The Journal of Immunology

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J Immunol 2006; 177:5736-5745; ;
doi: 10.4049/jimmunol.177.8.5736
http://www.jimmunol.org/content/177/8/5736

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Innate Immune Responses to Human Malaria: Heterogeneous Cytokine Responses to Blood-Stage *Plasmodium falciparum* Correlate with Parasitological and Clinical Outcomes[1]

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Taking advantage of a sporozoite challenge model established to evaluate the efficacy of new malaria vaccine candidates, we have explored the kinetics of systemic cytokine responses during the prepatent period of *Plasmodium falciparum* infection in 18 unvaccinated, previously malaria-naive subjects, using a highly sensitive, bead-based multiplex assay, and relate these data to peripheral parasite densities as measured by quantitative real-time PCR. These data are complemented with the analysis of cytokine production measured in vitro from whole blood or PBMC, stimulated with *P. falciparum*-infected RBC. We found considerable qualitative and quantitative interindividual variability in the innate responses, with subjects falling into three groups according to the strength of their inflammatory response. One group secreted moderate levels of IFN-γ and IL-10, but no detectable IL-12p70. A second group produced detectable levels of circulating IL-12p70 and developed very high levels of IFN-γ and IL-10. The third group failed to up-regulate any significant proinflammatory responses, but showed the highest levels of TGF-β. Proinflammatory responses were associated with more rapid control of parasite growth but only at the cost of developing clinical symptoms, suggesting that the initial innate response may have far-reaching consequences on disease outcome. Furthermore, the in vitro observations on cytokine kinetics presented here, suggest that intact schizont-stage infected RBC can trigger innate responses before rupture of the infected RBC. *The Journal of Immunology,* **2006, 177: 5736–5745.**

Research on the immunology of malaria infection has been driven by the need to develop a safe and effective vaccine to reduce malaria-associated morbidity and mortality in tropical countries, and has focused on adaptive immunity. By comparison, until recently, there has been rather little work characterizing innate immune responses to malaria, assessing their role in protection or their potential to modulate adaptive responses. Innate immune mechanisms represent the first line of defense against invading pathogens. For severe, acute infections such as malaria, the ability to mount an effective innate response may mean the difference between life and death.

The kinetics of primary malaria infections in mice suggests that innate responses are essential to limit the initial phase of parasite replication (1), and there is evidence from experimental human infections that parasite growth can be modulated very early during primary infections (2). In other experimental protozoal infections, innate responses have been shown to contribute to control of acute infection, synergize with chemotherapeutic agents, and augment partially effective vaccines (3). Thus, given the therapeutic and prophylactic implications, further understanding of the human innate response to malaria, and its role in determining the outcome of infection, is required.

Previous in vitro work has demonstrated that components of blood-stage parasites, including parasite-derived GPI, induce macrophages to produce IL-1β, IL-6, and TNF-α (4–7). IFN-γ is the key inducer of the immune effector mechanisms that are essential for initial control of both pre-erythrocytic and blood-stage malaria infections (8, 9), but there is evidence that IFN-γ levels need to be carefully balanced to avoid immune pathology (10, 11). In vivo, TNF-α production is associated with parasite clearance and resolution of fever (12), but elevated levels of TNF-α (13) and IL-6 (14, 15) have also been associated with cerebral malaria. A priori, one would expect a crucial role for IL-12—and possibly also IL-18—in initiation of the inflammatory cytokine cascade. Accordingly, associations have been reported between circulating levels of IL-12 and IL-18 and risk of severe *Plasmodium falciparum* malaria (14, 16–21), and, in a prospective epidemiological study, IL-12 production was positively associated with IFN-γ and TNF-α production and negatively associated with parasitemia (22). However, although an essential role has been shown for IL-12 in *P. falciparum*-induced IFN-γ production from human PBMC and NK cells (23–25), it has proved very difficult to detect IL-12 in vitro and little is known of its cellular origins. Evidence also suggests

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Received for publication April 28, 2006. Accepted for publication July 31, 2006.

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[1] This work was supported by grants from the Wellcome Trust (to E.M.R. and A.V.S.H.), the United Kingdom Medical Research Council (to E.M.R.) and the Malaria Vaccine Initiative at the Program for Appropriate Technology in Health, Seattle, WA (to A.V.S.H.). J.W. was funded by a Marshall Scholarship from the British Government, J.E.T. was funded by a scholarship from the Gates Malaria Partnership, and A.V.S.H. is a Wellcome Trust Principal Research Fellow.

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0022-1767/06/$02.00

that both TGF-β and IL-10 can be produced very rapidly, from innate sources, during murine malaria infections and are required to down-regulate potentially pathogenic inflammatory responses once parasitemia is brought under control (26–28); however, in both mice and humans, excessive concentrations of TGF-β and IL-10 early in infection inhibit type-1 immune responses and thus facilitate parasite growth (29, 30). Conversely, in clinical human infections, failure to produce sufficient TGF-β or IL-10 is associated with acute (31) and severe malaria (17, 32, 33), and severe malarial anemia (34, 35). Finally, high ratios of IFN-γ, TNF-α, and IL-12 to TGF-β or IL-10 are associated with decreased risk of malaria infection but increased risk of clinical disease in those who do become infected (22).

Although there are consistent themes running through these studies, there are also major gaps in our knowledge, especially with regard to the parasite molecules that induce early immune responses, the nature of the relevant host cells and receptors, and the order and timing of crucial events. Furthermore, it is becoming clear that innate responses can differ between individuals (25, 36) with potential clinical implications. To date, only two—rather small—studies have exploited the unique opportunity offered by experimental infection of malaria-naive volunteers (during the evaluation of antimalarial vaccines) to study the very early stages of the immune response to malaria (37, 38), and it has been difficult to draw any generalizable conclusions from them. Here, we present a detailed longitudinal analysis of 11 different cytokine responses in 18 experimentally infected individuals, using a highly sensitive, bead-based multiplex assay, and we compare these responses with peripheral parasite densities, as measured by quantitative real-time PCR (39). Furthermore, we have compared these findings with in vitro cytokine responses to *P. falciparum* blood-stage parasites in blood from another 11 malaria-naive donors.

Materials and Methods

Subject recruitment and experimental malaria infection

Blood donors for in vitro assays were 11 healthy, malaria-naive adult volunteers recruited at the London School of Hygiene and Tropical Medicine (LSHTM) through a volunteer blood donation system. The studies at LSHTM were approved by the LSHTM Ethical Review Committee, and informed consent was obtained from all volunteers.

Blood was also obtained from 18 healthy unvaccinated, malaria-naive subjects (median age, 22.5 years; range, 19–36 years) recruited in Oxford who were exposed to the bites of five *P. falciparum* (strain 3D7) sporozoite-infected *Anopheles stephensi* mosquitoes (each with 10^2–10^4 sporozoites per salivary gland) in the context of three phase IIa vaccine trials (40, 41, 62). Venous blood was collected on day 0, day 4, and twice daily from day 6.5 until the first microscopic detection of parasitemia, at which time subjects were cured with artemether/lumefantrine. Serum- and platelet-depleted plasma (42) was stored from each sample. Ethical approval was obtained from the Oxfordshire Research Ethics Committee, United Kingdom, and the Human Subjects Protection Committee (Seattle, WA).

