

# $\gamma\delta$ T cells and CD14<sup>+</sup> Monocytes Are Predominant Cellular Sources of Cytokines and Chemokines Associated With Severe Malaria

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**Background.** Severe malaria (SM) is associated with high levels of cytokines such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and interleukin 6 (IL-6). The role of chemokines is less clear, as is their cellular source.

**Methods.** In a case-control study of children with SM (n = 200), uncomplicated malaria (UM) (n = 153) and healthy community controls (HC) (n = 162) in Papua, New Guinea, we measured cytokine/chemokine production by peripheral blood mononuclear cells (PBMCs) stimulated with live *Plasmodium falciparum* parasitized red blood cells (pRBC). Cellular sources were determined. Associations between immunological endpoints and clinical/parasitological variables were tested.

**Results.** Compared to HC and UM, children with SM produced significantly higher IL-10, IP-10, MIP-1 $\beta$ m and MCP-2. TNF and MIP-1 $\alpha$  were significantly higher in the SM compared to the UM group. IL-10, IL-6, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-2 were associated with increased odds of SM. SM syndromes were associated with distinct cytokine/chemokine response profiles compared to UM cases. TNF, MIP-1 $\beta$ , and MIP-1 $\alpha$  were produced predominantly by monocytes and  $\gamma\delta$  T cells, and IL-10 by CD4<sup>+</sup> T cells.

**Conclusions.** Early/innate PBMC responses to pRBC in vitro are informative as to cytokines/chemokines associated with SM. Predominant cellular sources are monocytes and  $\gamma\delta$  T cells. Monocyte-derived chemokines support a role for monocyte infiltrates in the etiology of SM.

**Keywords.** cytokines; chemokines; severe malaria;  $\gamma\delta$  T cells; monocyte/macrophages.

Malaria remains a major cause of morbidity and mortality worldwide. 1%–2% of clinical *Plasmodium falciparum* infections lead to life-threatening severe malaria

that may involve impaired consciousness, coma, acute respiratory disease, severe anemia, metabolic acidosis, and multi-organ failure. Various parasite- and host-specific processes contributing to disease include microvasculature obstruction by parasites, local inflammatory infiltrates, and an inappropriate or uncontrolled systemic host immune response [1–3].

The early, inflammatory immune response to malaria involves monocytes, macrophages, CD4<sup>+</sup> T cells,  $\gamma\delta$  T cells, and natural killer (NK) cells [4–7] and may limit parasite growth. Cytokines such as interleukin 12 (IL-12), interferon  $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor (TNF) produced by innate immune cells in response to parasites are associated with reduced prospective risk of symptomatic and high-density infections and time-to-reinfection [8–11]. Such responses are however also proposed to contribute to severe malarial disease

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[1, 2]. High, circulating levels of the cytokines TNF, interleukin 1 (IL-1), IFN- $\gamma$ , interleukin 10 (IL-10) and interleukin 6 (IL-6) are observed in plasma or sera from severe malaria cases at presentation [12, 13]. Compared with cytokines, only a few studies have examined chemokines in human severe malaria [14–17].

Most studies examining the role of these mediators in severe malaria have measured levels in plasma/serum [15, 17–20]. This provides, at best, an indirect estimation of true cellular production. Plasma/serum TNF concentrations may be misleading as TNF also occurs in complex with soluble TNF receptor and thus is not bioavailable [21]. More accurate measures of cytokines and chemokines may be obtained by examining production directly from cellular sources. However, the composition of peripheral blood is highly altered during acute malaria due to leukocyte sequestration, and peripheral blood mononuclear cells (PBMCs) show pronounced anergy or hyporesponsiveness under these conditions [22–26].

To further elucidate critical cytokine and chemokine networks associated with susceptibility to severe disease and identify their cellular sources, we examined associations between risk of severe malaria and short-term IFN- $\gamma$ , IL-10, IL-1 $\beta$ , IL-6, interferon gamma-induced protein 10 (IP-10), TNF, macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ), and monocyte chemoattractant protein 2 (MCP-2) responses by PBMCs to *P. falciparum* infected red blood cells (pRBCs), within the context of a pediatric severe malaria case control study in an area of high malaria endemicity in Papua New Guinea. As clinically overt *P. falciparum* infection down-regulates cellular responsiveness and modifies the cellular composition of peripheral blood [22–26], we used convalescent samples, after homeostatic normalization of peripheral cellular composition. This is thought to provide a better estimate of an individual's intrinsic capacity to produce cytokines/chemokines [8, 24].

## MATERIALS AND METHODS

### Study Area, Design, and Participants

A severe malaria case-control study was conducted from 2006 to 2009 in Madang (pop. approximately 450 000), an area of holoendemic transmission of *P. falciparum* and *P. vivax* in Papua New Guinea [27, 28]. Modilon Hospital is the provincial hospital to which most children with severe illness are referred.

Based on sample availability, a sub-set of children in the main case-control study was used for this immunological study. Severe malaria (SM) cases included children aged 6 months to 10 years (n = 200) admitted to Modilon Hospital with a diagnosis of severe malaria according to World Health Organization (WHO) guidelines [29]. Briefly, this included children positive for asexual *Plasmodium* parasites by Giemsa-stained thick blood film or polymerase chain reaction (PCR) presenting with any of the following conditions: impaired consciousness

or coma (Blantyre Coma Score [BCS] <5[30]); prostration; multiple seizures; hyperlactatemia (blood lactate >5 mmol/L); severe anemia (hemoglobin <50 g/L); dark urine; hypoglycemia (blood glucose  $\leq$ 2.2 mmol/L); jaundice; respiratory distress; persistent vomiting; abnormal bleeding; or signs of shock. PBMCs were isolated from convalescent bleeds, collected 2–3 months after cases were discharged.

