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Malaria Blood Stage Parasites Activate Human Plasmacytoid Dendritic Cells and Murine Dendritic Cells through a Toll-Like Receptor 9-Dependent Pathway[1]

Sathit Pichyangkul,[2]* Kosol Yongvanitchit,* Utaiwan Kum-arb,* Hiroaki Hemmi,[†]
Shizuo Akira,[†] Arthur M. Krieg,[‡] D. Gray Heppner,[§] V. Ann Stewart,[§] Hitoshi Hasegawa,[¶]
Sornchai Looareesuwan,[‖] G. Dennis Shanks,* and R. Scott Miller*

A common feature of severe *Plasmodium falciparum* infection is the increased systemic release of proinflammatory cytokines that contributes to the pathogenesis of malaria. Using human blood, we found that blood stage schizonts or soluble schizont extracts activated plasmacytoid dendritic cells (PDCs) to up-regulate CD86 expression and produce IFN-α. IFN-α production was also detected in malaria-infected patients, but the levels of circulating PDCs were markedly reduced, possibly because of schizont-stimulated up-regulation of CCR7, which is critical for PDC migration. The schizont-stimulated PDCs elicited a poor T cell response, but promoted $\gamma\delta$ T cell proliferation and IFN-γ production. The schizont immune stimulatory effects could be reproduced using murine DCs and required the Toll-like receptor 9 (TLR9)-MyD88 signaling pathway. Although the only known TLR9 ligand is CpG motifs in pathogen DNA, the activity of the soluble schizont extract was far greater than that of schizont DNA, and it was heat labile and precipitable with ammonium sulfate, unlike the activity of bacterial DNA. These results demonstrate that schizont extracts contain a novel and previously unknown ligand for TLR9 and suggest that the stimulatory effects of this ligand on PDCs may play a key role in immunoregulation and immunopathogenesis of human falciparum malaria. *The Journal of Immunology,* 2004, 172: 4926–4933.

M alaria is still the world's leading parasitic disease. Each year, there are ~300–500 million episodes of acute malaria illness, and between 1 and 3 million people die from the disease, mostly children in Africa (1). The interaction of malaria parasites with the immune system of the host is complicated and poorly understood. Specific immunity to natural infection is acquired slowly despite a high degree of repeated exposure. Malaria parasites have devised many strategies to escape from the host's immune response, but the host innate immune system may have evolved the ability to detect parasite molecules. The parasites have a four-stage life cycle with a stage-specific expression of many proteins at each stage. These proteins tend to be highly polymorphic, and thus they are able to evade Ag-specific immunity. The majority of the malaria parasite's life cycle occurs within the RBC, thus excluding them both from the Ab attack and from killing by CTLs, as mature RBC do not express HLA molecules. Late blood stage parasites adhere to the endothelium of blood vessels, helping them to evade splenic clearance. The binding of blood stage parasites to myeloid dendritic cells (DCs)[3] inhibits cell maturation, leading to a suppression of the T cell response (2).

Malaria parasites are almost entirely confined to the bloodstream, suggesting that the blood stage parasites and their products continue to interact with host immune cells. Parasite products released from rupturing schizonts have been shown to activate cellular components of the innate immune system to produce proinflammatory cytokines (3). In human blood, there are two subpopulations of DC precursors, which can be identified by cell phenotype and morphology. CD11c$^+$ DCs are myeloid in appearance and express myeloid markers (CD13 and CD 33) (4–6), whereas CD11c$^-$ DCs have a negligible expression of myeloid markers, but express high levels of the IL-3R (CD123) (6). Due to their plasma cell-like morphology, CD11c$^-$ DCs have been called plasmacytoid DCs (PDCs) (6–8). CD11c$^+$ myeloid DCs and PDCs express different Toll-like receptors (TLRs) (9). In response to TLR2 and TLR4 ligands, CD11c$^+$ myeloid DCs produce TNF-α, IL-6, and IL-12 (10), whereas PDCs produce mainly IFN-α in response to TLR9 ligand (11). A recent study demonstrated that both CD11c$^+$ myeloid DCs and PDCs express TLR7, but their cytokine response to TLR7 ligand is subset specific, in that CD11c$^+$ myeloid DCs produce IL-12, and PDCs produce IFN-α (12). Taken together, these observations suggest that both DC subsets may be programmed to perform distinct functions through their lineage and their differences in TLR expression.

In addition to replenishing the pool of tissue-residing immature DCs, these circulating DC precursors could play a critical role in both innate and adaptive immunity against pathogens that can access the bloodstream, such as blood stage malaria. In the present

*Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; †Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; ‡Coley Pharmaceutical Group, Wellesley, MA 02481; §Department of Immunology, Walter Reed Army Institute of Research, Silver Spring, MD 20910; ¶Ehime University School of Medicine, Ehime, Japan; and ‖Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

[2] Address correspondence and reprint requests to Dr. Sathit Pichyangkul, Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok 10400, Thailand. E-mail address: sathitp@afrims.org

[3] Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow DC; MoDC, monocyte-derived DC; PDC, plasmacytoid DC; TLR, Toll-like receptor.

study we describe the interaction of *P. falciparum* blood stage schizonts with PDCs and show how such interaction can modulate other immunological responses.

Materials and Methods

Abs and reagents

mAbs against CD3, CD4, CD8, CD14, CD16, CD56, CD20, CD11c, CD11b, TCR-γδ, CD45RA, CD 45RO, CD69, CD123, and HLA-DR were purchased from BD Biosciences (San Jose, CA). mAbs against CD40, CD86, IFN-γ, and IL-4 were purchased from BD PharMingen (San Diego, CA). mAb against CCR7 was obtained from hybridoma clone CCR7.6B3 (13). Histopaque-1077, Percoll, *Escherichia coli* LPS, and *E. coli* DNA were purchased from Sigma-Aldrich (St. Louis, MO). Human GM-CSF, IL-4, and ELISA kits for human cytokines were obtained from R&D Systems (Minneapolis, MN). ELISA kits for mouse IFN-α and IL-12 p40 were purchased from PBL Biomedical Laboratories (New Brunswick, NJ) and Techne Laboratories (Minneapolis, MN), respectively. Human Flt3 ligand was obtained from PeproTech EC (London, U.K.). CpG ODN 2216 (ggGGGACGATCGTCGggggGG) and CpG ODN 2006 (tcgtcgttttgtcgttttgtcgtt) were obtained from Coley Pharmaceutical Group (Wellesley, MA). CpG ODN D19 (ggTGCATCGATGCAgggggG) was obtained from Hokkaido System Science (Sapporo, Japan). Sequences are shown 5′-3′; lowercase letters represent phosphorothioate linkage, and capital letters represent phosphodiester linkage 3′ of the base.

