Association of early interferon-γ production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children

MAJOR ARTICLE

Association of Early Interferon-γ Production with Immunity to Clinical Malaria: A Longitudinal Study among Papua New Guinean Children

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Background. Elucidating the cellular and molecular basis of naturally acquired immunity to *Plasmodium falciparum* infection would assist in developing a rationally based malaria vaccine. Innate, intermediate, and adaptive immune mechanisms are all likely to contribute to immunity. Interferon-γ (IFN-γ) has been implicated in both protection against and the pathogenesis of malaria in humans. In addition, considerable heterogeneity exists among rapid IFN-γ responses to *P. falciparum* in malaria-naive donors. The question remains whether similar heterogeneity is observed in malaria-exposed individuals and whether high, medium, or low IFN-γ responsiveness is differentially associated with protective immunity or morbidity.

Methods. A 6-month longitudinal cohort study involving 206 school-aged Papua New Guinean children was performed. Peripheral blood mononuclear cells collected at baseline were exposed to live *P. falciparum*–infected erythrocytes. Early IFN-γ responses were measured, and IFN-γ–expressing cells were characterized by flow cytometry. IFN-γ responsiveness was then tested for associations with parasitological and clinical outcome variables.

Results. Malaria-specific heterogeneity in early IFN-γ responsiveness was observed among children. High-level early IFN-γ responses were associated with protection from high-density and clinical *P. falciparum* infections. Parasite-induced early IFN-γ was predominantly derived from γδ T cells (68% of which expressed the natural killer marker CD56) and αβ T cells, whereas natural killer cells and other cells made only minor contributions. The expression of CD56 in malaria-responsive, IFN-γ–expressing γδ T cells correlated with IFN-γ responsiveness.

Conclusions. High, early IFN-γ production by live parasite–stimulated peripheral blood mononuclear cells is a correlate of immunity to symptomatic malaria in Papua New Guinean children, and natural killer–like γδ T cells may contribute to protection.

Natural immunity to malaria is eventually acquired in individuals who reside in regions of high endemicity [1], which provides the fundamental rationale for a malaria vaccine. The mechanisms of protective immunity remain unclear but are thought to involve antibody-dependent and T cell–dependent acquired immune mechanisms [2]. Activation of natural killer (NK) receptor– and γδ T cell receptor–dependent innate and intermediate immune mechanisms may also contribute to immunity to malaria, largely through rapid production of the proinflammatory cytokine IFN-γ [3].

IFN-γ regulates several hundred genes that are associated with immune system functions and is a hallmark Th1 cytokine. Rapid production of IFN-γ by innate and intermediate cells is therefore likely to direct downstream adaptive Th1 responses. During *Plasmodium falciparum* malaria infection, IFN-γ is proposed to directly mediate both antiparasitic and immunopathogenic effects in humans [4–7]. Understanding the basis for the dual role of this cytokine is therefore likely to be important for the development of novel therapeutics and vaccine-based interventions.

We have previously reported considerable heterogeneity in rapid IFN-γ responses to live *P. falciparum* parasites among PBMCs from malaria-naive donors [8]. This raises the question whether similar hetero-

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geneity is observed in malaria-exposed individuals and, accordingly, whether high, medium, or low IFN-γ responsiveness is differentially associated with protective immunity or pathogenic disease outcomes. To investigate this, we performed immunological assays in the context of a longitudinal cohort study involving 206 primary school–aged Papua New Guinean (PNG) children who were intensively observed for 6 months. Children of this age who reside in high-transmission areas are classified as semi-immune to *P. falciparum,* because they are thought to have acquired immunity to life-threatening severe malaria but are still susceptible to uncomplicated, symptomatic morbid episodes [1, 9]. The basic longitudinal study design, together with parasitological and clinical outcome variables, has been reported elsewhere [10] and was considered to provide an ideal methodology for the assessment of immunological parameters in relation to risk [11]. In the present study, we characterized the innate, intermediate, and adaptive cellular sources of early IFN-γ in semi-immune PNG children and tested for associations of early IFN-γ responses with protective immunity or risk of symptomatic *P. falciparum* morbid episode.