Uncomplicated malaria (UM) controls included children that had a positive rapid diagnostic test (ICT Diagnostics Malaria Combo Cassette ML02), were positive for asexual *Plasmodium* parasites by Giemsa-stained thick blood film or PCR, and displayed fever but no evidence of severe disease (n = 153). These children were recruited from immunization clinics, the hospital Paediatric Outpatient Clinic, and health centers. PBMC for elicitation assays were isolated from samples collected at 2–3 months after the malaria episode.

Healthy community (HC) controls were recruited from community immunization clinics surrounding Madang township and did not have acute illness or history of malaria within the previous 2 weeks (n = 162). PBMCs from these healthy children were isolated from bleeds taken at enrolment and were collected within 2–4 weeks of the enrolment of matched severe malaria cases. Both UM and HC were matched with SM cases by age, sex, and province of parents' birth.

### Ethics Statement

Written informed consent for participation was sought from the parent(s)/guardian(s) at recruitment. The study was approved by the 3 relevant HRECs (PNG Medical Research Advisory Committee, PNGIMR Institutional Review Board, and WEHI HREC).

### Laboratory Procedures

Giemsa-stained thick blood films were made during PBMC collection. Parasitemia was quantified by 2 independent microscopists with discrepancies resolved by a third. Parasite density was calculated per 200 leukocytes, assuming peripheral blood leukocyte counts of 8000/ $\mu$ L. The final density was the geometric mean of the 2 values. Published PCR methods [31, 32] were used for parasite detection in enrolment samples from individuals with severe and uncomplicated malaria. Hemoglobin levels were measured by Hemocue.

PBMC isolation was performed following blood collection. Blood was diluted 1:1 in phosphate-buffered saline (PBS) and PBMCs isolated by density centrifugation with Ficoll-Paque PLUS (Amersham). PBMCs were washed, resuspended at  $1 \times 10^7$  cells/mL in 90% fetal bovine serum (FBS)/10% dimethyl sulfoxide and frozen to  $-80^\circ\text{C}$  at  $1^\circ\text{C}$  per min in freezing containers for 24 hours (Nalgene), before transfer to liquid  $\text{N}_2$  for storage.

### Cultivation of *P. falciparum*

*P. falciparum* (3D7) was cultivated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ , 1%  $\text{O}_2$ , and 1%  $\text{N}_2$  at 4% hematocrit using  $\text{O}^+$  human erythrocytes and

10% O<sup>+</sup> human serum (Australian Red Cross Blood Service) in RPMI-1640 with 25 mM HEPES, 2 mg/mL glucose, 28 mM sodium bicarbonate, 25 mg/mL gentamicin, and 200 μM hypoxanthine. Sorbitol-synchronized, knob-selected, *Mycoplasma*-negative, schizont stage pRBCs were purified by using CS columns (Miltenyi Biotec).

### PBMC Stimulation Assays

Upon thawing, PBMCs were washed 3 times in complete medium (RPMI-1640, 5% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin, and 100 mg/mL streptomycin), counted using Turk's solution (Merck) and Trypan Blue (Sigma), and aliquoted into U-bottom 96-well plates ( $2 \times 10^5$  cells/well; 100 μL). Subsequently, 100 μL of purified pRBCs, or unparasitized red blood cells (uRBCs) ( $6 \times 10^5$  cells/well), 1% Phytohaemagglutinin (PHA; Gibco), or media only was added, and PBMC were cultured for 72 hours at 37°C, 5% CO<sub>2</sub>.

### Detection of Cytokines/chemokines by Cytometric Bead Array (CBA) and ELISA

After 72 hours, concentrations of IFN-γ, IL-10, IL-1β, IL-6, IP-10, TNF, MIP-1α, and MIP-1β were measured in culture supernatants using CBA Flex Sets (BD Biosciences). Samples were analyzed using an LSR II flow-cytometer, and initial data analysis was performed using BD FCAPArray Software. MCP-2 was detected by sandwich enzyme-linked immunosorbent assay (ELISA) using anti-human MCP-2 antibody (MAB281) and anti-human MCP-2 biotinylated antibody (BAF281; R&D Systems). To determine agonist-specific cytokine induction, background levels from medium alone were subtracted.

### Identification of Cellular Sources of Cytokines/Chemokines by Flow Cytometry

PBMCs were stimulated with pRBCs or uRBCs for 12 or 24 hours at 37°C in 5% CO<sub>2</sub>. For the final 8 hours of incubation, brefeldin A (10 μg/mL; Sigma) and GolgiStop (2 μM; BD Biosciences) were added. Cells were incubated for 10 minutes on ice with PBS containing 10 mM glucose and 3 mM EDTA to detach adherent cells. PBMCs were subsequently Fc-blocked with human immunoglobulin G (IgG; 10 μg/mL; Sigma) and surface stained in PBS containing 0.5% BSA and 2 mM EDTA on ice for 30 minutes with phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD56 (clone B159), PerCP Cy5.5-conjugated anti-CD4 (clone RPA-T4), allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-CD14 (clone MΦP9), Fluorescein isothiocyanate (FITC)-conjugated anti-γδTCR (clone 11F2; all from BD Biosciences), Brilliant Violet 570 conjugated anti-CD16 (clone 3G8; Biolegend), and Qdot 605-conjugated anti-CD8 (clone 3B5; Invitrogen). Aqua live/dead amine reactive dye (Invitrogen) was used for dead cell exclusion. Cells were fixed in 2% paraformaldehyde and permeabilized using Perm Buffer 2 (BD Biosciences). Intracellular staining with PE-Texas Red, (ECD)-conjugated anti-CD3 (clone UCHT1; Beckman Coulter), Alexa700-conjugated