Parasite cultures and parasite product preparation

A laboratory strain (TM267R) and different field isolates (GR, MRU, LA, and PH) of *P. falciparum* were cultured in group O$^+$ human RBC suspended in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated human serum. All parasite cultures were free of mycoplasma contamination, as detected by a commercially available PCR method (American Type Culture Collection, Manassas, VA). Schizont stage parasites were purified by sedimentation through 63% Percoll. This technique normally produces schizonts with a purity of ~80%. Schizonts were suspended in RPMI 1640 and subjected to two or three freeze-thaw cycles to obtain the lysate. The schizont-soluble fraction (particulate free) was obtained by centrifugation of the lysate at the highest speed for 5 min using a microcentrifuge (Eppendorf; Brinkmann Instruments, Westbury, NY). This centrifugation process was repeated two or three times until a clarified fraction was obtained. In some experiments this soluble fraction was heated at 100°C for 15 min or precipitated with ammonium sulfate (80%). The pellet from ammonium sulfate fractionation was dissolved in RPMI 1640 and dialyzed for 48 h, then the volume was adjusted to the original volume.

Cell purification and stimulation

Peripheral venous blood was obtained from healthy human donors. PBMC were obtained by centrifugation using Ficoll-Hypaque. PBMC preparations were separated into a T cell-depleted population and an enriched T cell population by rosetting with neuraminidase-treated sheep RBC. To purify the PDCs (CD123$^+$ DCs), T cell-depleted preparations were stained with mAbs against HLA-DR (FITC) and CD123 (PE). CD123^{bright} HLA-DR$^+$ cells were then sorted with FACSVantage (BD Biosciences, Mountain View, CA) (14). The sorted cells had a typical plasma cell-like morphology and did not express either CD11c or lineage marker. For myeloid CD11c$^+$ DC purification, T cell-depleted preparations were stained with a mixture of Abs, including mAbs against CD3, CD14, CD16, CD56, CD20 (FITC), and CD11c (PE). Lineage$^-$ and CD11c^{bright} cells were sorted with FACSVantage (BD Biosciences) (14). The sorted cells were analyzed and were found to express HLA-DR, but minimally expressed CD123 and had a typical myeloid morphology. γδT cells, NK cells, B cells, and monocytes were purified from PBMC as TCR-γδ$^+$, CD56$^+$, CD20$^+$, and CD14$^+$ cells, respectively. The αβT cells were purified by negative sorting of enriched T cell populations that had been stained with mAbs against CD56, CD20, CD14, and TCR-γδ (FITC). The purity of all sorted populations ranged from 92–97%. Different cell populations were stimulated with intact schizonts, schizont lysate, or soluble fraction derived from schizont lysate for 24–48 h. Schizont lysate and soluble fraction were used at concentrations equivalent to a cell:schizont ratio of 1:10 throughout the study. Cell activation was determined by monitoring the expression of costimulatory molecule (CD40, CD86), activation marker (CD69), or production of cytokines (IFN-α, TNF-α, IFN-γ). Activation of granulocytes was performed in whole blood cultures. After 1–2 h of incubation with soluble fraction, whole blood cells were stained with mAbs against CD16 (FITC) and CD11b/Mac-1 (PE), then treated with RBC lysing solution (BD Bio-

sciences). Leukocytes were analyzed by flow cytometry using a side light scatter/forward angle dot plot. CD16$^+$ cells in the granulocyte gate were identified as granulocytes, then analyzed for up-regulation of CD11b/Mac-1 expression. LPS (500 ng/ml) was used as a positive control for stimulation of CD11c$^+$ DCs, monocytes, and granulocytes, and CpG ODN 2216 and 2006 (10 μg/ml) were used as positive controls for PDC and B cell activation, respectively. For αβT and NK cell activation, PMA (50 ng/ml) and ionomycin (50 μg/ml) were used as a positive control.

To evaluate the up-regulation of CCR7 expression, enriched PDC populations from healthy donors were used. Lineage$^-$ CD11c$^-$ cells were sorted from T cell-depleted preparations that had been stained with FITC-conjugated mAbs against CD3, CD14, CD16, CD20, CD56, and CD11c. The sorted lineage$^-$CD11c$^-$ cells (consisted of 25–35% PDCs) were stimulated with intact schizonts for 48 h. The stimulated cells were stained with purified mAb against CCR7, followed by APC-conjugated second Ab, and finally, they were stained with mAbs against CD123 (PE) and HLA-DR (PerCP). CD123^{bright} HLA-DR$^+$ cells were gated as PDCs and then analyzed for CCR7 expression.

Allogeneic T cell response

Flow cytometrically sorted PDCs were stimulated with schizont lysate overnight. Monocyte-derived DCs (MoDCs) were prepared from flow cytometrically sorted CD14$^+$ cells and were cultured with 50 ng/ml GM-CSF and IL-4 for 5–7 days. They were then stimulated overnight with *E. coli* LPS (1 μg/ml). PDCs and MoDCs (control and stimulated) were plated at different cell concentrations in 96-well, U-bottom plates (Costar; Corning Glass, Corning, NY). Allogeneic naive CD4$^+$ T cells were obtained by negative sorting of enriched T cell populations that had been stained with mAbs against CD8 (FITC), CD20 (FITC), CD56 (FITC), CD45RO (FITC), and TCR-γδ (PE). This technique provides CD4$^+$CD45RA$^+$ naive T cells with a purity of >90%. Flow cytometrically sorted allogeneic naive T cells (1 × 10^5 cells/well) were added in culture wells that contained control and stimulated PDCs or MoDCs. The proliferative response was measured on day 5 by [^{3}H]thymidine uptake. Some of cell cultures were used to evaluate Th cell differentiation induced by schizont lysate-stimulated PDCs. On day 6, they were restimulated with PMA (50 ng/ml) and ionomycin (50 μg/ml) for 5 h, and brefeldin (10 μg/ml) was added during the last 3 h. The stimulated cells were then stained with mAb against CD4, fixed, and permeabilized (BD PharMingen), then stained with mAbs against IFN-γ and IL-4. CD4$^+$ T cells were gated, then analyzed for intracellular cytokines by FACSCalibur (BD Biosciences).

