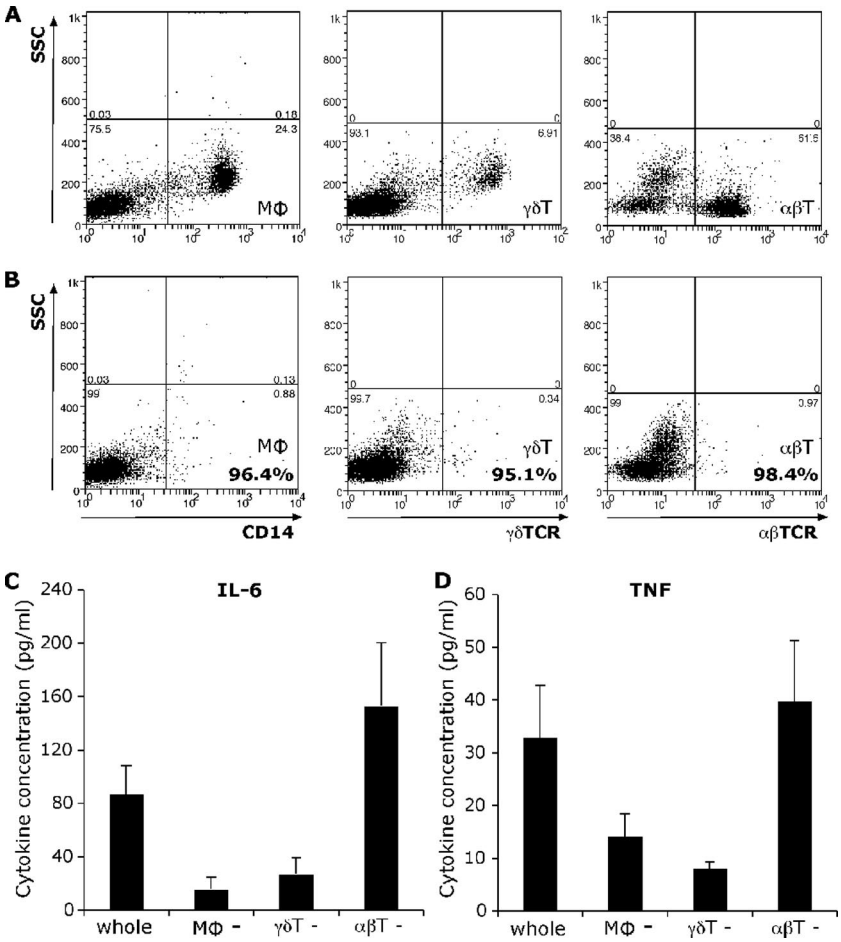

FIG. 4. Effects of selective cell depletion on *P. falciparum*-induced IL-6 and TNF production. (A and B) A total of 2×10^5 whole PBMCs (A) and 2×10^5 monocyte-depleted, γδ-T-cell-depleted, and αβ-T-cell-depleted PBMCs (B) were cultured in the presence of 6×10^5 iRBCs. Supernatants were harvested and assayed for IL-6 and TNF by CBA. (C and D) Mean IL-6 (C) and TNF (D) levels detected in iRBC-treated supernatants from whole and depleted PBMCs. The error bars indicate the standard error of the mean for nine individuals. SSC, side scatter; MΦ, macrophage; γδT, γδ-T cells; αβT, αβ-T cells; whole, whole PBMCs; MΦ−, macrophage depleted; γδT−, γδ-T cell depleted; αβT−, αβ-T cell depleted.

indicator and/or mediator in malaria-exposed children of this age, while TNF and IFN-γ might effectively control parasite replication and thereby reducing the incidence of clinical episodes.

IL-6 is produced mainly by monocytes/macrophages, but it can also be produced by T cells, endothelial cells, fibroblasts, epithelia, neutrophils, microglia, and astrocytes (17, 42) and, like IL-1 and TNF, is involved in induction of fever and the acute-phase response (42, 52). In this in vitro study of PBMC responses, depletion of monocytes/macrophages abrogated IL-6 production, suggesting that monocytes/macrophages are either a predominant source of *P. falciparum*-induced early IL-6 or are required for production of IL-6 by other cells. Depletion of γδ-T cells also reduced IL-6 production, suggesting that these cells are also involved in the production of *P. falciparum*-induced IL-6. Consistent with previous studies using naïve adult PBMCs (16, 47), γδ-T cells together with monocytes/macrophages appeared to be important for *P. falciparum*-induced TNF production in PBMCs from the malaria-exposed PNG children. However, based on the results of depletion studies alone, the possibility that these cells are simply required for TNF production as antigen-presenting cells cannot be ruled out. Interestingly, depletion of αβ-T cells did not appear to reduce *P. falciparum*-induced production of IL-6 and TNF in PBMCs from malaria-exposed children in this study. This suggests that the early production of these cytokines may be more dependent on innate cells, such as monocytes/macrophages and γδ-T cells, and may not have the same αβ-T-cell component as the production of IFN-γ (10).

Previous studies (20, 33) have detected increased plasma IL-6 levels in febrile, parasitemic individuals, and an increased plasma IL-6 level was also associated with fatal outcomes (7, 18). The observed association of IL-6 with increased risk of symptomatic malaria may therefore primarily reflect a role in the regulation or exacerbation of fever. However, IL-1, TNF, and IFN-γ can all induce IL-6 production (53), and in turn, IL-6 can regulate TNF and IFN-γ production (12). Thus, it is also possible that IL-6 hinders control of parasite density by downregulating TNF and IFN-γ pathways, thereby also increasing the risk of moderate-density infection and the devel-

opment of symptomatic malaria. Furthermore, IL-6 has been demonstrated to play a major role in T-cell activation by allowing effector T cells to overcome suppression mediated by T regulatory cells (Tregs) (36). IL-6 is potent in suppressing the TGF-β-driven induction of Foxp3 in naïve T cells and the differentiation of Th-17 cells and the strong induction of IL-17 (4). Thus, it is important for determining whether the immune response is dominated by proinflammatory Th-17 cells or protective Tregs. *P. falciparum*-induced IL-6 might therefore increase the risk of clinical malaria episodes by inhibiting the protective effects of Tregs and promoting the strongly proinflammatory Th-17 response.