P. falciparum *detection by PCR and microscopy*

At least 200 high power fields of a Giemsa-stained thick blood film were examined for malaria parasites by two expert slide readers (40, 41), and quantitative real-time PCR was used to detect *P. falciparum* DNA, as described previously (39). The lower limit of detection of the PCR is 20 parasites per milliliter.

P. falciparum *culture*

P. falciparum parasites (3D7 strain) were cultured in vitro as described previously (24) and were routinely shown to be mycoplasma free by PCR (BioWhittaker). Ring-stage parasites were purified by centrifugation through 5% sorbitol (Sigma-Aldrich), whereas trophozoite and schizont-infected erythrocytes (infected RBC (iRBC)[3]) were harvested from synchronized cultures by centrifugation through Percoll gradients (Sigma-Aldrich). Parasites were washed and resuspended in culture medium. Washed uninfected erythrocytes (uninfected RBC (uRBC)) were used as controls.

In vitro assays

Venous blood was collected into sterile, preservative-free sodium heparin (CP Pharmaceuticals) (10 IU/ml blood). For whole-blood assays, 20 μl of blood was aliquoted into sterile 96-well, round-bottom tissue culture plates (Nalge Nunc International) together with iRBC or uRBC (at final concentrations of 2×10^6, 2×10^4, or 2×10^2 iRBC/ml) or LPS (4 μg/ml; positive control) and diluted to a final volume of 200 μl/well with RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all Invitrogen Life Technologies). Plates were incubated at 37°C with 5% CO_2 for periods of up to 24 h. At each time point, supernatants were collected from one of nine identical plates and frozen at −80°C for cytokine analysis; cell pellets were resuspended in PBS to prepare thin blood smears, which were stained with Giemsa's stain and examined by light microscopy to determine the proportion of ring, trophozoite, and schizont stages in each culture. For PBMC cultures, blood was centrifuged over Histopaque 1077 (Sigma-Aldrich) and PBMC adjusted to a final concentration of 10^5 cells per well; all other aspects of the assay remained the same.

Multiplex analysis of serum cytokine concentration

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, GM-CSF, and TNF-α were assayed using multiplex luminescent beads (Beadlyte Human MultiCytokine Detection System 3; Upstate; for culture supernatants) (Lincoplex; Biogenesis; for serum) according to the manufacturer's instructions and analyzed with a Luminex 100 plate reader (Linco Research). A mixed cytokine mixture and aliquots of four experimental samples were run on each plate to assess interassay variability. Fluorescence intensity was transformed into cytokine concentration using StatLIA software (Brendan Scientific). The lower limit of detection was 3.2 pg/ml for each cytokine; values below this threshold were set to 3.2 pg/ml.

Cytokine ELISA

Spontaneously bioactive and total (latent plus bioactive) TGF-β were measured in platelet-poor plasma, exactly as described previously (30). The detection limit of the assay was 15 pg/ml; extrapolated values below this cut-off were set to 15 pg/ml. IL-1β, IL-6, IFN-γ, and TNF-α were assayed by sandwich ELISA using mAb pairs and standards (all BD Pharmingen), following a protocol described elsewhere (22). Where values exceeded the top of the standard curve, supernatants were retested at 1/10 dilution in RPMI 1640.

Flow cytometry

Heparinized whole blood, diluted 1/5 with serum-free culture medium (43), was incubated for 3–24 h (37°C, 4% CO_2) in the presence of either 1 μg/ml *Escherichia coli* LPS (Sigma-Aldrich), 5 μg/ml PHA (Sigma-Aldrich), 12.5×10^6 iRBC or 12.5×10^6 uRBC; RBC numbers were calculated to give an RBC-to-PBMC ratio of 5:1. Cells were washed with cold PBSA (PBS plus 1% heat-inactivated FCS (HI-FCS; Invitrogen Life Technologies) and 0.1% sodium azide), and incubated (20 min in the dark at 4°C) with fluorochrome-conjugated Abs to CD3 (FITC), CD8 (R-PE), HLA-DR (TRIC), Lin-1-FITC (containing Abs to CD3, CD14, CD16, CD19, CD20, and CD56), IgG1-FITC, IgG2a/κ-TRIC, IgG1-R-PE (all BD Biosciences). Red cells were lysed for 10 min (at room temperature (RT)) in the dark in a 20-fold volume of NH_4Cl (158 mmol/L), $KHCO_3$ (10 mmol/L), and Na_2EDTA (100 μmol/L) (pH 7.6). Cells were washed, fixed (2% paraformaldehyde in PBS (pH 7.5), 15 min in the dark at RT), washed again, and permeabilized in PBS plus 1% HI-FCS plus 0.1% NaN_3 plus 0.3% saponin (Sigma-Aldrich) (pH 7.5; 30 min at RT in the dark) in the presence of anti-IL-12-R-PE (Caltag), anti-IFN-γ-R-PE (BD Biosciences), or IgG1-R-PE isotype control Ab. After a final wash, cells were resuspended in PBSA and analyzed immediately by three-color flow cytometry (FACScan) using CellQuest data analysis software (both BD Biosciences). Gates were set to record events with forward- and side-scatter characteristics of viable cells; 100,000 gated events were analyzed per sample.

[3] Abbreviations used in this paper: iRBC, infected RBC; uRBC, uninfected RBC; RT, room temperature; CI, confidence interval; DC, dendritic cell.

Statistical analysis

Analysis of blood cytokine kinetics was performed using a mixed model with a random effect to allow for clustering of cytokine levels within subjects. Spearman's rank correlation coefficients were calculated on raw data. For in vitro cytokine measurements, values obtained after stimulation with iRBC and uRBC were compared using Wilcoxon's signed rank test. Flow cytometric analyses are expressed as the percentage of positively stained cells; values for cells stained with isotype-matched control Abs have been deducted. Paired *t* tests were used to compare scatter characteristics of IL-12-secreting cells after LPS and iRBC stimulation. Confidence intervals (CIs) were calculated using CIA 2.1 (T. Bryant, Southampton, U.K.).

The number of parasites emerging from the liver into the blood has been estimated as described (44).

Results

The course of blood-stage malaria following experimental sporozoite-induced infection

Eighteen subjects, all of whom were malaria-naive, nonvaccinated controls in three separate *P. falciparum* sporozoite challenge experiments (40, 41, 62), were each exposed to the bites of five infectious mosquitoes on day 0. Blood samples were collected before challenge on day 0, and then on day 4, day 6.5, and hence every 12 h until treatment was given; at each time point a clinical examination was performed, a thick blood film was examined for malaria parasites and quantitative PCR for parasite DNA was performed. The onset of fever (temperature ≥37.5°C or a report of febrile chills) correlated significantly with microscopic detection of parasitemia (correlation coefficient, 0.67; $p = 0.024$).