STUDY SUBJECTS, MATERIALS, AND METHODS

Study area, subjects, and design. A detailed description of the study area, subjects, and design was reported elsewhere [10]. A total of 206 children (age, 5–14 years) were enrolled from Mugil and Megiar Elementary Schools and Megiar Primary School in Madang Province, Papua New Guinea. All Mugil and Megiar Elementary School students who agreed to participate and a small number of first-grade Megiar Primary School children were enrolled. The sites were chosen on the basis of exclusive access to the Mugil Health Centre, which enabled a tight passive case detection system, as described elsewhere [10]. At baseline, 10 mL of peripheral blood was collected from each subject, and PBMCs were prepared. All participants were treated with 7-day artesunate monotherapy to clear existing malaria. After baseline, subjects were actively followed-up for 6 months for reinfection with malaria, which involved a clinical examination every 2 weeks, preparation of blood films, and collection of finger-prick blood samples to detect parasites by the species-specific PCR-based ligase detection reaction/fluorescent microsphere assay [10, 12]. Children with signs or symptoms of malaria were taken to the Mugil Health Centre, where they received a diagnosis and were treated. Clinical malaria was defined as an axillary temperature of >37.5°C (i.e., fever) and ⩾5000 *P. falciparum* parasites per μL of blood [13]. This study was approved by the institutional review boards of the PNG Medical Research Advisory Council (Madang, PNG) and The Walter and Eliza Hall Institute (Parkville, Australia) in accordance with the Helsinki Declaration [14].

PBMC preparation. Blood collected at baseline was diluted 1:1 in PBS, and PBMCs were separated by density centrifugation with Ficoll-Paque PLUS (Amersham). PBMCs were washed, resuspended at 1×10^7 cells/mL in 80% fetal bovine serum and 20% dimethyl sulfoxide, frozen to −80°C at 1°C per min in freezing containers (Nalgene), and transferred to liquid N_2.

***Cultivation of* P. falciparum.** *P. falciparum* (3D7) was cultivated at 37°C with 5% carbon dioxide, 1% oxygen, and 1% nitrogen at 4% hematocrit with use of O^+ human erythrocytes (Australian Red Cross Blood Service) in RPMI 1640 with 25 mM HEPES, 0.5% Albumax II, 2 mg/mL glucose, 28 mM sodium bicarbonate, 25 mg/mL gentamycin, and 100 μg/mL hypoxanthine. Sorbitol-synchronized, knob-selected, and *Mycoplasma*-negative live *P. falciparum* schizont-infected RBCs (iRBCs) were purified by magnetic cell sorting CS columns (Miltenyi Biotech).

IFN-γ induction assay. IFN-γ assays were done after completion of fieldwork, and investigators were blind to field outcomes. PBMCs were thawed, diluted 1:10 with complete medium (RPMI 1640, 5% heat-inactivated fetal blood serum, 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin), and pelleted (770 *g* for 10 min). PBMCs were washed twice, counted in Turk's solution (Merck) and Trypan Blue (Sigma), and aliquotted into U-bottom 96-well plates (2×10^5 cells/well; 100 μL). Subsequently, 100 μL of purified iRBCs and autologous uninfected erythrocytes (uRBCs) were added at a ratio of 3 iRBCs or uRBCs per PBMC. We used 100 μL of 4% phytohemagglutinin (PHA; Gibco) as a positive control for viability. Cultures were incubated for 72 h (at 37°C in 5% CO_2) to capture early responses from innate, intermediate, and adaptive immune cells. Golgistop (Pharmingen) was added for the last 4 h of incubation. Supernatants and cells were then harvested for IFN-γ analysis.

Detection of IFN-γ. After incubation of PBMCs with iRBCs, uRBCs, and PHA, IFN-γ was measured in supernatants with use of the BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (CBA). Samples were analyzed by FACSCalibur flow cytometry, and data analysis was performed with FCAPArray software (BD Biosciences). IFN-γ was also detected by sandwich ELISA and was normalized to CBA data for comparative analysis. Anti-human IFN-γ (NIB42) mAb was used for capture, and biotinylated anti-human IFN-γ (4S.B3) was used for detection (BD Biosciences). uRBC-induced IFN-γ was subtracted from iRBC- and PHA-induced IFN-γ.