anti-TNFα (clone MAb11; BD Biosciences), Allophycocyanin (APC)-conjugated IFNγ (clone B27; BD Biosciences), PE-conjugated anti-IP-10 (clone 6D4/D6/G2; BD Biosciences), Brilliant Violet 421-conjugated anti-IL-10 (clone JES3-9D7; Biolegend), PE-conjugated anti-MIP1α (clone 93 342; R&D Systems), and APC-conjugated anti-MIP1β (clone 24 006; R&D Systems) was performed on ice for 1 hour. Samples were analyzed on a 4-laser Fortessa flow cytometer. The gating strategy used to identify the different cell populations is provided in [Supplementary Figure 1](#). Data analysis was performed using FlowJo software (TreeStar). Positive populations were determined by a combination of fluorescence minus 1 (FMOs) and isotype controls. Positive responses were determined based on comparison to background cytokine and chemokine levels in cells incubated with uRBCs. Responding individuals were defined as having a frequency of cytokine or chemokine positive cells  $\geq 0.02\%$  for CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and γδ T cells, and  $\geq 1\%$  for CD14<sup>+</sup> cells following background subtraction or had a frequency of responding cells twice above the background.

### Statistical Analysis

Statistical analyses were performed using STATA v.9. Associations between categorical variables were assessed using  $\chi^2$  tests. Mann-Whitney and Kruskal-Wallis tests were performed for comparisons of 2 and 3 medians, respectively. Spearman rank correlations were calculated for associations between cytokine responses. Multinomial logistic regression analysis was used to determine odds ratios associated with unit increase in cytokine/chemokine response to pRBC, with and without adjustment for potential confounders. The association between cytokine/chemokine responses and BCS was assessed by non-parametric test for trend.

## RESULTS

### Characteristics of Study Participants

The epidemiology, population characteristics, and incidences of *P. falciparum* and *P. vivax* infections and disease in this severe malaria case-control study are reported elsewhere [27]. Demographic features and malariometric indices at enrolment for the 162 HC, 153 UM, and 200 SM cases included in this immunological substudy are shown in [Supplementary Table 1](#). Neither age in months ( $P = .79$ ) nor sex ( $P = .45$ ) differed significantly between the case-control groups in this substudy. A significant difference was observed between frequency distribution of ethnic groups ( $P = .045$ ). All individuals with severe or uncomplicated malaria included in this immunological substudy were positive by rapid diagnostic test and either light microscopy or PCR at enrolment. By light microscopy, 35.4% of HC controls, 87.6% of UM groups, and 98.5% of SM groups were positive for *P. falciparum*, *P. vivax* ( $P < .001$ ), or both species. The

residual 12.4% and 1.5% of individuals in the UM and SM groups, respectively, were assigned based on PCR positivity with appropriate clinical characteristics and responsiveness to antimalarials. At enrolment, median hemoglobin levels were significantly different between the 3 groups ( $P < .0001$ ) with markedly lower levels in the SM group (Supplementary Table 1).

At PBMC collection, 48% of UM and 35.9% of SM groups were positive for *P. falciparum*, *P. vivax*, or both (Supplementary Table 1). Median hemoglobin levels were significantly different between groups ( $P < .0001$ ; Supplementary Table 1).

### Clinical Features of Severe Malaria

Supplementary Table 2 provides a summary of clinical indices recorded for SM cases. Of those with severe malaria, 11.5% presented with respiratory distress, 10% with deep coma ( $\text{BCS} \leq 2$ ), 32% with impaired consciousness ( $\text{BCS} \leq 4$ ), 22.5% with severe anemia (hemoglobin  $< 50$  g/L), 19.1% with hyperlactatemia (blood lactate  $> 5$  mmol/L), and 10.7% with metabolic acidosis (plasma bicarbonate  $< 12.2$  mmol/L).

### Associations Between Severe Malaria and *P. falciparum* Induced Cytokine Responses

To determine whether *P. falciparum*-induced production of cytokines and chemokines by PBMCs is associated with malarial disease severity in PNG children, PBMCs from the HC, UM, and SM cases were co-cultured with pRBC at a ratio of 1:3, for 72 hours. This time point was chosen to maximize the elicitation outputs of innate immune response pathways while minimizing the relative contribution of acquired immune responses [6, 11]. Additionally, PBMC samples were cultured with PHA to assess cell viability and media to determine background cytokine production. Concentrations of cytokines and chemokines in cell culture supernatants were measured via FACS-based CBA or capture ELISA.

Cytokine and chemokine responses to pRBC and PHA are shown in Figures 1 and 2, respectively. Compared with UM and HC, SM cases had significantly higher IL-10, IP-10, MIP-1 $\beta$ , and MCP-2 responses to pRBC ( $P \leq .035$ ) (Figure 1). Median TNF ( $P = .0003$ ) and MIP-1 $\alpha$  ( $P = .0013$ ) responses to pRBC in the SM group were significantly higher than the UM group (Figure 1). Compared with UM and HC, SM cases had significantly higher IL-1 $\beta$ , IL-6, and MIP-1 $\alpha$  responses to PHA ( $P \leq .027$ ) (Figure 2). Median MIP-1 $\beta$  levels were significantly higher in the SM than HC group ( $P = .032$ ), and MCP-2 levels were significantly higher in the SM compared with the UM group ( $P = .0066$ ; Figure 2).