The time to first positive PCR—and the time at which parasite density first reached ≥50 iRBC/ml—was remarkably consistent among donors, with the mean being 7.6 and 7.9 days, respectively (Table I). Soon thereafter, however, considerable interindividual variability was observed, with maximal recorded parasitemia before treatment ranging from 388 iRBC/ml to 74,071 iRBC/ml. Interestingly, parasite densities oscillated, with peaks every 36 to 48 h, right from the beginning of the blood-stage infection, suggesting very rapid synchronization of parasite growth and sequestration of iRBC (Fig. 1).

Kinetics of the cytokine response to prepatent infection with P. falciparum

Serum from all 18 subjects was tested, by multiplex cytometric bead assay, for IL-2, IL-4, IL-6, IL-8, IL-10, IL-12-p70, IFN-γ, and TNF-α before infection (day 0), and then on day 4, day 6.5, and every 12 h until first detection of parasites by microscopy. In addition, for 12 of these subjects, IL-1β and GM-CSF were assayed by cytometric bead array and TGF-β (latent and bioactive) was measured by ELISA in platelet-depleted plasma. Because there were no significant changes in cytokine levels between days 0 and 4 (data not shown), individual baseline cytokine levels were defined for each cytokine for each volunteer, calculated as the mean of the day 0 and day 4 concentrations. An increase of at least 2-fold over the individual baseline value was regarded as significant up-regulation.

Table I. *Development of asexual P. falciparum parasitemia (as assessed by PCR) following sporozoite infection of 18 human volunteers*[a]

	Average	95% CI
First positive PCR (any value) (day)	7.61	7.26–7.96
First time >50 parasites/ml (day)	7.94	7.51–8.34
Highest parasite count (day)	11.14	10.63–11.65
Highest parasite count (GM)	4805/ml	2367–9754
Positive blood smear (day)	11.5	10.88–12.12
Parasite count at diagnosis (GM)	3106/ml	1202–8032

[a] Abbreviation: GM, Geometric mean.

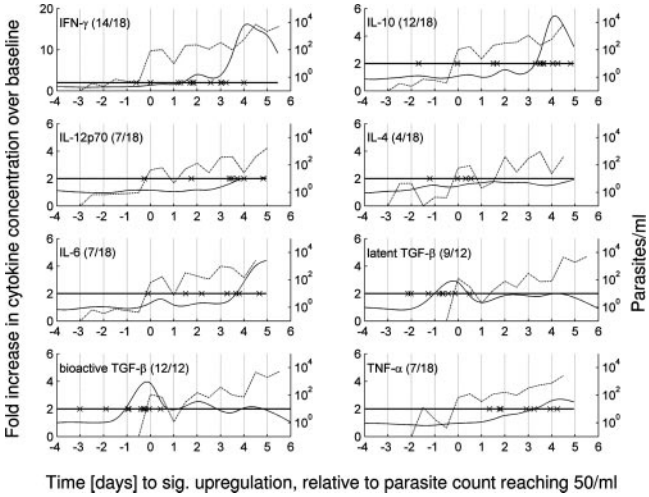

FIGURE 1. Kinetics of cytokine concentrations in peripheral blood after experimental malaria infection. In each graph, the solid line represents the magnitude of cytokine up-regulation (expressed as fold increase over baseline) for those subjects who showed at least a 2-fold increase in cytokine concentration over their own individual baseline at some time during the study. Curves were smoothed using a cubic smoothing spline with smoothing parameter 0.9 (with "0" being linear regression and "1" recreating the data). The horizontal line indicates the cut-off for up-regulation (2-fold) and crosses (×) on this line indicate the time of the first sample giving a value above the cut-off for each donor. The data are synchronized to the time of onset of parasitemia (0, first detection of ≥50 parasites/ml). The dotted line indicates the geometric mean parasitemia over the study period for cytokine positive subjects. The number of subjects contributing to each chart is shown.

Significant up-regulation of IL-1β, IL-2, IL-8, and GM-CSF was observed in only 0, 3, 2, and 0 subjects, respectively; these cytokines were therefore excluded from further analysis. For the remainder, Fig. 1 indicates the number of subjects for whom significant up-regulation of cytokine levels over baseline was seen and shows the kinetics and magnitude of cytokine up-regulation in these subjects alongside their parasite densities; the median time to up-regulation of each cytokine is shown in Fig. 2.

The first cytokine to show significant up-regulation in the majority of donors was TGF-β; latent TGF-β was significantly up-regulated ~18 h, and bioactive TGF-β was significantly up-regulated 6 h, before the first detection of ≥50 parasites/ml by PCR. TGF-β levels then oscillated, with a peak of TGF-β occurring approximately every 48 h. IL-4 was up-regulated only in four donors, but, where it was seen, it tended to coincide with first detection of ≥50 parasites/ml. This first wave of cytokine production was then followed by a second wave in which TNF-α, IL-6, IL-12, IL-10, and IFN-γ were all significantly up-regulated 2–3 days after parasites were first detected by PCR. Using a linear random effects model that allows for within-subject clustering, up-regulation of TGF-β and IL-4 occurred, on average, 2.93 days earlier than up-regulation of TNF-α, IL-6, IL-12, IL-10, and IFN-γ ($p < 0.001$; with a bootstrapped 95% CI of 2.3–3.5 days). Furthermore, up-regulation of IFN-γ was observed significantly earlier than up-regulation of either IL-12p70 or IL-10 (IFN-γ vs IL-12, 1.03 days, $p = 0.048$; IFN-γ vs IL-10, 0.95 days, $p = 0.028$; with p values adjusted for multiple comparisons using the Holm step-down method), suggesting that there are actually three waves of cytokine release. Interestingly, within donors, peak values of IL-10 were

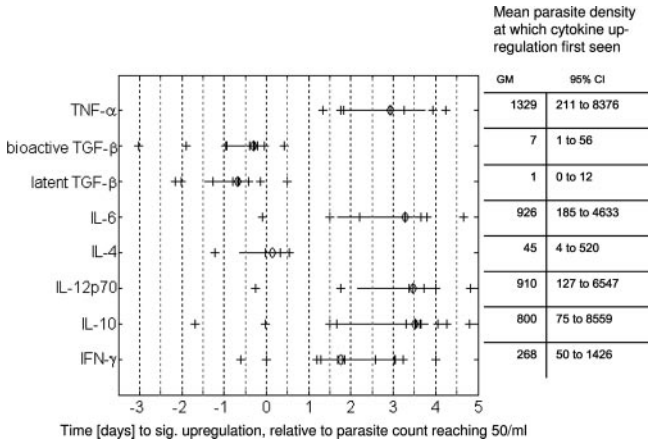

FIGURE 2. Sequential up-regulation of cytokines during primary blood-stage malaria infection. 1) The median (◇), interquartile range (horizontal line), and individual values (+) of the time to cytokine up-regulation (defined as plasma cytokine concentration at least twice the individual's baseline) after first detection of parasitemia (≥50 parasites/ml). 2) Geometric mean (95% CI) parasitemia at which 2-fold cytokine up-regulation was detected for responding subjects.

very closely correlated with those of IFN-γ (correlation coefficient, 0.716; $p = 0.001$).