Characterization of IFN-γ–expressing cells by flow cytometry. Intracellular IFN-γ was routinely undetectable in PBMCs yielding responses <3000 pg/mL. When PBMCs yielded ⩾3000 pg/mL, cells were stained for intracellular IFN-γ and various surface markers and were analyzed by flow cytometry. Anti–T cell receptor (TCR)-γ/δ fluorescein isothiocyanate, CD3 allophycocyanin, CD56 biotin, IFN-γ phycoerythrin, and $IgG_1\gamma$

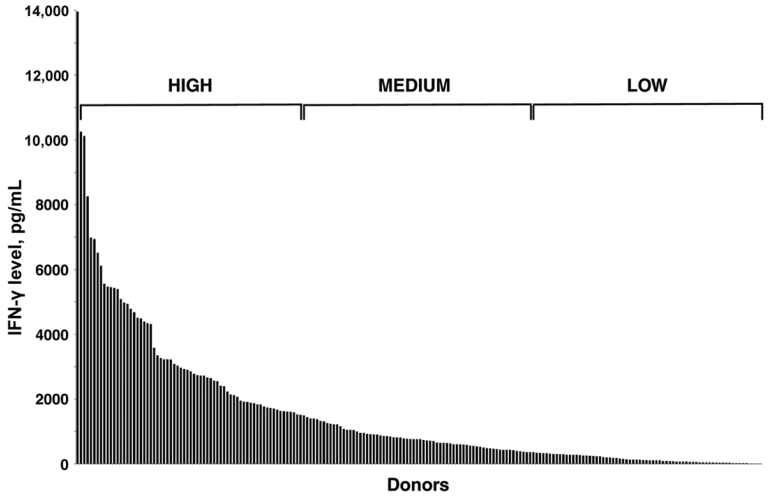

Figure 1. Plot indicating heterogeneity among semi-immune Papua New Guinean children in the early IFN-γ response of PBMCs to *Plasmodium falciparum* schizont-infected RBCs (iRBCs). The level of IFN-γ detected in supernatants after 72-h culture of 2×10^5 PBMCs with 6×10^5 iRBCs is shown. Children were classified in terciles as high (1495–13,943 pg/mL, medium (344–1494 pg/mL), or low (0–343 pg/mL) IFN-γ responders.

isotype control phycoerythrin were used for surface, intracellular, or isotype control staining (BD Biosciences). PBMCs were surface stained for 40 min, with a second 40-min incubation for biotin-labeled antibodies with PerCP-Cy-5.5–labeled streptavidin. PBMCs were then stained for intracellular IFN-γ with use of the BD Cytofix/Cytoperm kit (BD Biosciences). Cells were analyzed by FACSCalibur flow cytometry. Dead cells were excluded by forward/side scatter, and 100,000 live events were collected. Data analysis was performed with Weasel software, version 2.2.3.

Statistical analyses. IFN-γ values were $\log_{10}$ transformed, and geometric mean levels of IFN-γ production were compared by Student's *t* test. For analyses of association with risk of infection and disease, children were stratified into terciles of comparable size on the basis of the magnitude of IFN-γ production. For age associations, cutoff values were set at the median age of 9 years. The effect of IFN-γ production on incidence of disease was assessed by Poisson regression, whereas effects on time to first infection were assessed by Cox regression, as described elsewhere [10]. For Cox regression, children were considered to be at risk until they missed 2 follow-up visits, were re-treated, or withdrew [10]. For Poisson regression, children were considered to be at risk until they withdrew or completed follow-up [10]. Estimates of the effects on incidence of disease were adjusted for previously identified confounders [10]. Spearman's rank correlations (ρ) were used to assess correlations between IFN-γ heterogeneity and CD56 expression on IFN-γ–expressing γδ T cells or resting frequencies of γδ T cell, NK cell, αβ T cell ($CD3^+$ γδ TCR^- cell), or other cell populations.

RESULTS

***Reinfection with* P. falciparum.** The epidemiology, population characteristics, and incidences of *P. falciparum* infection and disease have been reported in detail elsewhere [10]. Twelve children were excluded from reinfection analysis because of suspected *P. falciparum* treatment failure [10]. During the 6-month follow-up period, 185 (95.4%) of 194 children were reinfected with *P. falciparum,* as diagnosed by ligase detection reaction/fluorescent microsphere assay [10]. Of the 206 study children, 80 (38.8%) experienced at least 1 symptomatic *P. falciparum* episode [10]. The high rates of reinfection and clinical episodes, together with clear age-dependent acquisition of clinical immunity to *P. falciparum* observed in the cohort [10], result in a study that is well suited to investigate associations of immunological parameters with risk of infection and/or morbid episodes.