Multinomial logistic regression analysis was performed to investigate associations between severe malaria and unit increases (100 pg/mL for IL-10 and 1000 pg/mL for all others) in cytokines or chemokines produced (Table 1). Parasite-elicited IL-

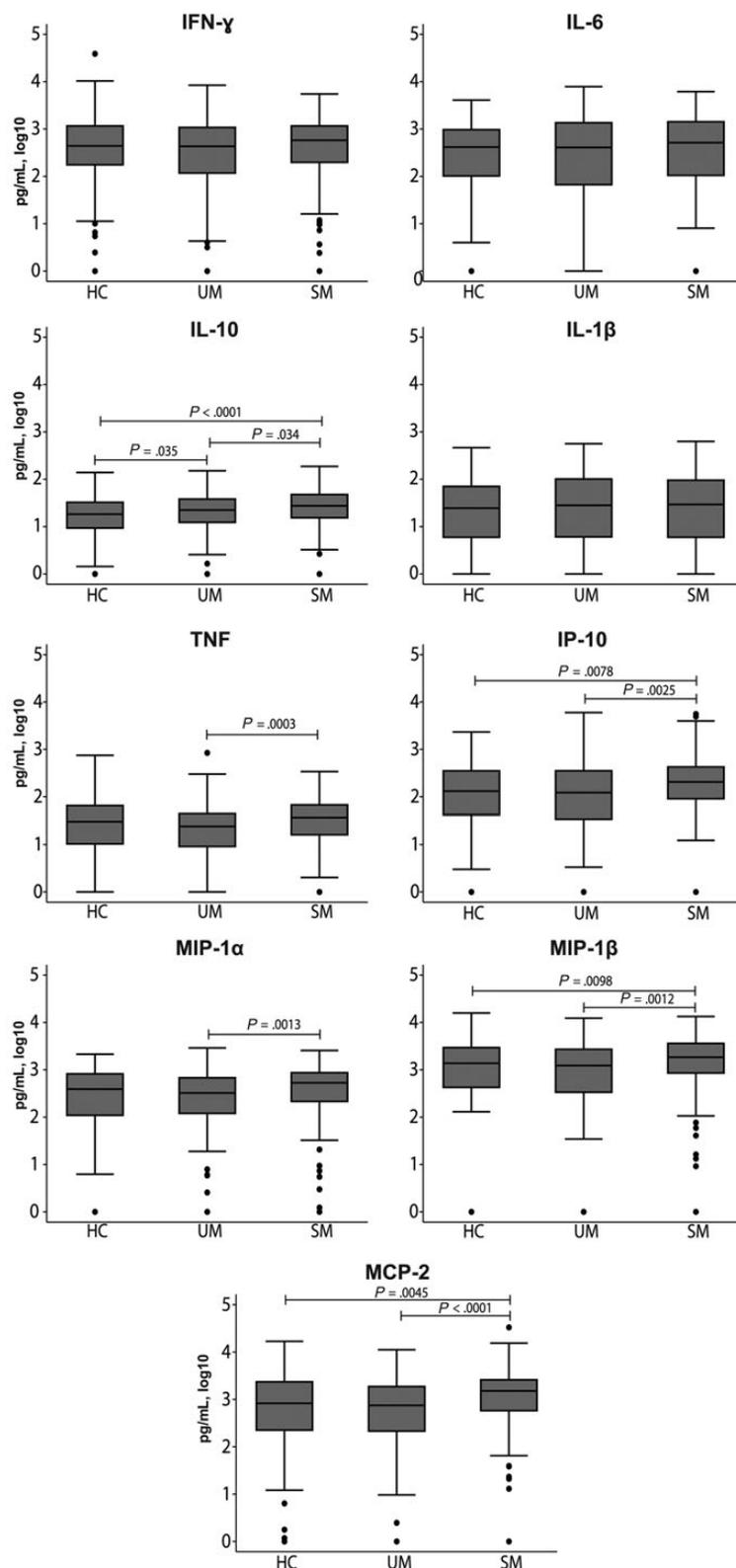
10, IL-6, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-2 was associated with increased odds of severe malaria (odds ratio [OR] 1.01–5.74,  $P \leq .04$ ). Adjusting for the covariates age, sex, ethnicity, and parasite positivity (any species) at the time of PBMC sampling did not alter the magnitude of the associations between cytokine responses and odds of severe malaria (Table 1).