TNF is a major effector cytokine and is implicated in mediating both protection and pathogenicity during malaria infection (42). In this study, the observed association of TNF with reduced risk of symptomatic malaria supports the hypothesis that TNF has a role in limiting parasite density and providing protection from mild symptomatic malaria episodes. Similarly, several previous studies have reported an association between a high level of TNF production and parasite clearance (21, 22, 32), and the percentage of *P. falciparum*-specific T cells expressing TNF was associated with a reduced rate of reinfection in a cohort of malaria-exposed children in Gabon (40). Moderate, rapid *P. falciparum*-induced TNF production could mediate protective effects through increased expression of Fc receptors and macrophage phagocytosis (28). TNF also primes neutrophils (52), regulates macrophage IL-12 production, and is a cofactor for IL-12-induced IFN-γ production (28). However, high plasma TNF levels have also been associated with clinical episodes (33) and severity of disease (2, 7, 15, 20, 24, 27, 35, 39, 44). It is not clear if these associations result from a harmful effect of TNF directly or from its ability to stimulate the production of other factors, such as IFN-γ, IL-1, IL-6, nitric oxide, and reactive oxygen intermediates (6, 42). The triggers and cellular sources of TNF, the presence of other cytokines (such as IL-10, IL-4, and TGF-β), and the host genotype are likely to influence the biological role of this cytokine during malaria infection.

All *P. falciparum*-induced cytokine responses were significantly positively correlated with each other, as previously reported (7, 8), and it is possible that there might be synergy between certain cytokines, leading to greater biological effects (7). In particular, production of IFN-γ and TNF are highly correlated, and these cytokines appear to be broadly interchangeable as predictors of reduced risk in the multicytokine models. This suggests that there may be a close relationship with regard to their regulation and effector mechanisms. We have previously shown that in the majority of children, γδ-T cells and αβ-T cells produce *P. falciparum*-specific IFN-γ, with minor contributions from NK cells and other cells (10). The TNF and IL-6 responses observed here also appear to depend, at least in part, on γδ-T cells, as well as monocytes/macrophages. Taken together, these findings suggest a key role for cells of the innate, intermediate, and adaptive arms of the immune system and, in particular, an important role for γδ-T cells in the early cellular immune response to *P. falciparum* in a cohort of children who are in the process of acquiring immunity. In addition, there was a demonstrated age-dependent increase in early PBMC production of IL-2, IL-4, TNF, and IFN-γ. This finding is consistent with several previous studies (8, 41) and suggests that early cytokine responses appear to mature or strengthen with age and might therefore play an important role in the age-dependent acquisition of immunity to malaria. The associations of TNF and IL-6 with the incidence of clinical malaria were influenced by adjustment for age and cumulative exposure to *P. falciparum*, but, importantly, the effects did not disappear, suggesting that these responses do not merely reflect age and exposure.

Early immune effector mechanisms have been proposed to underlie density-dependent regulation of parasitemia in individuals repeatedly exposed to malaria infection (5), leading to oscillation of peripheral parasite densities between a lower level (at which early effector mechanisms are not induced) and a higher level (at which these mechanisms are triggered) (49). The fact that low-density *P. falciparum* infections at baseline did not appear to influence the subsequent ability of these cells to respond to stimulation in vitro support this hypothesis. Furthermore, increased levels of TNF relative to IL-6 levels were observed to be strongly associated with decreased risk of high-density clinical episodes and modestly associated with decreased risk of moderate-density *P. falciparum* reinfections but not to be associated with reinfection per se. Similarly, we previously reported that a high level of IFN-γ production was associated with protection against high-density symptomatic *P. falciparum* episodes, as well as moderate-density *P. falciparum* infections, but not with protection against low-density infections (10). Taken together, these associations are consistent with the fact that the corresponding in vivo cellular immune responses may have density-dependent activation thresholds, serving to regulate parasite densities and progression to symptomatic episodes.

In conclusion, this study demonstrates that early cellular cytokine responses to live, intact *P. falciparum* may be important determinants of subsequent risk of clinical episodes in a cohort of PNG children who are in the process of acquiring clinical immunity to *P. falciparum*. In response to *P. falciparum*, children able to produce increased amounts of TNF and IFN-γ relative to the amount of IL-6 experienced a reduced incidence of *P. falciparum* clinical episodes, implicating TNF and IFN-γ as correlates of protection and IL-6 as a correlate of risk. These results support the hypothesis that cytokines such as TNF and IFN-γ have a role in the regulation of parasite densities and that IL-6 has a role as an important febrile mediator. Incorporation of autologous plasma into the cellular assays described here may provide interesting insights into the cooperation of humoral and cellular immune responses in association with clinical outcomes. Both γδ-T cells and monocytes/macrophages were shown to be important for *P. falciparum*-induced TNF and IL-6 production in these malaria-exposed children. Further characterization of the cellular source of these cytokines in malaria-exposed children and their tight regulation during *P. falciparum* infection is also likely to yield useful insights. Similarly, it will be important to assess these early to intermediate cellular responses in younger children who have not yet begun acquiring significant clinical immunity to *P. falciparum* malaria, especially considering that such children will be the target of future vaccine interventions.

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