Heterogeneity in* P. falciparum*–induced IFN-γ responsiveness among semi-immune PNG children. PBMCs from all children produced IFN-γ in response to PHA, which demonstrated that all samples were viable. After stimulation of PBMCs with iRBCs, heterogeneity in IFN-γ responsiveness was observed among children, enabling stratification into high (1495–13,943 pg/mL), medium (344–1494 pg/mL), or low (0–343 pg/mL) responders (figure 1). Importantly, this heterogeneity was malaria specific (figure 2; coefficient of variance of $\log_{10}$-transformed responses, 0.29 iRBCs vs. 0.08 PHA). In addition, iRBC- and PHA-elicited IFN-γ responses increased significantly with age ($P < .001$) (figure 2). uRBC-induced IFN-γ was negligible (median value, 11.3 pg/mL; interquartile range,

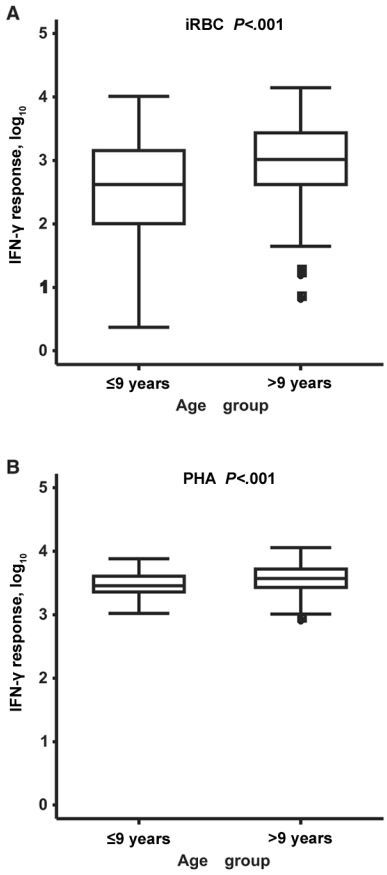

Figure 2. Box plots indicating that heterogeneity in IFN-γ responsiveness among semi-immune Papua New Guinean children is malaria specific. Samples consisting of 2×10^5 PBMCs were cultured for 72 h in the presence of 6×10^5 *Plasmodium falciparum* schizont-infected RBCs (iRBCs) or 2% phytohemagglutinin (PHA). IFN-γ was then detected in supernatants. The whisker box plots illustrate a high degree of variation in iRBC-induced IFN-γ responses *(A)*, but not in PHA-induced IFN-γ responses *(B)*. The level of IFN-γ produced in response to iRBCs and PHA increases with age ($P < .001$, by Student's *t* test).

0–76.5 pg/mL) compared with iRBC-induced IFN-γ (median value, 751.4 pg/mL; interquartile range, 244.4–1953.3 pg/mL).

Higher IFN-γ responses are associated with protection from clinical and high-density* P. falciparum *infections. Because there was significant heterogeneity in malaria-specific IFN-γ responsiveness, we sought to determine whether this was associated with altered risk of clinical malaria. Children who experienced clinical episodes had significantly lower IFN-γ responses at baseline than did those who did not (geometric mean response, 359 pg/mL vs. 686 pg/mL; $P = .01$). Children with low IFN-γ responses thus had a significantly higher risk of symptomatic malaria, compared with medium and high responders (incidence rate ratio, 1.85; 95% CI, 1.25–2.74; $P = .002$). There was however no difference in risk between medium and high IFN-γ responders ($P = .31$). The increased risk of illness in low responders remained statistically significant even with adjustment for age (incidence rate ratio, 1.61; 95% CI, 1.07–2.42; $P = .02$). This is equivalent to an age-adjusted 30% reduction (incidence rate ratio, 0.70; 95% CI, 0.51–0.94; $P = .02$) in risk of clinical disease with a 10-fold increase in IFN-γ production in children with an IFN-γ response of ⩽1000 pg/mL, with no further significant reduction in risk in children with responses >1000 pg/mL.