### Associations Between Cytokine/chemokine Responses to *P. falciparum* and Severe Malaria Syndromes

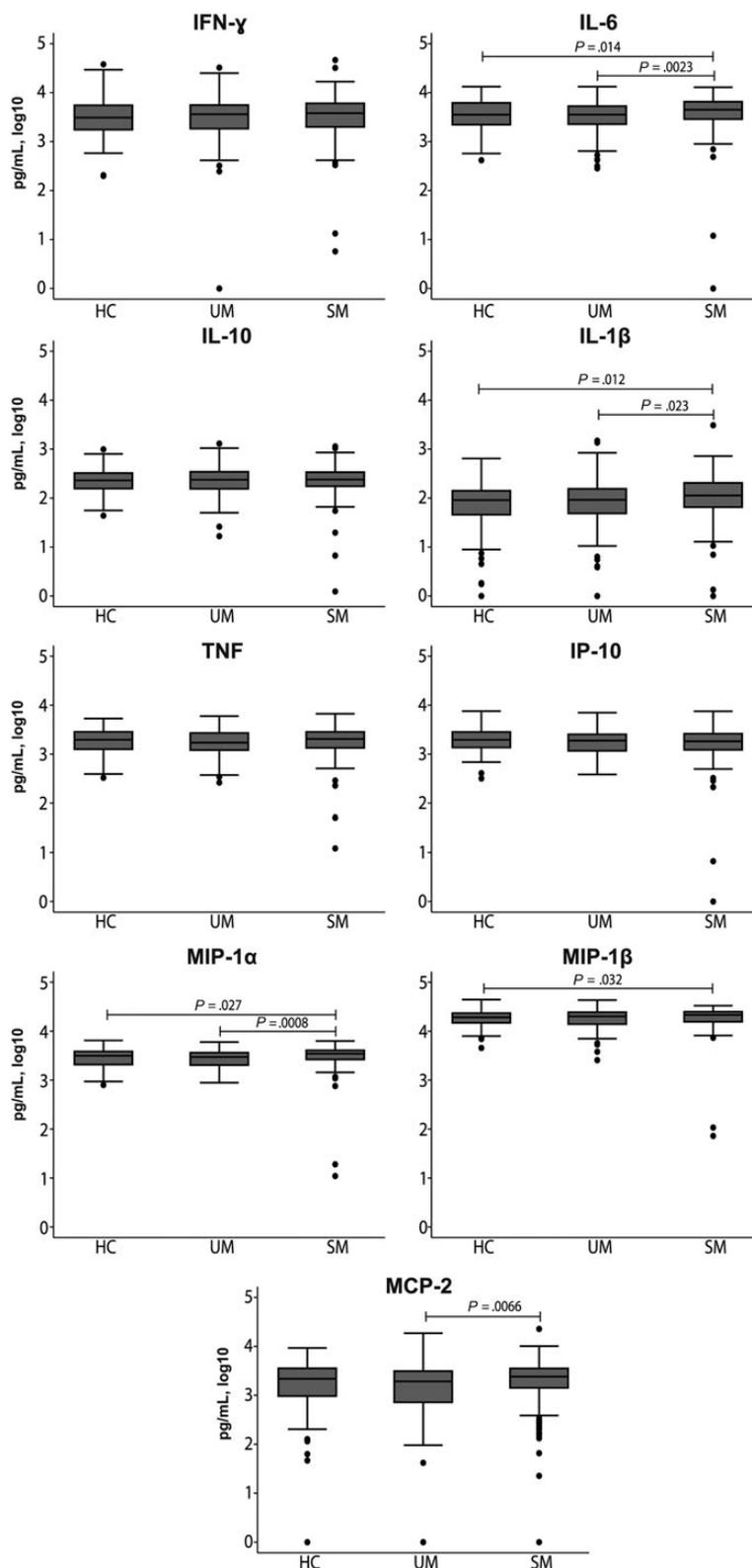
Severe malaria is associated with diverse but overlapping syndromes, including severe anemia, respiratory distress, coma, hyperlactatemia and metabolic acidosis. We investigated associations between various severe malaria clinical syndromes and individual cytokine and chemokine responses to pRBC, using UM controls as the comparator (Table 2). SM cases with respiratory distress ( $n = 23$ ) had significantly higher IL-6 and MIP-1 $\alpha$  responses to pRBC compared with UM controls ( $P \leq .032$ ). SM cases with severe anemia ( $n = 45$ ) had significantly higher TNF ( $P = .048$ ) and MCP-2 ( $P = .0049$ ) responses; those with deep coma ( $n = 20$ ) had higher IFN- $\gamma$ , IP-10, TNF, MIP-1, MIP-1 $\beta$ , and MCP-2 responses to pRBC ( $P \leq .022$ , Table 2). SM cases with metabolic acidosis ( $n = 21$ ) had higher IL-10, MIP-1 $\beta$ , and MCP-2 ( $P \leq .034$ , Table 2), and those with hyperlactatemia ( $n = 38$ ) had higher IL-10, IL-6, TNF, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-2 responses ( $P \leq .046$ , Table 2). Additionally, associations between BCS and cytokine and chemokine responses to pRBC were assessed. The BCS of uncomplicated malaria controls ( $\text{BCS} = 5$ ) and severe malaria cases ( $\text{BCS} = 0$  to 5) was significantly negatively associated with TNF ( $P = .008$ ) and MIP-1 $\alpha$  responses ( $P = .038$ ; Figure 3), suggesting a relationship between coma scores and elevated production of these factors. No other cytokine/chemokine responses were significantly associated with BCS.

### Cellular Sources of Cytokines and Chemokines

PBMCs from the top 32 responders for IP-10, TNF, IFN- $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , and IL-10 were applied into short-term elicitation assays with pRBCs and uRBCs to determine the cellular sources of these cytokines and chemokines.  $\gamma\delta$  T cells were the major source of IFN- $\gamma$  in 27/32 donors tested, with a contribution by CD4 $^+$  T cells in 13/32 donors tested (Figure 4 and Table 3).  $\gamma\delta$  T cells were also an important source of TNF (24/32), MIP-1 $\alpha$  (23/32), and MIP-1 $\beta$  (28/32). CD14 $^+$  cells (monocyte/macrophages) were also responsible for production of TNF, MIP-1 $\alpha$ , and MIP-1 $\beta$  in 26/29, 22/29 and 23/29 donors, respectively (Figure 4 and Table 3). Notably, NK cells were not major sources of TNF and IFN- $\gamma$ , in agreement with prior studies [6, 11]. In a moderate proportion of donors, NK cells produced MIP-1 $\alpha$  and MIP-1 $\beta$ . IL-10 was detected in a number of cell populations, predominantly CD4 $^+$  T cells. IP-10 from CD14 $^+$  cells was only detected in one donor individual.



**Figure 1.** *Plasmodium falciparum*-elicited cytokine and chemokine responses in samples from healthy community controls and convalescent samples from individuals with uncomplicated malaria or severe malaria. Peripheral blood mononuclear cells were stimulated with *P. falciparum* parasitized red blood cells. Cytokine/chemokine measurements (pg/mL) were log<sub>10</sub> transformed after adding 1 to the original concentration value. Horizontal lines represent medians; boxes represent 25th and 75th percentiles; whiskers represent the 5th and 95th percentiles. Statistically significant differences ( $P \leq .05$ ) are indicated.  $P$  values were calculated by Mann–Whitney test. Abbreviations: HC, healthy community controls; IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; SM, severe malaria; TNF, tumour necrosis factor; UM, uncomplicated malaria.