Individual heterogeneity in cytokine responses to P. falciparum *infection*

The aggregate data presented in Figs. 1 and 2 hide some interesting and potentially important differences in the cytokine response between individuals (Fig. 3). In 6 of 18 donors (group 1), IFN-γ was the first proinflammatory cytokine to be up-regulated after sporozoite infection, reaching levels up to 7.9-fold (range, 4- to 14-fold) higher than baseline levels (e.g., Fig. 3, *a* and *b*). In five of these six cases, this was followed by a significant increase in IL-10 (average increase, 4.2-fold; range, 1.0- to 6.5-fold), but there was no detectable induction of IL-12.

Another six subjects (group 2) showed a similarly early IFN-γ response that was followed by up-regulation of IL-12p70 (e.g., Fig. 3, *c* and *d*). Although up-regulation of IL-12p70 was modest (average increase, 2.7-fold; range, 2- to 3.3-fold), in these subjects a second wave of IFN-γ was detected, with an average 43-fold increase (range, 20- to 82-fold) above baseline, which was accompanied by very marked increases in IL-10 (average increase, 7.6-fold; range, 2.6- to 15.6-fold).

Conversely, in the remaining six subjects (group 3), proinflammatory cytokines were not noticeably up-regulated at any time during the observation period (maximum detected IFN-γ concentration; average, 1.5-fold; range, 1- to 2.5-fold increase over baseline). In these subjects, a sharp, transient increase in bioactive TGF-β (average, 14.6-fold; range, 3.5- to 35.5-fold) was observed within 12 h of first PCR detection of parasites, and virtually no increase in IL-10 was seen (average, 1.4-fold; range, 1- to 2.4-fold increase over baseline (Fig. 3, *e* and *f*); some of this data has been reported previously by us (30)).

To determine whether the observed differences might be merely a function of the infective dose of sporozoites received by each volunteer, the number of parasites emerging from the liver into the blood was estimated for each group, as previously described (44).

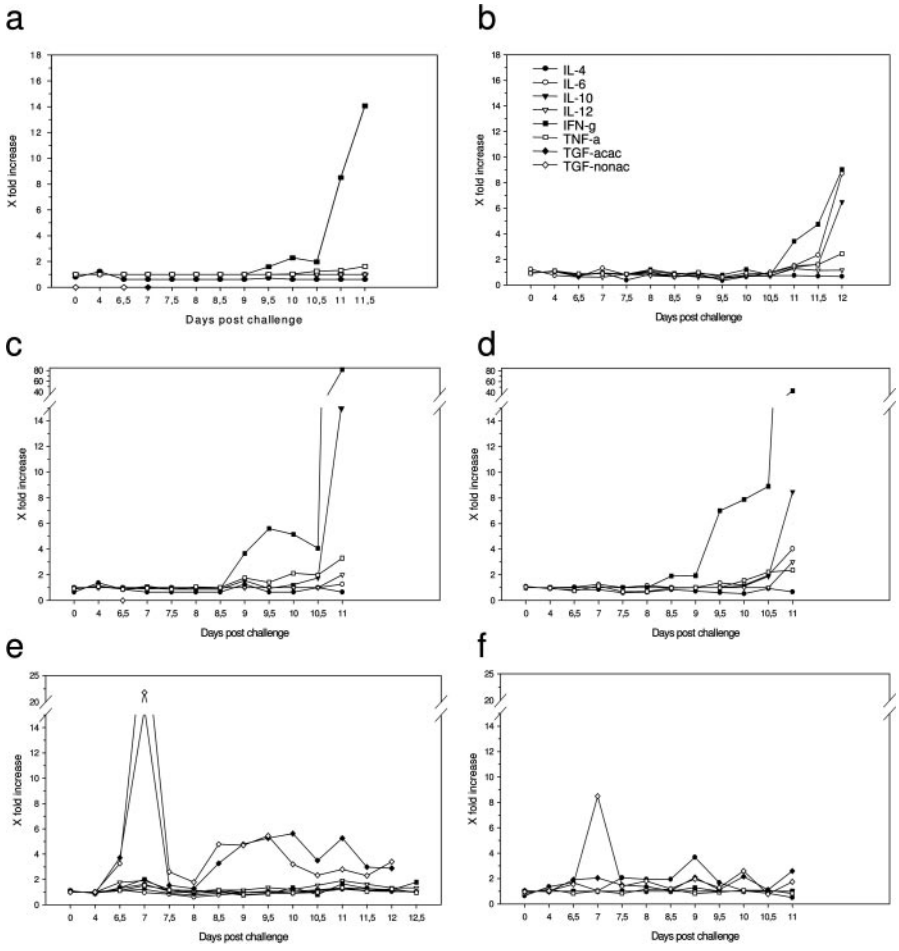

FIGURE 3. Individual patterns of plasma cytokine concentrations in malaria-infected subjects. Fold up-regulation of each cytokine above baseline at each time point after sporozoite infection. *a*, This subject shows up-regulation of IFN-γ only. *b*, This subject up-regulates IFN-γ and then IL-10. *c* and *d*, These two subjects develop very high concentrations of IFN-γ in combination with >2-fold up-regulation of IL-12 and subsequent up-regulation of IL-10. *e* and *f*, In these two examples, early up-regulation of TGF-β occurs and there is no subsequent proinflammatory response and no up-regulation of IL-10.

The averages were 136,460 (IFN-γ-high group), 144,003 (IFN-γ-intermediate group), and 122,940 (IFN-γ-low group). At the 5% level, the Kruskal-Wallis test showed no significant difference in the infecting parasite dose among the three groups. Similarly, there was no significant difference among the three groups in the time of first parasite detection by PCR ($p = 0.082$).

Four of the 18 subjects significantly up-regulated IL-4. Three of these fell into group 2 because they had significantly increased levels of IL-12p70 and a 20- to 45-fold increase in IFN-γ (Fig. 3, *c* and *d*); the fourth subject fell in group 3, showing no up-regulation of any proinflammatory cytokine or IL-10. Three of the four subjects with raised IL-4 levels were also among those tested for TGF-β, and, interestingly, all three had significantly elevated TGF-β levels at parasite emergence (average, 7.72-fold; range, 3.3- to 11.4-fold).