γδ T cell response

Enriched T cells (2 × 10^5 cells/well) were cocultured with flow cytometrically sorted PDCs (4 × 10^4 cells/well) in the presence of schizont lysate. After 40 h of incubation, cell cultures were treated with brefeldin (10 μg/ml) and incubated for another 8 h. Cells were stained with mAbs against CD3 (PerCP) and TCR-γδ (PE), fixed, and permeabilized (BD PharMingen), then stained with mAb against IFN-γ (FITC). CD3$^+$ TCR-γδ$^+$ cells were gated as γδ T cells and analyzed for intracellular IFN-γ by FACSCalibur (BD Biosciences). To evaluate the proliferative response, cell cultures were incubated until day 7, then stained with mAbs against CD3 (PerCP) and TCR-γδ (PE). The increase in γδ T cells in cell cultures was determined by flow cytometry.

Mice

C57BL/6 mice were obtained from CLEA Japan (Tokyo, Japan). MyD88-deficient (MyD88$^{-/-}$) mice were established as described previously (15) and backcrossed over eight generations with C57BL/6. TLR9-deficient (TLR9$^{-/-}$) mice were generated as described previously (11) and backcrossed eight times with C57BL/6 mice. TLR7-deficient (TLR7$^-$/Y) mice were generated as described previously (16) and backcrossed with B6 mice three generations.

Preparation and analysis of mouse bone marrow DCs

Bone marrow cells were prepared from femora and tibia and passed through nylon mesh. Then cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2-ME, and 100 ng/ml human Flt3 ligand. After 7 days, the cells were harvested and used as bone marrow DCs (BMDCs; 80–90% CD11c$^+$) for additional experiments. B220$^+$ and B220$^-$ cells were purified by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, Flt3 ligand-induced BMDCs were incubated with anti-B220 microbeads for 20 min. After washing, B220$^+$ cells were selected by MACS LS column. The B220$^-$ cell fraction was stained with anti-CD11c beads, and B220$^-$CD11c$^+$ cells were then selected by MACS LS column.

The purity of B220$^+$ cells was >85%, and the purity of B220$^-$ cells exceeded 90%. BMDCs, B220$^+$ cells, or B220$^-$ cells were seeded into 96-well plates at a concentration of 2×10^6 cell/ml and stimulated with schizont-soluble fraction, heated schizont-soluble fraction, ammonium sulfate fraction (at an equivalent concentration of cell:schizont, 1:10), CpG ODN D19 (3 μM), or LPS (100 ng/ml) for 24 h. The culture supernatants were collected and analyzed for IFN-α and IL-12p40 production by ELISA. To evaluate surface expression levels of costimulatory molecules, cells were stained with biotinylated anti-CD40 and FITC-labeled anti-CD86, developed with PE-conjugated streptavidin, and analyzed by flow cytometry.

Patients and blood samples

Blood samples were collected from 30 malaria-infected patients (15 complicated and 15 noncomplicated patients) who were admitted at Bangkok Hospital of Tropical Medicine. Ethical approval and informed consent were obtained before blood donation. Complicated malaria was defined by the presence of asexual forms of *P. falciparum* in the blood and one or more of the following symptom: cerebral malaria; hyperparasitemia (>200,000 parasites/μl); spontaneous bleeding from gum, nose, gastrointestinal tract etc.; repeated generalized convulsion; impaired consciousness; prostration; inability to swallow or retain oral medication; hyperpyrexia; or clinical laboratory tests showing severe anemia (hematocrit, <15%; hemogbulin, <5 g/dl, renal failure (serum creatinine >265 μmol/l) or hypoglycemia (whole blood glucose, < 2.2 mmol/l). Patients with none of the above abnormalities were considered to have noncomplicated falciparum malaria. Fifteen control samples were obtained from healthy adult volunteers. For the detection of circulating PDCs, 200 μl of whole blood was stained with an mAb mixture containing lineage-specific mAbs (anti-CD3, anti-CD14, anti-CD16, anti-CD56, anti-CD20; FITC), mAb anti-HLA-DR (PerCP), and mAb anti-CD123 (PE). These stained cells were treated with RBC lysing solution (BD Biosciences), then washed with PBS, and finally reconstituted in 1% paraformaldehyde. Fifty thousand stained cells were analyzed using FACSCalibur (BD Biosciences) and lineage$^-$ HLA-DR$^+$CD123^{bright} cells were identified as PDCs. The absolute numbers of circulating PDCs were obtained by multiplying the percentage of PDCs by the numbers of leukocytes per milliliter of blood.

Detection of cytokines

The production of cytokines in cell cultures were measured by ELISA, as were the serum cytokine levels in healthy donors and malaria-infected patients.

Statistical analysis

The data were analyzed using SigmaStat (Jandel Scientific, San Rafael, CA). The Student's *t* test was used for parametric data, and the Mann-Whitney rank-sum test was used for nonparametric data. A value of $p \leq$ 0.05 was considered significant.