**Figure 2.** Phytohaemagglutinin-elicited cytokine and chemokine responses in samples from healthy community controls and convalescent samples from individuals with uncomplicated malaria or severe malaria. Peripheral blood mononuclear cells were stimulated with phytohaemagglutinin. Cytokine/chemokine measurements (pg/mL) were log<sub>10</sub> transformed after adding 1 to the original concentration value. Horizontal lines represent medians; boxes represent 25th and 75th percentiles; whiskers represent the 5th and 95th percentiles. Statistically significant differences ( $P \leq .05$ ) are indicated.  $P$  values were calculated by Mann–Whitney test. Abbreviations: HC, healthy community controls; IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; SM, severe malaria; TNF, tumour necrosis factor; UM, uncomplicated malaria.

**Table 1. Multinomial Logistic Regression Analysis of Cytokine/Chemokine Responses to *P. falciparum* Infected Red Blood Cells**

Cytokine/Chemokine	Odds of Severe Malaria (Healthy Controls as Base Outcome)			Odds of Severe Malaria (Uncomplicated Malaria as Base Outcome)		
	OR <sup>a</sup>	95% CI	P Value	OR <sup>a</sup>	95% CI	P Value
IFN- $\gamma$	0.90	[.78, 1.03]	.13	0.97	[.83, 1.14]	.71
IL-10	5.68	[2.31, 13.98]	<.001	2.42	[1.10, 5.35]	.029
IL-1 $\beta$	8.30	[.63, 109.75]	.11	0.84	[.089, 7.89]	.88
IL-6	1.32	[1.07, 1.63]	.009	1.003	[.85, 1.19]	.97
IP-10	1.40	[.94, 2.09]	.096	1.07	[.79, 1.45]	.68
TNF	1.69	[.089, 32.1]	.73	3.33	[.13, 88.6]	.47
MIP-1 $\alpha$	1.41	[.95, 2.10]	.092	1.77	[1.15, 2.72]	.009
MIP-1 $\beta$	1.08	[.99, 1.17]	.094	1.10	[1.004, 1.21]	.04
MCP-2	1.11	[1.00, 1.24]	.056	1.28	[1.11, 1.47]	.001
Cytokine/Chemokine	aOR <sup>b</sup>	95% CI	P Value	aOR <sup>b</sup>	95% CI	P Value
IFN- $\gamma$	0.89	[.77, 1.03]	.12	0.96	[.81, 1.12]	.57
IL-10	5.59	[2.25, 13.89]	<.001	2.31	[1.08, 5.17]	.043
IL-1 $\beta$	11.71	[.84, 163.19]	.067	1.01	[.10, 9.97]	.99
IL-6	1.33	[1.08, 1.65]	.008	1.03	[.87, 1.23]	.72
IP-10	1.38	[.92, 2.07]	.12	1.05	[.76, 1.44]	.78
TNF	1.59	[.081, 31.16]	.76	2.99	[.11, 78.27]	.51
MIP-1 $\alpha$	1.42	[.95, 2.12]	.09	1.86	[1.20, 2.91]	.006
MIP-1 $\beta$	1.08	[.99, 1.18]	.09	1.10	[1.00, 1.21]	.04
MCP-2	1.10	[.99, 1.23]	.086	1.29	[1.11, 1.49]	.001

Abbreviations: IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; OR, odds ratio; pRBC, *P. falciparum* infected red blood cell; TNF, tumour necrosis factor.

<sup>a</sup> Odds ratios (per 1000 pg/mL increase in cytokine response to pRBC) were calculated using multinomial logistic regression. For IL-10, this was calculated per 100 pg/mL increase in cytokine response to pRBC.

<sup>b</sup> Adjusted Odds ratios (per 1000 pg/mL increase in cytokine response to pRBC) were calculated using multinomial logistic regression, adjusting for age, sex, province of parents' birth (ethnicity), and parasite positivity at the time of PBMC sampling. For IL-10, this was calculated per 100 pg/mL increase in cytokine response to pRBC.

## DISCUSSION

PBMC elicitation has recently been shown to predict risk of acute malaria morbidity in longitudinal cohort studies [10, 11] but has not yet been applied to association analysis in relation to severe disease. The low incidence of severe malaria precludes prospective assessment of risk for immunological variables, necessitating case-control designs. Clinical malaria at presentation profoundly changes the cellular composition of the peripheral leukocyte compartment and induces cellular anergy/hyporesponsiveness [22–26]. These changes homeostatically renormalize during convalescence [8, 24]. Therefore, we compared cytokine/chemokine production from PBMCs drawn from convalescent children previously diagnosed with either severe or uncomplicated malaria, with healthy community controls. The very low SM case-fatality rates in PNG preclude bias due to fatality. PBMCs from SM individuals had significantly higher IL-10, IP-10, MIP-1 $\beta$ , and MCP-2 responses to pRBC compared with the UM and HC individuals and

significantly higher TNF and MIP-1 $\alpha$  responses compared with UM individuals. Interestingly, with the exception of IL-10, no differences were observed between the HC and UM groups, indicating that recent, uncomplicated malaria infection and treatment does not bias PBMC-derived innate cytokine/chemokine production. This comparability between UM and HC groups suggests the elevated cytokine/chemokine responses observed in the SM group do not simply reflect recent malaria exposure and treatment but rather reflects an intrinsic immunological responsiveness associated with susceptibility to SM.