We then examined whether IFN-γ responses were associated with a reduced risk of acquiring a new *P. falciparum* infection that exceeded a density threshold. Accordingly, there was a significantly reduced risk of experiencing a moderate-density infection (>500 parasites per μL of blood; hazard ratio, 0.63; $P = .03$) and high-density infection (⩾5000 parasites per μL of blood; hazard ratio, 0.47; $P = .008$) but not low-density infection (<500 parasites per μL of blood) in children with high IFN-γ responses, compared with the risk in children with low IFN-γ responses (figure 3). Children with medium IFN-γ responses were not statistically significantly protected against low-density and moderate-density infections, but they tended to have a lower risk for high-density infections (hazard ratio, 0.64; $P = .09$). The association of high IFN-γ responses with protection against high-density infection remained borderline statistically significant even with adjustment for age (hazard ratio, 0.57; $P = .06$) (figure 3). Importantly, unlike iRBC-induced IFN-γ responses, there were no statistically significant associ-

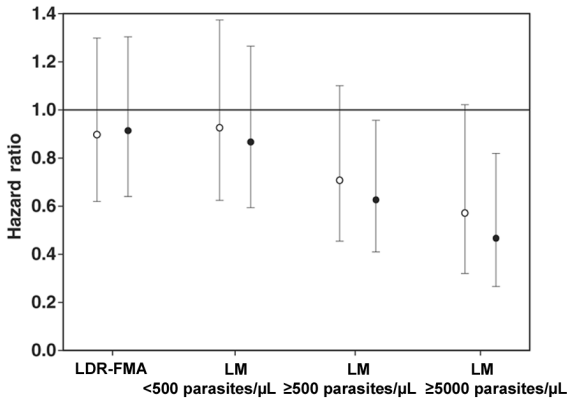

Figure 3. Plots indicating that children with high IFN-γ responses have a lower risk of experiencing a high-density and clinical episode of *Plasmodium falciparum* infection. Hazard ratios and 95% CIs for high-tercile versus low-tercile IFN-γ responders from Cox regression of time to first *P. falciparum* infection above variable parasite density thresholds (adjusted for known spatial confounder [10]) are shown. Parasite density is given per μL of blood. Filled circles indicate unadjusted values; open circles indicate values adjusted for age effect. Hazard ratio was defined as the ratio of hazard of having a *P. falciparum* infection versus high IFN-γ responsiveness. LDR-FMA, parasites detected by the PCR-based ligase detection reaction/fluorescent microsphere assay [12]; LM, parasites detected by light microscopy.

ations between PHA-induced IFN-γ responses and risk of malaria infections (at any density) or disease, which indicates that the associations between iRBC-induced IFN-γ and protection from clinical and high-density infections are malaria specific.

***γδ T cells and αβ T cells predominate over NK cells in the early IFN-γ response to* P. falciparum.** We have shown elsewhere that the predominant early IFN-γ–expressing cells in the majority of malaria-naive individuals are γδ T cells, with minor contributions made by NK cells and naive αβ T cells [8]. Importantly, γδ T cells also appear to be fundamental to early IFN-γ production in semi-immune PNG children (figure 4). Across all of the children analyzed, γδ T cells and αβ T cells comprised a mean of 42.4% and 33.5% of the total IFN-γ–expressing cell population, respectively, whereas NK cells and other undefined cells comprised only 14.7% and 9.4%, respectively. When individual responses from each child were examined (figure 4), γδ T cells and αβ T cells were the predominant cellular sources of IFN-γ (constituting ⩾50% of the total IFN-γ–expressing cells) in 14 (48.3%) and 13 (44.8%) of the 29 children analyzed, respectively. NK cells predominated in only 2 (6.9%) of 29 children (figure 4).

In addition, we have previously shown that the majority of malaria-responsive γδ T cells from malaria-naive donors express NK receptors [8]. CD56 (a pan–NK cell marker) was