Results

Blood stage schizonts of P. falciparum activate human PDCs

Given the role of PDCs in innate and adaptive immunity (17, 18) and their continuous exposure to blood stage malaria in the bloodstream, we investigated the interaction of PDCs with blood stage schizonts. Highly purified PDCs (>95%) were obtained from peripheral blood of healthy individuals. Cocultures of PDCs with different concentrations of intact schizonts for 48 h led to cell activation. Unlike CpG ODN 2216, which is potent for PDC activation, blood stage schizonts stimulated the PDCs to up-regulate the expression of CD86, but not CD40, and also to induce IFN-α, but not TNF-α, production (Fig. 1, *a* and *b*). The viability of PDCs was maintained in both schizont- and CpG ODN 2216-treated cell cultures (Fig. 1*c*). These PDCs, when stimulated with schizonts, developed into blast cells, but never fully differentiated into mature PDCs associated with DC-like morphology (Fig. 1*d*). The ability of schizonts to stimulate IFN-α production from PDCs is not restricted to only a laboratory strain (TM267R), but was also observed in the field isolates from different geographical regions (GR, MRU, LA, PH; Table I). In addition to intact schizonts, IFN-α-inducing activity was also observed in schizont lysate and in the soluble fraction prepared from the lysate (Table I).
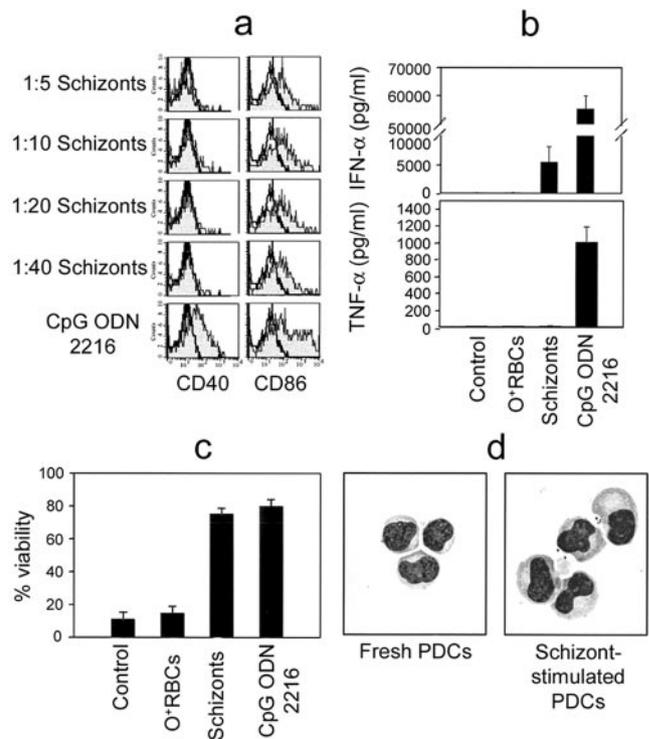

FIGURE 1. *P. falciparum* blood stage schizonts (TM267R) activate PDCs. Purified PDCs (2×10^5 cells/ml) were stimulated with different ratios of cell:schizont (1:5, 1:10, 1:20, and 1:40) or CpG ODN 2216 (10 μg/ml) for 48 h. Cell surface expression of CD40 and CD86 before (solid line) and after (shaded area) stimulation was analyzed by flow cytometry (*a*). Data are representative of the five experiments. Supernatants of PDC cultures that had been stimulated with schizonts (1:10) or CpG ODN 2216 (10 μg/ml) for 48 h were measured for IFN-α and TNF-α. Data are the mean $\pm$ SEM of three separate experiments (*b*). The viability of stimulated cells and control cells from cytokine production experiments was quantitative after 48 h, as determined by trypan blue exclusion. Data are the mean $\pm$ SEM of three separate experiments (*c*). *d*, Giemsa staining of freshly isolated PDCs and schizont-stimulated PDCs at 48 h.

Role of schizont-stimulated PDCs in T cell and γδ T cell responses

We next evaluated the ability of the schizont-stimulated PDCs to prime naive CD4$^+$ T cells. In line with the enhanced expression of CD86, but not CD40, and the fact that this population lacks mature morphology, stimulated PDCs induced a poor allogeneic naive CD4$^+$T cell response compared with monocyte-derived dendritic cells (MoDCs) or LPS-stimulated MoDCs from the same donor (Fig. 2*a*). Intracellular cytokine staining (IFN-γ and IL-4) demonstrated that the schizont-stimulated PDCs did not induce allogeneic naive CD4$^+$T cell response of either Th1 or Th2 bias (Fig. 2*b*).

Circulating γδ T cells markedly increase during malaria infection (19, 20). We therefore also investigated the role of the schizont-stimulated PDCs in specifically promoting γδ T cell responses. Cocultures of enriched T cell populations containing γδ T cells with PDCs in the presence of schizont lysate for 7 days led to a significant increase in the numbers of γδ T cells in cell cultures compared with enriched T cell cultures stimulated with schizont lysate in the absence of PDCs. Fig. 2*c* shows a representative histogram. The mean $\pm$ SEM of four experiments was 15.6 $\pm$ 3.2 vs 0.8 $\pm$ 0.3 (*p* = 0.014). IFN-γ response of γδ T cells was also evaluated by intracellular staining at 48 h of incubation. The percentage of γδ T cells expressing IFN-γ in schizont lysate-stimulated enriched T cell cultures was minimal (7.2 $\pm$ 1.3). When

Table I. *IFN-α production in PDC cultures stimulated with schizonts from different strains of* P. falciparum[a]

Parasites	IFN-α Production (pg/ml)
None	<50
GR schizonts (Thai isolate)	15,887
MRU schizonts (Burma isolate)	10,919
LA schizonts (Lao isolate)	9,820
PH schizonts (Philippine isolate)	4,364
TM267R schizonts (laboratory strain)	9,540
TM267R schizont lysate	12,181
TM267R soluble fraction	8,234

[a] PDCs (2×10^5 cells/ml) were stimulated with schizonts from different strains of *P. falciparum* at a PDC:schizont ratio of 1:10. Schizont lysate and soluble fraction were also used at an equivalent concentration. After 48 h of incubation, culture supernatants were harvested and assayed for IFN-α production.

PDCs were present in those cultures, the numbers of γδ T cells that were positive for IFN-γ was significantly increased by almost 3-fold (19.8 ± 4.0; $p = 0.018$; $n = 5$; Fig. 2d).

Characterization of soluble molecule from schizont lysate

Having established that the soluble fraction from schizont lysate can activate PDCs to produce IFN-α (similar to intact schizonts), we then determined whether this soluble fraction could activate other immune cells. Fig. 3 demonstrates that the schizont-soluble fraction selectively activated PDCs (assayed by CD86 expression and IFN-α production) and γδ T cells (assayed by CD69 expression and IFN-γ production). No activation of the other immune cells by these soluble molecules was observed, including CD11c$^+$ DCs (assayed by CD86 expression and TNF-α production), monocytes (assayed by TNF-α production), granulocytes (assayed by CD11b expression), and NK, B, and αβT cells (assayed by CD69 expression).