Association between cytokine responses, clinical and parasitological parameters

In the majority of subjects, increasing cytokine concentrations were coincident with the rise in asexual parasitemia, suggesting that there is a causal relationship between onset of blood-stage infection, initiation of the immune response, and subsequent parasite growth rates. Furthermore, peak concentrations of IL-12p70, IFN-γ, and TNF-α, as well as the ratio of TNF-α to bioactive TGF-β, were positively correlated with the length of time to first detection of 1000 parasites/ml (correlation coefficients of 0.53, 0.53, 0.66, and 0.67, respectively; $p < 0.036$ in all cases). Similarly, the ratio of IFN-γ to IL-10 was negatively associated with the maximum parasitemia during the first 12 days postinfection (correlation coefficient, −0.57; $p = 0.022$). However, higher ratios of IL-12 or TNF-α to IL-10 were also correlated with more rapid onset of any clinical symptoms (including headache or malaise; correlation coefficient, −0.53, $p = 0.034$, and −0.68, $p = 0.004$, respectively), and fever/feverishness was predominantly reported from subjects who showed significant up-regulation of IFN-γ (10 of 12 in groups 1 and 2, respectively, compared with 1 of 6 in group 3; $p = 0.013$, Fisher's exact test).

In vitro cytokine responses to P. falciparum*-infected RBC*

Due to the potential difficulties of interpreting some of the in vivo cytokine data (because biologically relevant concentrations of each cytokine differ and concentrations in serum or plasma may correlate only poorly with concentrations of bioactive cytokine within tissues), we have conducted a parallel in vitro analysis of the early cytokine response to iRBC. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α, and GM-CSF were measured, by cytometric bead assay and/or ELISA, in whole blood and in PBMC from up to 11 malaria-naive donors after stimulation for up to 24 h with bacterial LPS, uRBC, or iRBC. The only difference noted between cytokine responses detected in whole blood compared with PBMC culture supernatants was that IFN-γ responses were blunted and delayed in whole blood (data not shown), consistent with previous reports (45); however, significant up-regulation of IFN-γ was seen in whole blood under the conditions used here. In all cases, cytokine responses were only detected with parasite concentrations of at least 2×10^6 iRBC/ml (no significant response was detected at concentrations of 2×10^2 or 2×10^4 iRBC/ml).

In contrast to in vivo observations, levels of in vitro iRBC-induced IL-2, IL-4, and IL-12p70 remained at background level (<2-fold increase compared with uRBC) throughout the 24-h time course (data not shown).

Significant secretion of IL-1β, TNF-α, IL-6, IFN-γ, IL-10, IL-8, and GM-CSF was detected within 24 h of iRBC stimulation (Fig. 4). The earliest cytokine to be detected was TNF-α, which was

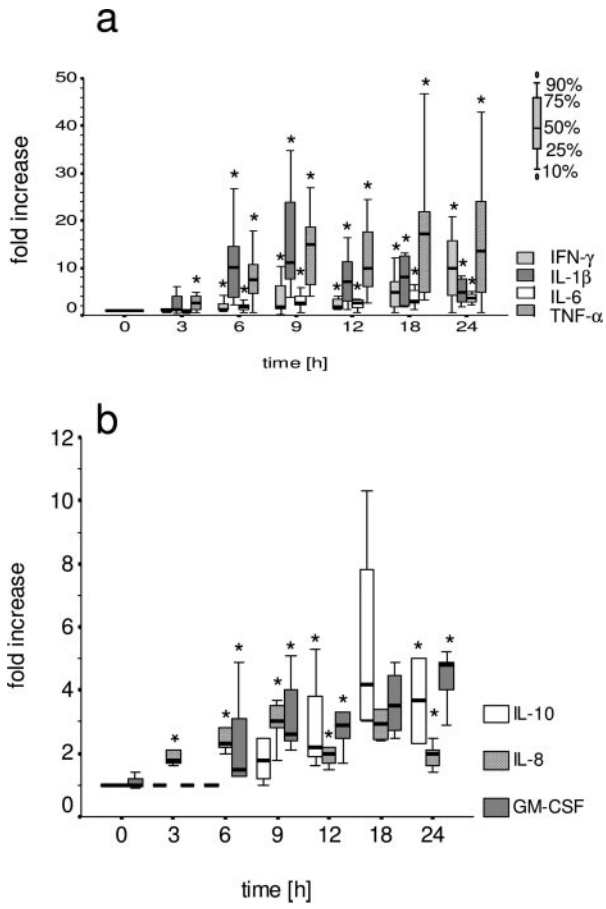

FIGURE 4. Cytokine production in vitro in response to *P. falciparum*-infected RBC. Median increase in cytokine concentration after in vitro stimulation of whole blood with iRBC (values are shown as fold up-regulation over concentrations in wells incubated with uRBC). *a*, Concentrations of IFN-γ, IL-1β, IL-6, and TNF-α were tested for 11 donors at all time points. *b*, Concentrations of IL-10, IL-8, and GM-CSF were tested for six donors at all time points. ∗, Significantly increased cytokine response ($p \leq 0.05$) for samples stimulated with iRBC compared with uRBC stimulation.

significantly up-regulated (in comparison to levels in control cultures with uRBC) within 3 h and reached maximal levels (10- to 20-fold up-regulation) within 9 h of incubation. IL-1β responses mirrored those of TNF-α, although not being statistically significantly up-regulated until ~6 h, and again peaked (>10-fold up-regulation) at 9 h. IFN-γ was modestly, but significantly, up-regulated within 6 h of stimulation by iRBC and continued to increase steadily, reaching >10-fold up-regulation after 24 h. GM-CSF levels mirrored those of IFN-γ, being significantly up-regulated at 6 h and still continuing to increase at 24 h, although the degree of up-regulation was less marked (4-fold). IL-10 was not significantly up-regulated until 12 h after iRBC stimulation and plateaued between 18 and 24 h, whereas IL-8 was modestly (but significantly) up-regulated from 3 h onward.

The kinetics of the cytokine response to iRBC was remarkably similar to the response to LPS (e.g., Fig. 5, comparing TNF-α, IL-6, and IFN-γ responses to LPS and iRBC) with the exception that IL-10 was up-regulated slightly earlier by LPS (within 9 h), and significant up-regulation of IL-12p70 (within 6 h) was observed with LPS but not with iRBC (data not shown). The similarity in the kinetics of the LPS and iRBC responses strongly suggested that iRBC must be able to trigger innate responses