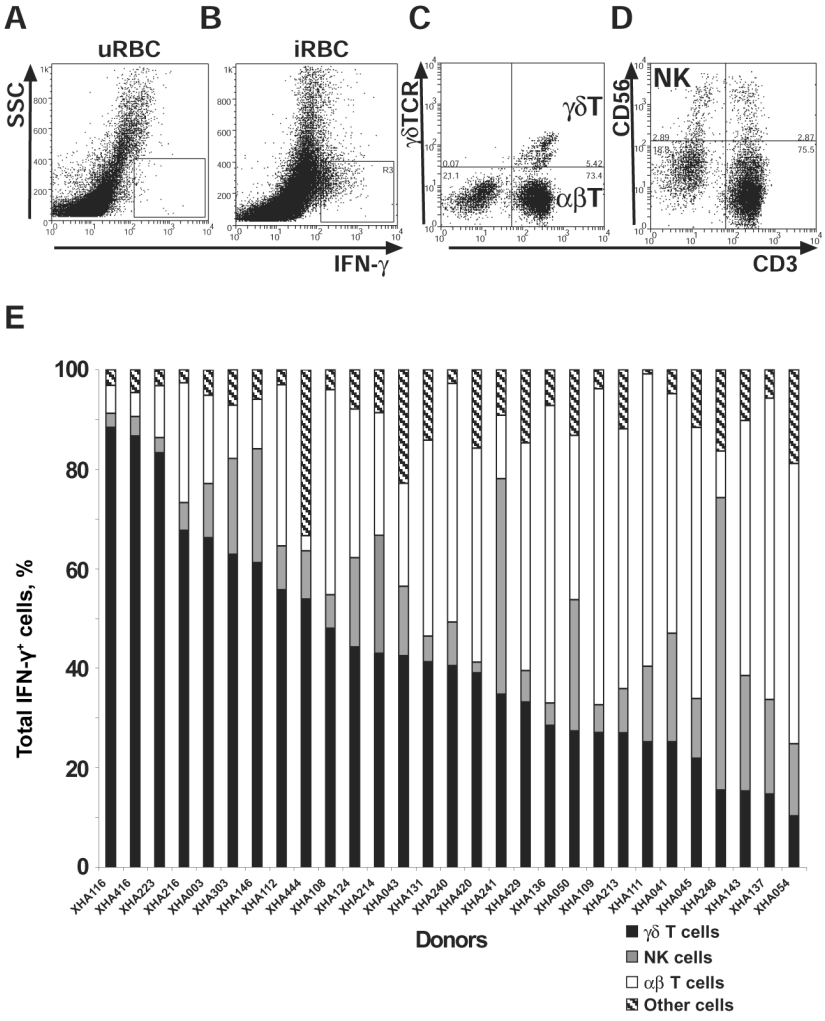

Figure 4. Phenotypic characterization of the cellular sources of early IFN-γ after stimulation of PBMCs from semi-immune Papua New Guinean children with *Plasmodium falciparum* schizont-infected RBCs (iRBCs). Samples consisting of 2×10^5 PBMCs were cultured for 72 h in the presence of 6×10^5 autologous uninfected erythrocytes (uRBCs) or 6×10^5 iRBCs, were harvested, and were stained for intracellular IFN-γ, CD3, γδ T cell receptor (TCR), and CD56. An appropriate isotype-matched control was included to define the IFN-γ$^+$ cell gate. No IFN-γ$^+$ cells were detected in uRBC-treated PBMCs *(A).* IFN-γ$^+$ cells were gated *(B).* Dead cells were gated out by forward and side scatter (SSC), and live γδ T cell and αβ T cell populations *(C)* and natural killer (NK) cell populations *(D)* were defined using quadrants, as shown. The percentage contribution of γδ T cells, NK cells, αβ T cells, and other cells to the total IFN-γ$^+$ cell population was determined *(E).* All plots shown in panel E were derived from iRBC treated PBMCs *(B).*

included in the phenotypic analysis, and a mean of 68% of the IFN-γ–expressing γδ T cells from the semi-immune PNG children expressed this marker (figure 5). A mean of 18% of the IFN-γ–expressing αβ T cells expressed CD56. Importantly, as for malaria-naive adults [8], resting frequencies of γδ T cells, NK cells, αβ-T cells, or other cells did not correlate with the overall level of IFN-γ produced. Interestingly, however, there was a weak positive correlation ($\rho = 0.39$; $P = .03$) between the level of IFN-γ secretion and differential expression of CD56 on activated IFN-γ–expressing γδ T cells, suggesting that this NK receptor may be partially involved in determining the level of the early IFN-γ response to iRBCs and, thus, protection from clinical and high-density *P. falciparum* infection.