We were interested in whether the immunostimulatory activity for PDCs and γδ T cells, found in the schizont-soluble fraction, was due to the same or different molecules. As shown in Table II, heating the soluble fraction at 100°C for 15 min completely abolished the activation of PDCs, but not the activation of γδ T cells. The stimulatory molecule(s) that activated PDCs, but not γδ T cells, was precipitated by ammonium sulfate (80%). *E. coli* DNA, a known activator of PDCs, did not stimulate IFN-γ production from γδ T cells. Heating *E. coli* DNA at 100°C for 15 min had no effect on its ability to activate PDCs to produce IFN-α (Table II).

Role of MyD88 and TLR9 in schizont-stimulated PDCs

TLR9 and TLR7 have been shown to mediate immune cell activation in response to CpG DNA and the antiviral compounds, imidazoquinolines, respectively (11, 16). Some TLR ligands, such as CpG DNA, show some species specificity in their effects, so it was important to confirm that the schizonts would activate mouse DCs as well as human.

In the presence of Flt3 ligand large numbers of CpG DNA responsive DCs can be generated in vitro from mouse bone marrow cells (21, 22). We first evaluated the responses of Flt3 ligand-induced mouse BMDCs to schizont-soluble fraction, heated schizont-soluble fraction, and ammonium sulfate fraction. These BMDCs demonstrated up-regulation of CD40 and CD86 expression in response to schizont-soluble fraction and ammonium sulfate fraction, but not to heated schizont-soluble fraction (Fig. 4a). To examine the role of the TLR-MyD88 signaling pathway in murine schizont-stimulated DCs, we tested schizont-soluble fraction on Flt3 ligand-induced BMDCs derived from TLR7-deficient (TLR7$^-$/Y), TLR9-deficient (TLR9$^{-/-}$), and MyD88-deficient
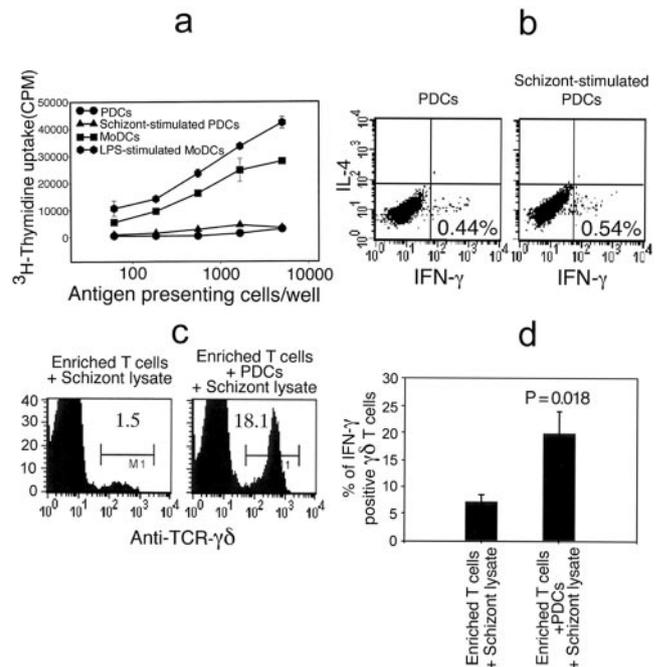

FIGURE 2. The stimulatory capacity of schizont-stimulated PDCs on allogeneic naive T cells and autologous γδ T cells. Control PDCs, schizont lysate-stimulated PDCs, control MoDCs, and LPS-stimulated MoDCs were used to prime naive CD4$^+$ T cells for 5 days, and the proliferative response was then measured (*a*). The production of IFN-γ and IL-4 was measured by intracellular staining in stimulated CD4$^+$ T cells after priming with control PDCs or schizont lysate-stimulated PDCs. The percentage of cytokine-producing T cells is indicated (*b*). Enriched T cell and enriched T cell plus PDC cultures were stimulated with schizont lysate. The increase in the number of γδ T cells in cell cultures was determined after 7 days of incubation. Numbers in each histogram represent the percentage of γδ T cells in the CD3$^+$ T cell fraction. The percentage of γδ T cells in unstimulated enriched T cell cultures or unstimulated enriched T cell plus PDC cultures was <2. Data are from one of four separate experiments (*c*). The increase in γδ T cells that were positive for intracellular IFN-γ was measured after day 2. Data are the mean ± SEM of five separate experiments (*d*). The percentage of γδ T cell expressing IFN-γ in unstimulated enriched T cell cultures or unstimulated enriched T cell plus PDC cultures was <0.5.

(MyD88$^{-/-}$) mice. Wild-type and TLR7$^-$/Y BMDCs were found to be activated by schizont-soluble fraction as indicated by up-regulation of CD40, CD86 expression, and IL-12 production (Fig. 4, *b* and *c*). However, MyD88$^{-/-}$ and TLR9$^{-/-}$ BMDCs demonstrated negligible up-regulation of CD40, CD86 expression, and IL-12 production (Fig. 4, *b* and *c*). The data indicate that TLR9-MyD88 signaling pathway is a critical requirement for schizont-induced DC activation. LPS, which was used as a positive control for BMDCs, induced enhanced expression of CD40 and CD86, but had no effect on the IL-12 production in MyD88$^{-/-}$ BMDCs (Fig. 4, *b* and *c*). The data confirmed that LPS-induced cell activation is MyD88 independent, but that MyD88 is critical for LPS-induced cytokine production (16, 23).