Increased production of IL-6, IL-10, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-2 was associated with severe malaria compared to either or both of the control groups. Excessive production of pro-inflammatory cytokines may contribute to disease in a number of ways, as reviewed [1–3]. Chemokines may contribute to severe disease by recruiting leukocytes to sites of parasite sequestration. Chemokines MIP-1 $\alpha$  and MIP-1 $\beta$  may contribute to inflammation by inducing macrophage proliferation and stimulating the secretion of TNF, IL-6, and IL-1 $\alpha$ . To date,

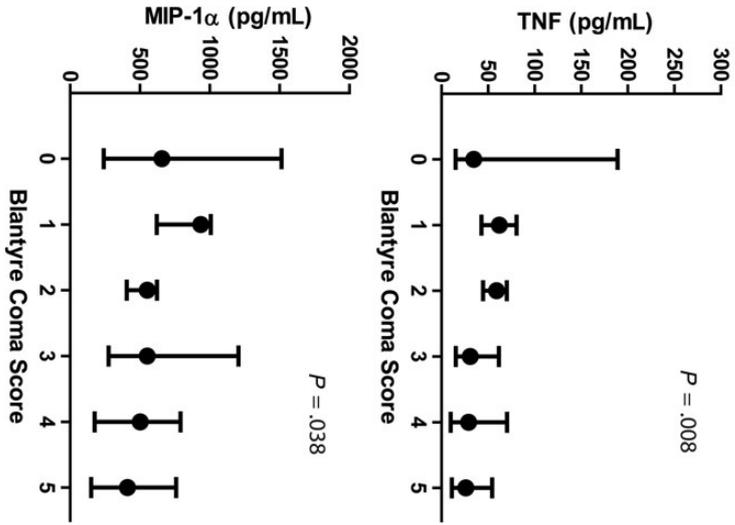
**Table 2. *P. falciparum* Infected Red Blood Cell-elicited Cytokine and Chemokine Responses According to Severe Malaria Syndromes**

Cytokine/ Chemokine	Uncomplicated Malaria			Respiratory Distress			Severe Anemia			Severe Deep Coma			Metabolic Acidosis			Hyperlactatemia		
	n	Value	<i>P</i> Value <sup>a</sup>	n	Value	<i>P</i> Value <sup>a</sup>	n	Value	<i>P</i> Value <sup>a</sup>	n	Value	<i>P</i> Value <sup>a</sup>	n	Value	<i>P</i> value <sup>a</sup>	n	Value	<i>P</i> Value <sup>a</sup>
IFN- $\gamma$	153	430 [116,1078]	.23	23	576 [328,1176]	.23	45	599 [215,1010]	.26	20	853 [472,1358]	.022	21	456 [234,1157]	.57	38	726 [264,1181]	.13
IL-10	153	21.4 [11.3,37.2]	.23	23	30.1 [15.0,45.9]	.23	45	25.6 [17.1,36.7]	.27	20	23.4 [13.7,34.6]	.78	21	44.3 [16.2,67.8]	.0078	38	38.1 [21.7,58.8]	.0013
IL-1 $\beta$	153	27.2 [5.05,100]	.26	23	49.1 [13.4,113]	.26	45	35.6 [5.48,75.9]	.73	20	29.7 [4.77,59.8]	.74	21	46.6 [5.48,108]	.59	38	66.5 [10.2,121]	.075
IL-6	153	408 [66.0,1354]	.032	23	1290 [391,2372]	.032	45	492 [154,1608]	.45	20	433 [156,1099]	.998	21	780 [176,1872]	.17	38	1046 [216,1867]	.033
IP-10	153	121 [33.3,352]	.47	23	113 [76.1,421]	.47	45	151 [75.7,423]	.086	20	386 [111,562]	.012	21	251 [59.1,521]	.21	38	214 [88.9,421]	.096
TNF	153	23.2 [8.11,43.2]	.052	23	38.2 [21.3,52.5]	.052	45	30.9 [17.2,43.8]	.048	20	54.4 [37.9,71.3]	.0007	21	35.8 [20.6,62.2]	.054	38	40.1 [20.0,69.0]	.0059
MIP-1 $\alpha$	153	323 [119,677]	.016	23	545 [314,835]	.016	45	460 [276,753]	.059	20	592 [433,778]	.016	21	666 [194,935]	.11	38	648 [416,912]	.0013
MIP-1 $\beta$	153	1225 [335,2718]	.11	23	1820 [739,3746]	.11	45	1628 [1102,3077]	.051	20	2343 [1191,4134]	.020	21	2367 [866,4051]	.034	38	2065 [866,3542]	.046
MCP-2	146	749 [213,1870]	.18	21	1259 [311,2160]	.18	44	1353 [642,2754]	.0049	19	1966 [514,2750]	.011	21	2060 [808,3317]	.0015	37	1792 [623,2795]	.0064

Data are presented as median [interquartile range] and represent comparisons between individuals with uncomplicated malaria and individuals with different severe malaria syndromes.

Abbreviations: IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; TNF, tumour necrosis factor.

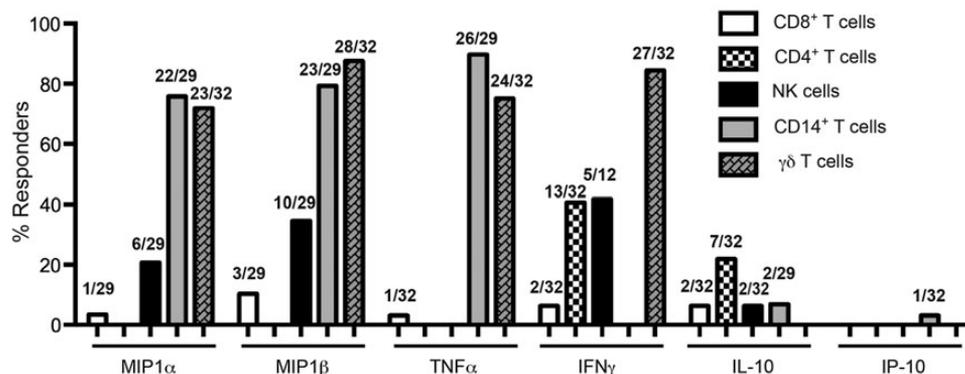
<sup>a</sup> *P* values are the result of Mann-Whitney tests.