DISCUSSION

In contrast with late IFN-γ responses (i.e., >120 h coculture), which reflect adaptive immunity [4, 15, 16], early IFN-γ responses to *P. falciparum* (i.e., <72 h coculture) encompass cellular responses from innate, intermediate, and adaptive arms of the immune system [8, 17, 18]. It has been proposed that early IFN-γ responses to *P. falciparum* contribute to protection and pathogenesis [19, 20]. However, *P. falciparum*–induced early IFN-γ production has not been assessed in relation to risk. Longitudinal cohort studies are powerful tools for associating immune end points with disease outcomes. Therefore, to define a correlate of risk or correlate of immunity, we coupled a robust immunological assay of whole parasites and PBMCs with a prospective treatment reinfection study that intensively observed 206 semi-immune PNG children for 6 months [10].

PBMCs were collected from each child at baseline and were stimulated with live iRBCs, and immune responses were measured. Like malaria-naive adults [8], semi-immune children exhibited heterogeneity in early IFN-γ responses. This heterogeneity was malaria specific. Children with low IFN-γ responses were significantly more likely to experience clinical episodes of *P. falciparum* infection than were other children, whereas high responders showed protection against moderate-density and high-density *P. falciparum* infections but not against low-density infections. IFN-γ, triggered by newly established blood stage infections, may assist in limiting the replication rate of parasites and in keeping densities below pyrogenic thresholds. With adjustment for differences in risk with age, high levels of IFN-γ remained associated with a decrease in the risk of high-density and clinical infections, which indicates that early IFN-γ production by PBMCs stimulated with live parasites provides a true correlate of protection from symptomatic morbid episodes associated with high-density parasitaemia in semi-immune children.

The cellular sources of IFN-γ are remarkably similar between malaria-naive adults and semi-immune children. As has been

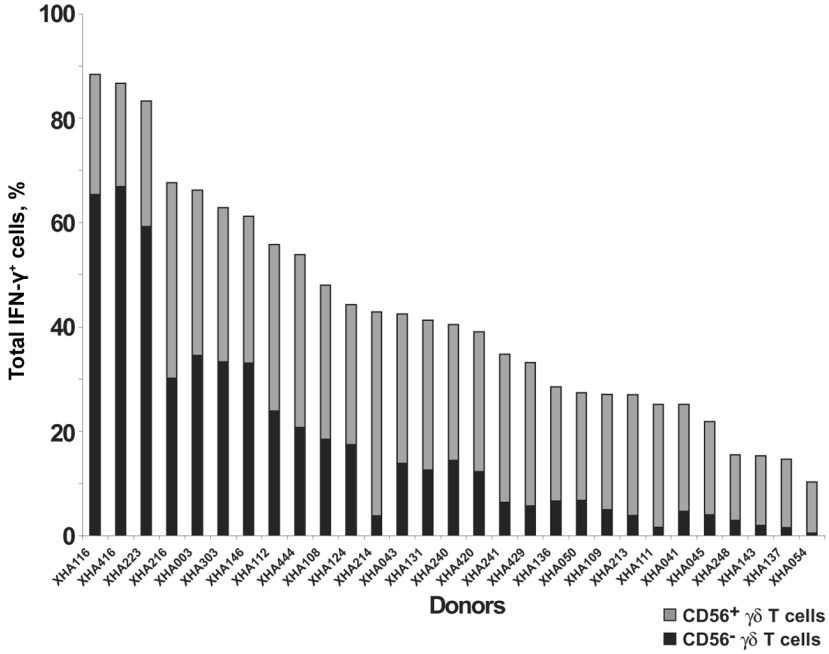

Figure 5. Graph indicating that the majority of the malaria-responsive γδ T cells express the natural killer receptor CD56. Samples consisting of 2×10^5 PBMCs were cultured for 72 h in the presence of 6×10^5 autologous uninfected erythrocytes (uRBCs) or 6×10^5 *Plasmodium falciparum* schizont-infected RBCs (iRBCs). PBMCs were harvested and stained for intracellular IFN-γ, CD3, γδT cell receptor (TCR), and CD56. An appropriate isotype-matched control was included to define the IFN-γ$^+$ cell gate. IFN-γ$^+$ cells were gated as shown in figure 4, and the percentage contribution of CD56$^+$γδTCR$^+$ γδ T cells and CD56$^-$ γδTCR$^+$ γδ T cells to the total IFN-γ$^+$ cell population was determined.