The TLR9-mediated response to CpG DNA is sensitive to chloroquine (24, 25). To determine whether the TLR9-dependent, schizont-mediated immune cell activation is also chloroquine sensitive, we evaluated the response of human PDCs in the presence of chloroquine. As expected, chloroquine markedly inhibited the IFN-α production from human PDCs stimulated with either a schizont-soluble fraction or CpG ODN 2216 (Fig. 4d). The same concentrations of chloroquine had negligible effect on the LPS-induced TNF-α production from human monocytes.
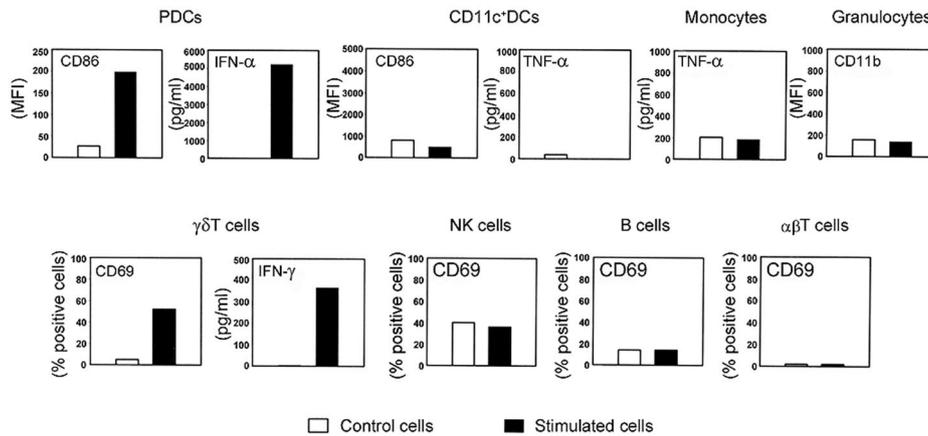
FIGURE 3. Effect of schizont-soluble fraction on different immune cells. Different purified immune cells (2×10^5 cells/ml), except granulocytes, which were used as a whole blood culture, were stimulated with the schizont-soluble fraction at an equivalent concentration of cell:schizont of 1:10. After 24 h of incubation, cell activation was evaluated. For granulocyte activation, whole blood cultures were stimulated with the schizont-soluble fraction for 1 h, and $CD16^+$ cells in a granulocyte gate were analyzed for activation. The activation of PDCs was monitored by CD86 expression and IFN-α production. The activation of $CD11c^+$DCs was monitored by CD86 expression and TNF-α production. The activation of monocytes was monitored by TNF-α production. The activation of granulocytes was monitored by CD11b expression. The activation of $\gamma\delta$ T cells was monitored by CD69 expression and IFN-γ production. The activation of NK cells, B cells, and $\alpha\beta$T cells was monitored by CD69 expression.

Flt3 ligand-induced mouse BMDCs can be divided into two subsets; PDCs and myeloid DCs according to B220 expression (21, 22). We tested the responses of these subpopulations to schizont-soluble fraction. Both $B220^+$ PDCs and $B220^-$ myeloid DCs demonstrated up-regulation of CD40, CD86 expression, and IL-12 production in response to schizont-soluble fraction (Fig. 5, *a*, *b*, and *d*). Neither subpopulation produced detectable levels of IFN-α (Fig. 5*c*).

Elevated serum IFN-α and decreased circulating PDCs in P. falciparum *patients*

To obtain some indication of whether PDCs are activated during *P. falciparum* infection, serum samples and blood cells were taken from patients and then analyzed for IFN-α levels as well as the number of circulating PDCs compared with healthy controls. Both complicated and noncomplicated malaria-infected patients demonstrated a significant increase in their serum levels of IFN-α and a marked reduction of circulating PDCs (Fig. 6, *a* and *b*). However, PDCs are known to actively migrate (7, 26), thus the reduction of circulating PDCs observed in this study may reflect their migration to secondary lymphoid tissues. To evaluate this possibility, we investigated the expression of CCR7, which plays a critical role in DC migration. The freshly isolated PDCs from healthy donors

showed negligible expression of CCR7; however, upon stimulation with schizonts in vitro, the expression of CCR7 on PDCs was up-regulated (Fig. 6*c*).

Discussion

PDCs are normally found in blood, bone marrow, and the T cell area of secondary lymphoid tissues. They represent a unique cell subset that has an important role in the innate immunity because of their ability to produce high levels of IFN-α in response to viral (7, 27) or microbial DNA or CpG DNA stimulation (28, 29). Although unstimulated PDCs express very low levels of costimulatory molecule, activated PDCs can serve as efficient APCs for the adaptive immune response (17, 18). We have demonstrated in this study that *P. falciparum* schizonts, both from a laboratory strain and from field isolates from different geographical regions, activated human PDCs. The schizont-stimulated PDCs demonstrated a phenotype of incomplete mature cells, as they expressed CD86, but not CD40, and did not have a typical DC-like morphology. They produced IFN-α, but not TNF-α.

Blood stage parasites have the ability to adhere to host cells via surface adhesion molecules and to modulate cell functions. For example, suppression of the Ag-presenting capability of MoDCs by blood stage parasites requires the adhesion of malaria parasites to target cells (2). However, the observed PDC activation did not require adhesion of schizonts. The soluble fraction from schizont lysate had similar IFN-α-inducing activity, as did the intact schizonts when cultured with PDCs, suggesting that soluble molecules derived from rupturing schizonts were responsible for the observed effect.

Our observation that PDCs promoted $\gamma\delta$ T cell proliferation and IFN-γ production in response to stimulation with schizonts may have further physiological significance and points to a possible role for PDCs in mediating the innate immune response to the schizonts. A marked increase in circulating $\gamma\delta$ T cells has been reported in malaria patients (19, 20). The increase in $\gamma\delta$ T cell responses observed in this study could be via mediators released from the schizont-stimulated PDCs. IFN-α has been shown to activate $\gamma\delta$ T cells (29), but we failed to inhibit the increase in numbers of $\gamma\delta$ T cells by treatment with neutralizing anti-IFN-α Ab or a mixture of neutralizing anti-IFN-α Ab and anti-IFN-β Ab (data

Table II. *Partial characterization of schizont soluble molecules*[a]

Samples and Treatments	PDCs (IFN-α, pg/ml)	$\gamma\delta$ T Cells (IFN-γ, pg/ml)
None	<50	<15
Schizont soluble fraction	5,493	236
Heated schizont-soluble fraction (100°C, 15 min)	<50	305
Ammonium sulfate (80%)	7,068	<15
E. coli DNA (5 ug/ml)	17,085	<15
Heated *E. coli* DNA (100°C, 15 min)	27,447	<15