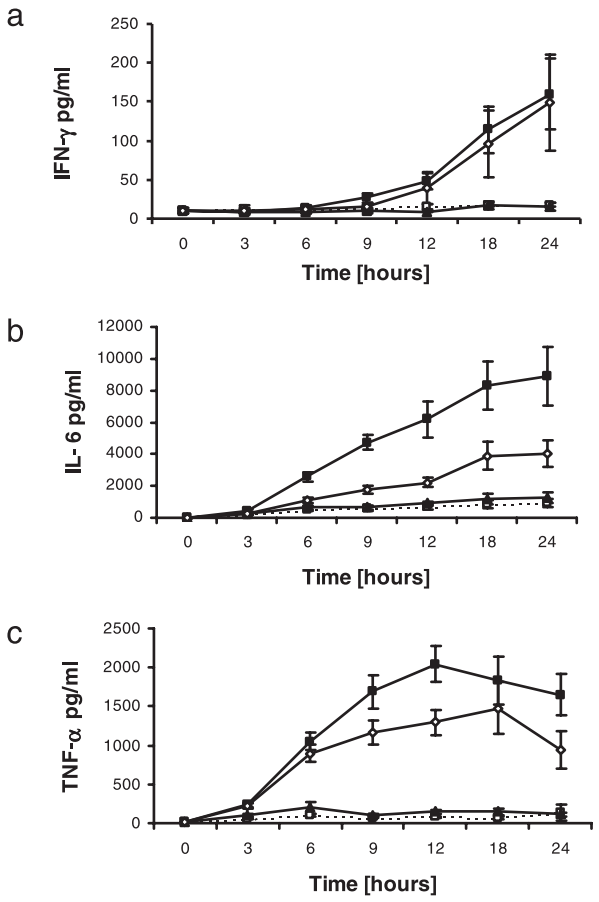

FIGURE 5. In vitro cytokine responses to *P. falciparum*-infected RBC and LPS. Whole blood was stimulated with LPS, growth medium, 2×10^6 iRBC or equivalent numbers of uRBC for various time periods. The mean with SE is shown for $n = 11$ subjects in each case. *a*, IFN-γ. *b*, IL-6. *c*, TNF-α. □, Growth medium; ■, LPS; ▲, uRBC; ◇, iRBC.

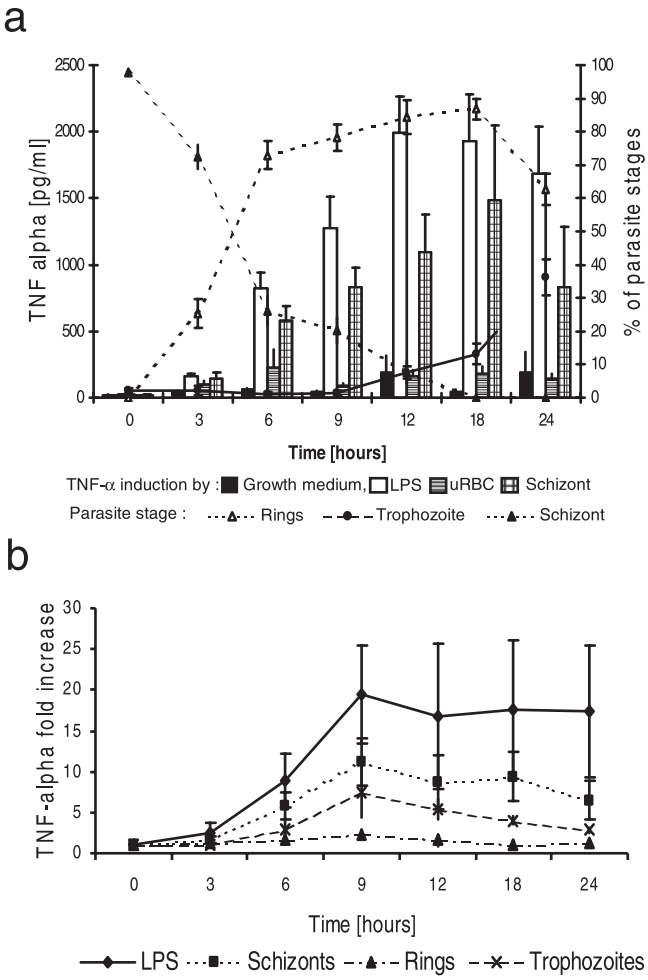

FIGURE 6. Schizont-infected, but not ring- or trophozoite-infected, RBC induce TNF-α secretion in whole blood. *a*, TNF-α production in response to no stimulation (GM), LPS, uRBC, or iRBC. All iRBC were at late schizont stage when added at 0 h. Dotted lines indicate the proportions of ring-, trophozoite-, and schizont-infected RBC in the cultures at each time point. *b*, TNF-α production (fold increase over uRBC values) in response to 2×10^6 purified ring-, trophozoite-, or schizont-infected RBC added to the culture at 0 h; LPS data are fold increase over no stimulation. The mean (SEM) for six donors is shown in each plot; essentially identical data were obtained in an independently repeated experiment with five donors.

immediately on being added to the PBMC cultures. This was unexpected because it is generally assumed that schizont rupture is the key event in triggering innate immune responses (6, 7, 46), and large-scale schizont rupture did not occur until 3–6 h after adding of iRBC to the cultures (Fig. 6*a*). Furthermore, although ring-stage parasites did not induce cytokine responses, marked induction of TNF-α was observed when trophozoites (24 h postinvasion) were added to the cultures (Fig. 6*b*). Taken together, these data indicate that schizont-stage (and to a lesser extent trophozoite-stage) *P. falciparum* iRBC can trigger innate responses before rupture of the infected RBC.

IL-12 production in whole blood in response to iRBC as determined by flow cytometry

Although IL-12p70 was found to be modestly up-regulated in plasma of sporozoite-challenged volunteers, we were unable to detect any significant production of IL-12p70 in supernatants from in vitro assays. To further investigate this discrepancy, and to clarify the source of IL-12 in *P. falciparum* infection, whole blood was incubated without stimulation or with LPS, iRBC, or uRBC for up to 24 h, and cells were then stained with a mixture of Abs (Lin-1) for lineage surface markers, for surface MHC class II (HLA-DR), and for intracellular IL-12. Lin-1^+ cells include B cells, T cells, NK cells, and monocyte-macrophages; only dendritic cells (DCs) are expected to be HLA-DR^+Lin-1^-.

On average HLA-DR^+Lin-1^- DC constituted 1.3% (+0.21%) of unstimulated, 1.2% (+0.19%) of LPS-stimulated, and 1.15% (+0.23%) of *P. falciparum*-stimulated cells within the live gate and did not change significantly during the 24 h of in vitro culture. IL-12-positive cells were gated (Fig. 7, *a–c*) and analyzed for HLA-DR and Lin-1 (*d* and *e*); all IL-12-positive cells were HLA-DR^+. After stimulation with LPS, maximal numbers of IL-12^+ cells (0.9%; range, 0.6–1.3% of cells within the live gate) were seen after 18 h (Fig. 7*b*); the majority of IL-12^+ cells were Lin-1^+ (*d*) and varied in their scatter characteristics (*g*), but a significant proportion (5.6%; range, 3.7–9.3% of IL-12^+ cells) were Lin-1^-HLA-DR^{high} and had the low scatter characteristics (*f*) typical of DCs (47).