reported elsewhere, γδ T cells represent the major cellular source of innate IFN-γ in malaria-naive individuals, with minor contributions from NK cells and αβ T cells [8, 18]. Interestingly, semi-immune PNG children and malaria-naive adults exhibit very similar low frequencies of NK cell–dominant IFN-γ responses (6.9% and 6.7%, respectively), suggesting a minor role for peripheral NK cells in the innate IFN-γ response to *P. falciparum.* Conversely, both malaria-naive adults and a large proportion of semi-immune children exhibit predominant γδ T cell responses. Thus, these intermediate cells may remain important throughout the acquisition of immunity to *P. falciparum.* Peripheral blood γδ T cells possess a junctionally diverse TCR that requires gene rearrangement, but these cells can respond to antigens independent of major histocompatibility complex presentation [21]. γδ T cells are, therefore, described as being at the interface between innate and adaptive immune systems. During acute *P. falciparum* infections, γδ T cells upregulate activation markers and increase in the circulation [22–25]. γδ T cells are also cytotoxic for *P. falciparum* merozoites in vitro [26, 27]. Furthermore, γδ T cells express CD45 RO and possess a memory phenotype [21]. Thus, some of the responding γδ T cells from the semi-immune children may represent malaria-specific memory γδ T cells.

The majority of IFN-γ–expressing γδ T cells from malaria-naive individuals express NK receptors [8]. In the *Plasmodium berghei* murine malaria model, innate IFN-γ production and both pathogenesis and protection are controlled by cells expressing NK complex–encoded receptors [28–30]. A mean of 68% of IFN-γ–expressing γδ T cells from the semi-immune children expressed the pan NK marker CD56. The frequency of circulating γδ T cells expressing CD56 and CD57 is elevated in cases of *P. falciparum* infection [31]. Furthermore, expression of CD56 on malaria-responsive IFN-γ–expressing γδ T cells correlated with the magnitude of IFN-γ responses in semi-immune children. Similarly, in malaria-naive individuals, expression of the NK complex–encoded receptor CD94 correlated with IFN-γ output [8]. Thus, NK receptor expression on NK-like γδ T cells may partially control IFN-γ responsiveness to *P. falciparum,* as does NK complex expression in *P. berghei* murine malaria [28, 29].

As expected, the contribution of αβ T cells to early IFN-γ production was greater in semi-immune children than it was in malaria-naive adults [8]. Antigen-specific αβ T cells from exposed individuals mount IFN-γ recall responses [32]. The αβ T cell responses observed here likely constitute rapid activation of malaria-specific memory αβ T cells. Antigen-specific $CD4^+$ T cells help antibody production [2]. In addition, evidence from humans [33] and mice [34] suggests that antibody-independent $CD4^+$ T cells may be important in immunity to malaria, although the mechanisms remain obscure. Interactions among innate, intermediate, and adaptive arms of the immune system may be important for protection against *P. falciparum,* because contributions from γδ T cells, αβ T cells, and (to a small extent) NK cells were observed in our study.

Innate and intermediate immune cells, such as NK cells and γδ T cells, represent the first line of defense and are able to shape downstream adaptive immune responses. In a study of peptide-specific T cell responses, late IFN-γ production was also associated with reduced incidence and time to reinfection with *P. falciparum* [4]. Plasma IFN-γ concentrations were also lower in children with cerebral malaria than in those with uncomplicated malaria, which is consistent with a protective role for this cytokine [35], and polymorphisms in the IFN-γ receptor 1 gene promoter were associated with protection from cerebral malaria and death [36]. The protective effects of this cytokine may result from increased macrophage activation and enhanced parasite clearance through phagocytosis and production of radicals [37]. Additionally, IFN-γ may induce cytophilic antibodies and enhance opsonization and antibody-dependent cellular cytotoxicity [38].

In summary, we undertook a longitudinal cohort study to investigate associations of early IFN-γ production with protective or pathogenic outcomes of *P. falciparum* infection. We found that high, early IFN-γ production is a correlate of immunity to symptomatic morbid malaria in semi-immune PNG children. IFN-γ was associated with protection from high-density infection but not from low-density infection or from the establishment of infection. γδ T and αβ T cells were the predominant sources of early IFN-γ, suggesting that IFN-γ–associated protection is mediated, in part, by γδ T and αβ T cells. The αβ T cells are likely to be malaria-specific memory T cells, whereas the γδ T cells may be previously unprimed cells, memory cells, or both. CD56 expression on γδ T cells correlated with the magnitude of IFN-γ responses, suggesting that $CD56^+$, NK-like γδ T cells may contribute to protection from uncomplicated, symptomatic malaria in humans.

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