[a] The PDCs (2×10^5 cells/ml) and $\gamma\delta$ T cells (5×10^5 cells/ml) were stimulated with the schizont-soluble fraction (the same concentration as described), heated schizont-soluble fraction, fraction from ammonium sulfate precipitation, *E. coli* DNA, or heated *E. coli* DNA. After 24 h of incubation, culture supernatants from PDC and $\gamma\delta$ T cell cultures were harvested and assayed for production of IFN-α and IFN-γ, respectively. Results from one of two experiments with similar results are shown.
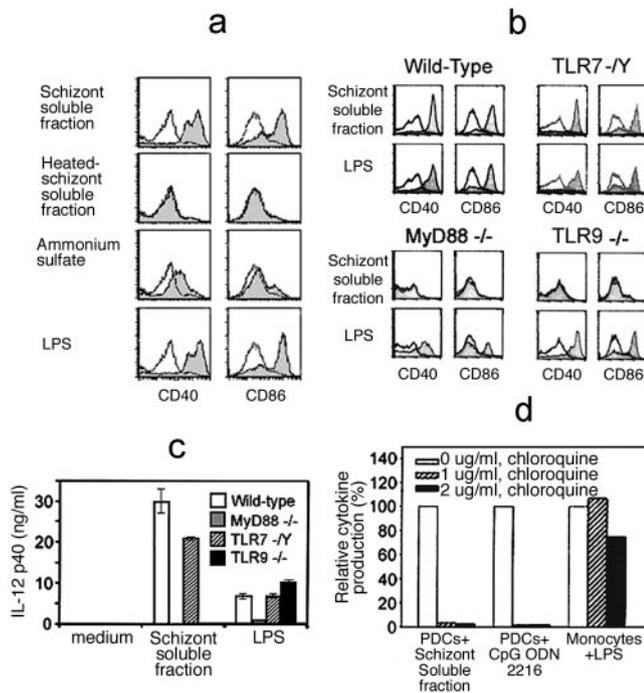
FIGURE 4. Schizont-soluble fraction-induced cell activation is TLR9-MyD88 dependent and sensitive to chloroquine. Flt3 ligand-induced BM-DCs from wild-type (*a* and *b*), TLR7-/Y, MyD88$^{-/-}$ (*b*), and TLR9$^{-/-}$ (*b*) mice were stimulated with the schizont-soluble fraction, the heated schizont-soluble fraction, the ammonium sulfate fraction, or LPS for 24 h. The cells were then analyzed for the expression of CD40 and CD86 by flow cytometry. The solid line represents control cells, and the shaded area represents stimulated cells. The culture supernatants were harvested, and IL-12 p40 production was determined (*c*). Purified human PDCs or monocytes were preincubated for 1 h with various concentrations of chloroquine before addition of the schizont-soluble fraction, CpG ODN 2216 (10 μg/ml), or LPS (100 ng/ml). After 48 h, culture supernatants from PDC and monocyte cultures were harvested, and the production of IFN-α and TNF-α, respectively, was determined (*d*).

FIGURE 5. Activation of murine B220$^+$ PDCs and B220$^-$ myeloid DCs by the schizont-soluble fraction. Purified B220$^+$ and B220$^-$ DCs were stimulated with the schizont-soluble fraction or CpG ODN D19 for 24 h. B220$^+$ PDCs (*a*) and B220$^-$ myeloid DCs (*b*) were analyzed for the expression of CD40 and CD86 by flow cytometry. The solid line represents control cells, and the shaded area represents stimulated cells. The culture supernatants were harvested, and IFN-α (*c*) and IL-12 p40 (*d*) production was determined.

not shown). It is possible that PDC-produced IFN-α could nevertheless drive $\gamma\delta$ T cell proliferation if this was directly transferred between the cells. The possible requirement of cell contact between PDCs and $\gamma\delta$ T cells could not be ruled out.

The increased $\gamma\delta$ T cell response in malaria could have both beneficial and deleterious effects. $\gamma\delta$ T cells have been shown to directly inhibit the growth of blood stage parasites in vitro (30). However, a marked increase in $\gamma\delta$ T cell activity associated with high levels of proinflammatory cytokines can cause pathology (31). In the mouse model the accumulation of $\gamma\delta$ T cells in the brain is found to be associated with cerebral malaria (32).

The soluble fraction from *P. falciparum* schizont lysate was highly specific in its immune effects; it directly activated only two innate immune cell populations, PDCs and $\gamma\delta$ T cells, but not CD11c$^+$ DCs, monocytes, granulocytes, NK cells, B cells, or $\alpha\beta$T cells. We have previously shown that a nonprotein component of schizont lysate-containing phosphate groups activates $\gamma\delta$ T cells (33). In this study we have identified additional immunostimulatory components from schizonts that are capable of stimulating PDCs to produce IFN-α. Other products from the parasite, such as glycosylphosphatidylinositol and malaria pigment, are also known to activate human monocytes to produce cytokines (34, 35).