For *P. falciparum*-stimulated cells, the proportion of IL-12^+ cells also peaked after 18 h, with 0.8% (range, 0.6–1.04%) of cells within the live gate staining positively for IL-12 (Fig. 7*c*). However, all of these cells were Lin-1^+ and HLA-DR^{low} (Fig. 7*e*) with the scatter characteristics of large granular cells (*h*), characteristic for activated monocytes. Overall, IL-12^+ cells induced by iRBC had significantly higher forward and side scatter than LPS-induced

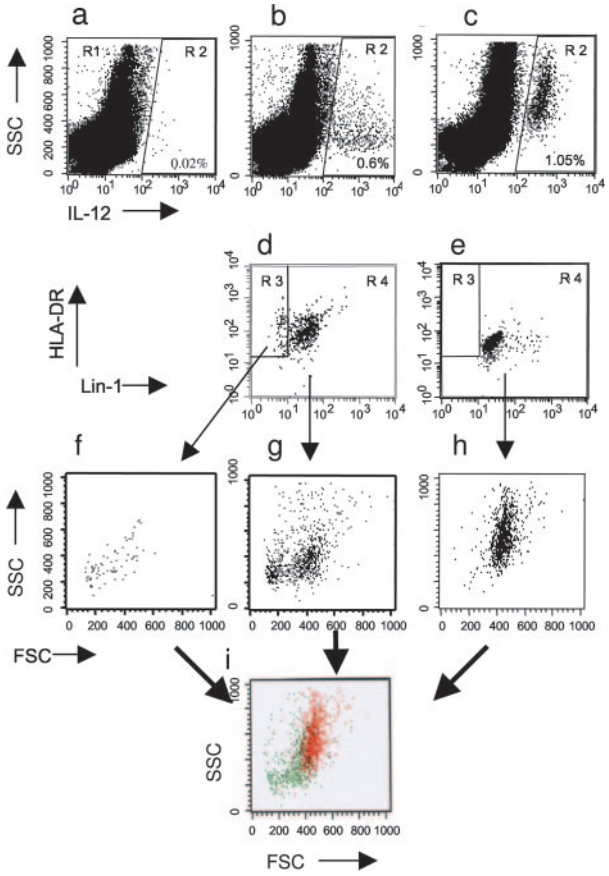

FIGURE 7. Characterization of IL-12-producing cells. *a–c*, Whole blood was stimulated for 18 h with uninfected RBC (*a*), LPS (*b*), or *P. falciparum* iRBC (*c*), cells stained for Lin-1, HLA-DR, and intracellular IL-12, and IL-12$^+$ cells gated (R2). *d* and *e*, LPS-stimulated (*d*) and *P. falciparum*-stimulated (*e*) IL-12$^+$ cells were analyzed for HLA-DR and Lin-1. *f–h*, Scatter characteristics of LPS-induced IL-12$^+$ DCs (*f*), LPS-induced IL-12$^+$ non-DCs (*g*), and *P. falciparum*-induced IL-12$^+$ cells (*h*). *i*, Overlay of light scatter characteristics of LPS-induced (green) and *P. falciparum*-induced (red) IL-12$^+$ cells (from R2 gates). Data are representative of at least three independent experiments.

IL-12$^+$ cells (geometric mean, forward scatter: iRBC, 440 (CI 95%, 302–578); LPS, 317 (CI 95%, 285–349); $p = 0.019$; side scatter: iRBC, 581 (CI 95%, 383–778); LPS, 354 (CI 95%, 300–408); $p = 0.008$); this difference can be seen in an overlay of the IL-12$^+$ cells induced by LPS and iRBC (Fig. 7*i*).

Discussion

Most knowledge about innate immune responses to human malaria is derived either from in vitro studies or from cross-sectional epidemiological studies conducted at a time when the infection has already led to disease. The former has allowed the potential role of different cell populations such as dendritic cells (48), γδ T cells (46), and NK cells (24, 25, 36) to be evaluated; the latter has been useful to uncover associations between immune responses and disease outcome (14, 18–20) but cannot provide information about the sequence of events between infection and onset of disease. Here, we have taken advantage of experimental, sporozoite-induced malaria infections to describe the kinetics of the systemic cytokine response during the prepatent period of *P. falciparum* infection in 18 unvaccinated, previously malaria-naive subjects and have compared this with the cytokine responses triggered in vitro from whole blood or PBMC by parasite-infected RBC.

Perhaps the most intriguing observation is that in vivo systemic responses to infection over a period of 10–14 days vary in both quality and magnitude between subjects despite similar numbers of merozoites emerging from the liver. Subjects could be divided into distinct groups according to the strength of their proinflammatory response. One group developed moderately up-regulated levels of IFN-γ and IL-10, but did not produce detectable IL-12p70. A second group produced detectable levels of circulating IL-12p70 and developed very high circulating levels of IFN-γ and IL-10. Although the average time until parasites were first detected by microscopy was identical in both groups (11.6 days), we cannot exclude the possibility that the first group would have developed a cytokine profile similar to that observed for group 2, had a longer observation period been possible. In contrast, the third group failed to up-regulate any significant proinflammatory responses, but showed the highest levels of TGF-β at parasite emergence from the liver. These cytokine responses had clear clinical and parasitological consequences. A predominantly proinflammatory response was associated with more rapid control of parasite growth but only at the cost of developing clinical symptoms. These observations strongly support the conclusions of several correlative clinical and epidemiological studies that have also suggested that proinflammatory responses might be causally associated with both clearance of parasites and clinical disease (14, 22, 33). Conversely, subjects in group 3 who had no detectable inflammatory response but the highest levels of TGF-β were less able to control parasite growth (30) but hardly ever reported a fever or feverishness, again supporting data from studies in malaria endemic populations that anti-inflammatory activity is associated with less severe clinical symptoms (22). Intriguingly, as we have already reported, circulating bioactive TGF-β at the time of parasite emergence from the liver is associated with the induction of Foxp3 and of CD4$^+$CD25$^+$ regulatory T cells (30), suggesting that the initial innate response to malaria infection may determine long-term immunological responses and thus have important clinical consequences.

An interesting subset of four subjects developed raised plasma IL-4 levels; three of these subjects subsequently showed significant induction of IL-12p70, and were among those individuals with the highest IFN-γ responses. Despite this clear proinflammatory response, all of the IL-4 responders that were also tested for TGF-β ($n = 3$) also showed significantly increased plasma TGF-β levels, although to a lesser extent (7.72-fold for IL-4 responders vs 14.6-fold in group 3). In mice, IL-4 in combination with TGF-β has been described as an alternative pathway to induce IFN-γ responses from CD4$^+$ T cells (49), and pretreatment of human PBMC with IL-4 before LPS stimulation has been shown to enhance their subsequent production of IL-12p70 (50, 51), suggesting that the association we observed here between IL-4, TGF-β, IL-12p70, and IFN-γ production may be causal.

The kinetics of the observed cytokine responses in vivo suggests that merozoites derived from both liver schizonts and iRBC may trigger the initial cytokine response (Fig. 2).