**Figure 3.** Association between BCS and *Plasmodium falciparum*-elicited TNF and MIP-1 $\alpha$  responses in convalescent samples from individuals with severe or uncomplicated malaria. Children with severe malaria were assigned a BCS from 0–5 and children with uncomplicated malaria were assigned a BCS of 5. For BCS 0: n = 4; BCS 1: n = 3; BCS 2: n = 13; BCS 3: n = 15; BCS 4: n = 29; BCS 5: n = 290. Symbols: median. Error bars: 25th and 75th percentiles. *P* values were calculated using a nonparametric test for trend. Abbreviations: MIP, macrophage inflammatory protein; TNF, tumour necrosis factor.

there are few studies that have addressed the role of chemokines in severe human malaria. High serum MIP-1 $\alpha$  and MIP-1 $\beta$  have been described in acute *P. falciparum* malaria [16], and IP-10 is associated with an increased risk of cerebral malaria mortality [33, 34]. However, measuring serum chemokines levels may be subject to similar caveats identified for cytokines. Therefore, a novel finding of this study is the association between SM and high PBMC-derived chemokine responses, providing support for a direct role for these mediators in pathogenesis.

We show here the predominant cell populations producing cytokines and chemokines associated with severe malaria are CD14<sup>+</sup> and  $\gamma\delta$  T cells. A role for CD14<sup>+</sup> cells in the pathogenesis of severe malaria is consistent with observations of placental monocyte infiltration during malaria in pregnancy, and placental plasma concentrations of chemokines such as MIP-1 $\alpha$  associated with this condition [35]. Monocyte infiltrates are also reported from a proportion of brain autopsies from fatal cerebral malaria cases.



**Figure 4.** Cellular Sources of *Plasmodium falciparum*-elicited cytokines and chemokines in convalescent samples from individuals with severe malaria. Peripheral blood mononuclear cells from the top 32 responders for MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF, IFN- $\gamma$ , IL-10, and IP-10 were stimulated with *P. falciparum* parasitized red blood cells to determine the cellular source of these cytokines/chemokines. Numbers indicate the number of responders/total samples tested where a responding individual was defined as having a frequency of cytokine or chemokine positive cells  $\geq 0.02\%$  for CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and  $\gamma\delta$  T cells and  $\geq 1\%$  for CD14<sup>+</sup> cells following background subtraction or had a frequency of responding cells twice above the background. Abbreviations: IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; NK cells, natural killer cells; TNF, tumour necrosis factor;  $\gamma\delta$ , gamma delta T cells.

In peripheral blood,  $\gamma\delta$  T cells express typical surface markers associated with conventional T cells and can display memory-like features including prolonged recall responses upon reinfection [36–39]. This study demonstrates that  $\gamma\delta$  T cells are the dominant source of MIP-1 $\alpha$  and MIP-1 $\beta$  associated with SM. The functionally diverse nature of  $\gamma\delta$  T cells and their specificity for restricted TCR ligands suggest this population may be an attractive target for preventive vaccination, that is, inducing a functional bias in  $\gamma\delta$  T-cell cytokine/chemokine output by vaccination with conserved malarial ligands may protect against severe disease.  $\gamma\delta$  T cells have been explored as immunotherapeutic targets in cancer settings with varying success (reviewed in [40]).

In conclusion, these findings have significant implications for understanding susceptibility to severe malarial disease and the development of possible vaccination for preventing severe malaria. The association between elevated cytokine/chemokine production by innate immune cells and risk of developing severe malaria suggest that intrinsic differences in an individual's immunological responsiveness can influence susceptibility to severe disease and are consistent with a role for monocyte activation and recruitment in severe malaria. Further studies may determine if this differential susceptibility is genetically linked and whether modulating  $\gamma\delta$  T cells by prior exposure to their conserved antigenic targets can prevent severe disease.

**Table 3.** Frequency of Cytokine and Chemokine Responding Cells to *P. falciparum* Infected Red Blood Cells

Cytokine/Chemokine	CD4 <sup>+</sup> T cells		CD8 <sup>+</sup> T cells		$\gamma\delta$ T cells		CD14 <sup>+</sup> T cells		NK cells	
	n <sup>a</sup>	Frequency	n <sup>a</sup>	Frequency	n <sup>a</sup>	Frequency	n <sup>a</sup>	Frequency	n <sup>a</sup>	Frequency
IFN- $\gamma$	13	0.02–0.3	2	0.08–0.4	27	0.1–2.4	0	ND	5	0.05–1.3
TNF	0	ND	1	0.1	24	0.12–1.2	26	1.95–29.7	0	ND
IL-10	7	0.05–0.8	2	0.09–0.3	0	ND	2	1.0–2.9	2	0.7–1.1
MIP-1 $\alpha$	0	ND	1	0.3	23	0.4–3.9	22	6.0–60.9	6	0.9–2.0
MIP-1 $\beta$	0	ND	3	1.65–5.9	28	0.3–5.7	23	8.0–64.6	10	2.0–14.8
IP-10	0	ND	0	ND	0	ND	1	1	0	ND

Data are presented as minimum-maximum frequency (%) of each cell population in responding individuals.

Abbreviations: IFN, interferon; IL, interleukin; IP, interferon gamma-induced protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; ND, not detected; TNF, tumour necrosis factor.

<sup>a</sup> n = Number of Responders. Responding individuals were defined as having a frequency of cytokine or chemokine positive cells  $\geq 0.02\%$  for CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells and  $\gamma\delta$  T cells and  $\geq 1\%$  for CD14<sup>+</sup> cells following background subtraction or had a frequency of responding cells twice above the background.

## Supplementary Data

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

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