The innate immune response against *P. falciparum*, especially involving TLRs and their corresponding ligands, is as yet poorly understood. A recent study in the mouse model of *P. berghei* blood
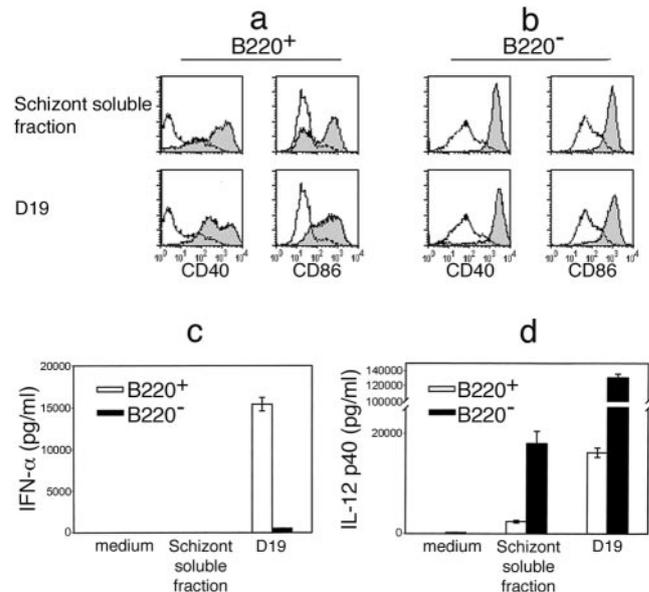
stage infection demonstrated that the IL-12-mediated liver injury depends on the TLR-MyD88-dependent pathway (36). TLR2, TLR4, and TLR6 do not seem to be involved (36). In this study we have shown that schizont-soluble fraction from *P. falciparum* induced maturation of mouse bone marrow B220$^+$ and B220$^-$ DCs and stimulated IL-12, but not IFN-α, production. The TLR9-MyD88 signaling pathway is required for the response to schizont-soluble products, as the response is absent in mice genetically deficient in TLR9. To date, the only known TLR9 ligand is CpG motifs in pathogen DNA. In addition to species specificity, the ability of CpG DNA to induce cell maturation and IFN-α production is also dependent on the characteristic sequence of CpG DNA (37). It is possible that the schizont-soluble fraction exerts an opposite effect in human cells compared with that in murine cells. Our findings suggest that in human cells the schizont-soluble fraction is a potent stimulator of IFN-α production, but a weak inducer of cell maturation (induces CD86, but not CD40, expression). Conversely, our findings also suggest that in murine cells, the same schizont-soluble fraction is a weak stimulator of IFN-α production, but a strong inducer of cell maturation (induces both CD86 and CD40 expression). We have not yet fully characterized the nature of the parasite molecule(s) involved in PDC activation, but our data indicate that it is unlikely to be DNA. First, although *E. coli* DNA triggered the expected large amounts of IFN-α production, comparable to that induced by the schizont-soluble fraction, schizont DNA induced very little IFN-α production, even when added at concentrations far exceeding the level present in the schizont-soluble fraction (data not shown). Second, the majority of the IFN-α-inducing activity was heat labile, unlike DNA. Finally, the activity could be precipitated with ammonium sulfate, indicating that it is not DNA, and suggesting instead that it may be associated with protein. Taken together, these findings suggest that a novel and previously unknown TLR9 ligand is present in schizonts and is responsible for IFN-α production. It is also possible that one or
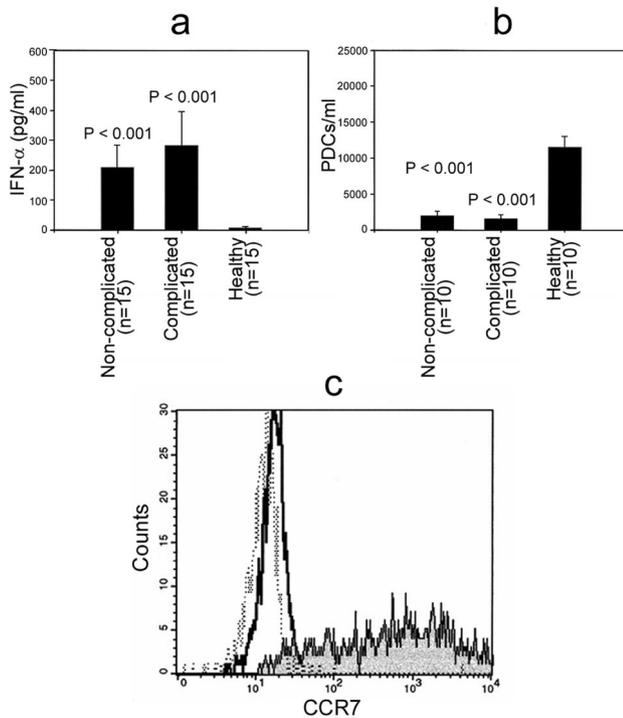
FIGURE 6. Malaria patients have an increase in serum levels of IFN-α, but a decrease in the number of circulating PDCs. Serum levels of IFN-α in malaria patients and healthy controls were measured by ELISA. Data are the mean $\pm$ SEM (*a*). The percentage of circulating PDCs was assessed by flow cytometry. The absolute number per milliliter of blood was calculated by multiplying the percentage of PDCs by the number of leukocytes per milliliter of blood. Data are the mean $\pm$ SEM (*b*). Schizont-stimulated PDCs demonstrated up-regulation of CCR7 expression compared with freshly isolated PDCs (*c*). PDCs used in this experiment were obtained from healthy donors. The dotted line is the isotype control. The solid line is the freshly isolated PDCs, and the shaded area is schizont-stimulated PDCs.

more parasite protein(s) could form a complex with DNA and synergistically costimulate PDCs to produce IFN-α. A two-signal engagement pathway has been recently implicated in the autoreactive B cell response, in which binding of the Ab to the B cell receptor may enhance the uptake of chromatin-containing immune complexes, and this is thought to result in the effective delivery of the chromatin fragment to the endosome-associated TLR9 (25). Clearly, the interaction of pathogen-derived molecules with TLRs has many complexities that remain to be elucidated.

During the last 20 years, there have been at least four reports documenting *P. falciparum*-mediated IFN-α response both in vitro and in vivo (38–41). Our study confirms those observations and demonstrates for the first time the important role of schizont products in PDC activation. An increase in IFN-α production was detected in both complicated and noncomplicated malaria-infected patients. The possible role of PDCs in human malaria is further supported by the findings of a significant reduction of circulating PDCs in patients. One possible mechanism for the loss of PDCs in the blood is due to the mobilization of cells to secondary lymphoid tissues such as the spleen, where marked immunological activity has been observed (42). The migration capability of PDCs requires chemokine stimulation and induction of CCR7 (43). Our study demonstrates that schizont-stimulated PDCs up-regulate the expression of CCR7, providing a potential explanation for the decrease in blood PDCs seen in malaria infection.

A possible scenario for *P. falciparum*-mediated PDC activation in vivo may start with the rupture of blood stage schizonts, releas-

ing soluble molecules that activate PDCs via the TLR9-MyD88 signaling pathway. The stimulated PDCs produce IFN-α and enhance the response of $\gamma\delta$ T cells that have been simultaneously primed with a schizont-derived phosphorylated molecule. Further characterization of the complex nature of parasite ligands that activate PDCs will provide new insight into innate immunity and its role in the pathogenesis of malaria infection.

Acknowledgments

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