Given the timing of detectable IFN-γ up-regulation, beginning on average 1.75 days after parasite emergence from the liver, NK cells (24) and γδ T cells (46) are likely to be the primary sources of this cytokine. NK cell IFN-γ responses may be particularly relevant to our ex vivo observations—where high IFN-γ responses were seen in only one-third of donors and exclusively in those donors up-regulating IL-12p70—because they can be very rapid (within 6–15 h (24)), very heterogeneous between donors (36), and highly dependent upon IL-12p70 and IL-18 (24, 25, 52) (K. C. Newman, D. S. Korbel, and E. M. Riley, submitted for publication). The notion that IL-12 is a key factor determining the overall

magnitude of the IFN-γ response is in line with immuno-epidemiological data suggesting a correlation between bioactive IL-12 and clinical and parasitological outcomes of malaria infection (14, 16–22).

Intriguingly, in our ex vivo study, up-regulation of IL-12p70 was detected significantly later than up-regulation of IFN-γ and was not detected at all in supernatants of in vitro-activated PBMC. Although it is possible that early, transient, and low-level IL-12 responses might have been missed (as suggested by the flow cytometric analysis in which IL-12-positive cells could be detected after 18 h of incubation with iRBC), it also seems likely that an initial wave of IFN-γ markedly up-regulates the IL-12 response and that this in turn may further augment the IFN-γ response, leading to a positive feedback loop from which very high concentrations of proinflammatory cytokines can result. In contrast, previous in vitro studies have reported rapid in vitro induction of IL-12p40 upon stimulation of PBMC with iRBC (4, 46), raising the interesting possibility that one of the first cytokines to be induced by iRBC may be IL-23 (a heterodimer composed of p40 and p19) or possibly even IL-27, which contains a p40-related protein (53). Intriguingly, IL-27 has been shown to induce IFN-γ production by human NK cells independently of, and before IL-12 (54), suggesting that the potential role of this cytokine in the early response to iRBC requires further investigation. Our observation that human DCs do not produce IL-12 is consistent with previous reports (48), and our finding that monocyte-macrophages are the major source of iRBC-induced IL-12p70 likely explains the requirement for this cell type for induction of optimal NK cell IFN-γ responses (K. C. Newman, D. S. Korbel, and E. M. Riley, submitted for publication).

Apart from providing IL-12p70, monocyte-macrophages also contribute to NK cell activation in a cell contact-dependent manner (K. C. Newman, D. S. Korbel, and E. M. Riley, submitted for publication), pointing out the importance of mechanisms facilitating close contact of both cell types. Intriguingly, the parasite metabolite hemozoin can mediate recruitment of monocytes (55), most likely via an IL-8-dependent mechanism (56), and NK cells have been shown to produce IL-8 in response to iRBC stimulation (52). In our in vitro experiments, IL-8 was among the earliest cytokines to be significantly up-regulated (at 3 h), whereas significant IL-8 increase could only be measured in two of the sporozoite-infected donors. Pulling these data together, hemozoin-induced IL-8 production by NK cells, leading to recruitment of monocytes that in the presence of iRBC activate NK cells and produce IL-12, may portray a pathway used by NK cells to enhance their IFN-γ production.

Both in vitro and in vivo, IL-10 levels were highly correlated with levels of IFN-γ, confirming previous clinical reports that IL-10 is up-regulated in concert with IFN-γ (33). The sequential up-regulation of IFN-γ followed by IL-10, both in vivo and in vitro, and the close correlation between peak concentrations of IFN-γ and IL-10, suggests that IL-10 may be up-regulated as a direct consequence of IFN-γ production as part of a homeostatic feedback mechanism to limit IFN-γ-mediated pathology, as is seen in murine malaria infections (26).

The sequence of events we observed in vitro is slightly different from that obtained ex vivo. It is unlikely that these differences are parasite related, because the parasite stocks used in both studies were derived from the cloned parasite line 3D7. Rather, the heterogeneity of responses seen ex vivo may represent gradual divergence of responses over several days and/or varying interactions between parasites and immune cells in lymphoid organs, in particular the spleen, or other tissues. Furthermore, we cannot exclude that the immune response to blood-stage parasites is affected in some way by the interaction between sporozoites or sporozoite-infected hepatocytes and immune cells in the liver. Our observation that parasite-derived molecules, including thrombospondin-related proteins such as TRAP, which is highly expressed in sporozoites, or its homolog MTRAP, which is abundantly expressed in blood-stage merozoites (57), can contribute to activation of latent TGF-β to its bioactive form (58), may be relevant to this discussion as variation between individuals in their initial TGF-β response was highly correlated with their subsequent immune response and the course of parasitemia (30).

In the in vitro studies, TNF-α was induced with identical kinetics to the LPS-induced response following stimulation with schizont- and trophozoite-infected, although not ring-stage-infected, RBC. Both free GPI and hemozoin, which have been identified as ligands for TLRs 2, 4, and 9 (59, 60), accumulate as parasites mature and are thus likely to be present in much greater concentration within trophozoite- or schizont-infected cells. *P. falciparum*-derived hemozoin binds to TLR-9 on murine myeloid DCs that subsequently produce large amounts of TNF-α (60). Intriguingly, in our study, TNF-α was up-regulated before any significant schizont rupture leading us to speculate that, in contrast to what has previously been suggested (6, 7, 46), large-scale schizont rupture may not be essential for induction of innate immune responses to malaria. Possible alternative explanations include activation of monocyte-macrophages following phagocytosis of iRBC or leakage of soluble molecules from damaged—but still intact—iRBC.

The rapid production of macrophage activating cytokines such as TNF-α and IFN-γ that we observed is consistent with earlier reports showing that PBMC from nonimmune donors produce parasite growth-inhibiting molecules within 24 h of coculture (61).

In summary, we have shown that early immune responses to *P. falciparum* in previously malaria-naive subjects are heterogeneous, with particularly noticeable variation in the speed and strength of the inflammatory cytokine response, leading to clear parasitological and clinical consequences. This in vivo heterogeneity confirms the relevance of similar findings from in vitro studies (25, 36) (K. C. Newman, D. S. Korbel, and E. M. Riley, submitted for publication). Furthermore, data generated from this longitudinal study of prepatent malaria infection have generated a number of novel hypotheses that warrant further investigations in vitro. Our study also shows that in vitro studies need to be accompanied by detailed clinical and experimental studies in infected individuals to obtain a comprehensive understanding of the ways in which innate immune responses can be triggered, how they are regulated, and how they modify the resulting infection and adaptive response.

Acknowledgments

We are grateful to all of the subjects who volunteered to take part, to Fiona Thompson, Susie Dunachie, Ian Poulton, Daniel Korbel, and Carolynne Stanley for help with collecting the blood samples, to Angela Hunt-Cooke and Simon Correa for slide reading and help with processing blood samples, and to Rikke F. Andersen for help with *P. falciparum* PCR. We thank Jacqui Mendoza and Geoff Butcher (Imperial College, London, U.K.) and Dr. Jack Williams (Walter Reed Army Institute of Research, Silver Spring, MD) for supplying *P. falciparum*-infected mosquitoes.

Disclosures

The authors have no financial conflict of interest.

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