Apoptosis and dysfunction of blood dendritic cells in patients with falciparum and vivax malaria

Alberto Pinzon-Charry,[1,2] Tonia Woodberry,[3] Vivian Kienzle,[1] Virginia McPhun,[1,5] Gabriela Minigo,[3] Daniel A. Lampah,[4] Enny Kenangalem,[4] Christian Engwerda,[1] J. Alejandro López,[1,2] Nicholas M. Anstey,[3] and Michael F. Good[1,5]

[1]Queensland Institute of Medical Research, Brisbane QLD 4006, Australia
[2]School of Biomolecular and Physical Sciences, Griffith University, Brisbane QLD 4111, Australia
[3]Menzies School of Health Research, Charles Darwin University, Casuarina NT 0811, Australia
[4]District Health Authority and Menzies School of Health Research-National Institute of Health Research and Development Program, Timika, Papua 99962, Indonesia
[5]Institute for Glycomics, Griffith University, Gold Coast QLD 4222, Australia

Malaria causes significant morbidity worldwide and a vaccine is urgently required. *Plasmodium* infection causes considerable immune dysregulation, and elicitation of vaccine immunity remains challenging. Given the central role of dendritic cells (DCs) in initiating immunity, understanding their biology during malaria will improve vaccination outcomes. Circulating DCs are particularly important, as they shape immune responses in vivo and reflect the functional status of other subpopulations. We performed cross-sectional and longitudinal assessments of the frequency, phenotype, and function of circulating DC in 67 Papuan adults during acute uncomplicated *P. falciparum*, *P. vivax*, and convalescent *P. falciparum* infections. We demonstrate that malaria patients display a significant reduction in circulating DC numbers and the concurrent accumulation of immature cells. Such alteration is associated with marked levels of spontaneous apoptosis and impairment in the ability of DC to mature, capture, and present antigens to T cells. Interestingly, sustained levels of plasma IL-10 were observed in patients with acute infection and were implicated in the induction of DC apoptosis. DC apoptosis was reversed upon IL-10 blockade, and DC function recovered when IL-10 levels returned to baseline by convalescence. Our data provide key information on the mechanisms behind DC suppression during malaria and will assist in developing strategies to better harness DC's immunotherapeutic potential.

CORRESPONDENCE
Alberto Pinzon-Charry:
albertoP@qimr.edu.au
OR
Michael F. Good:
Michael.good@griffith.edu.au

Abbreviations used: ΔMFI, difference in mean fluorescence intensity; HRP2, histidine-rich protein II; mDC, myeloid DC; pDC, plasmacytoid DC; *Pf*, *Plasmodium falciparum*; *Pv*, *Plasmodium vivax*.

Plasmodium falciparum (*Pf*) and *P. vivax* (*Pv*) infections cause significant morbidity and mortality worldwide (WHO Expert Committee on Malaria, 2000). The scaling up of control methods, including water and vector control, intermittent preventive therapies (Kobbe et al., 2007), indoor residual spraying (Kolaczinski et al., 2007), and artemisinin combination therapies (Bhattarai et al., 2007), have reduced the incidence of *Pf* but not *Pv* malaria in many parts of the world (Nosten et al., 2000; Guerra et al., 2010; Oliveira-Ferreira et al., 2010). Moreover, antimalarial drug resistance continues to emerge and malaria remains a major public health problem (Snow et al., 2005). A malaria vaccine would represent the most cost-effective strategy to tackle resistance and protect humans from this disease. Consequently, numerous candidate vaccines are in development for *Pf* and *Pv* malaria (Pinzon-Charry and Good, 2008) but an effective vaccine remains elusive. Importantly, *Pf* and *Pv* infections impair the generation of recall cellular (Walsh et al., 1995) and humoral responses (Williamson and Greenwood, 1978) affecting the generation of any type of vaccine immunity. Given the central role of DCs in directing primary and vaccine-induced immune responses (Banchereau et al., 2000), improved understanding

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Table 1. Study cohort

	Subjects	Age	Sex	Parasites/µl	HRP2	Temperature
		median (*IQR*)	*M/F*	*median* (*IQR*)	*median ng/ml* (*IQR*)	*median °C* (*IQR*)
Pf	42	23 (18–35)	27/15	5,655 (533–18,250)	107 (1.06–9.28)[a]	36.2 (36.1–37.9)
Pv	25	23 (19–31)	9/16	3,207 (1,020–8,067)	n.d.	36.6 (36.3–37.1)
U	17	27 (20–24)	14/3	n.d.	n.d.	36 (35.5–36.3)

[a]36 subjects. 10 of the 42 *Pf* patients were analyzed at days 7 and 28 after treatment. No peripheral parasitemia or HRP2 was detected on any *Pf* patient at days 7 or 28 after treatment. There were no significant differences between any of these groups. n.d., not detected. U, uninfected.

of DC biology during acute malaria is paramount to improve vaccination outcomes.

Circulating DCs are particularly important as they replenish tissue-residing DCs and shape immune responses in vivo (Banchereau et al., 2000). These cells can be identified as mononuclear cells expressing MHC-II molecules (HLA-DR) but lacking common lineage markers (Savary et al., 1998). This blood DC compartment includes two different subsets discernible into myeloid DC (mDC) or plasmacytoid DC (pDC) based on their reciprocal expression of CD11c or CD123 antigens (Robinson et al., 1999). Moreover, mDC can be further subdivided into three subtypes based on the respective expression of CD141, CD16, and CD1c antigens (Piccioli et al., 2007; Jongbloed et al., 2010). Despite their importance, most studies have only assessed blood DC's numerical phenotype and few functional studies have been reported to date. Numerical characterizations indicate that children with *Pf* display less activated mDC during acute uncomplicated infection (Urban et al., 2001a) yet increased numbers of mDC during severe infection (Urban et al., 2006). Adults with *Pf* exhibit reduced pDC during acute uncomplicated, severe (Pichyangkul et al., 2004), or pregnancy malaria (Diallo et al., 2008). Similarly, adults infected with *Pv* display reduced mDC and pDC numbers (Jangpatarapongsa et al., 2008). We have also demonstrated numerical blood DC reductions and increased apoptosis before patent parasitemia in healthy donors undergoing an experimental low-dose *P. falciparum* challenge (Woodberry et al., 2012). Further functional characterization, in contrast, has mainly been undertaken using in vitro or murine DC models (Urban et al., 2001b; Perry et al., 2005; Elliott et al., 2007; Bettiol et al., 2010). However, rodent DCs or DCs generated in vitro after prolonged culture with cytokines are unlikely to reflect the functional status of human DC populations circulating in vivo.

To address these questions, evaluate the role of different *Plasmodium* species on circulating DC, and determine the type and duration of DC impairment after antimalarial treatment, we performed cross-sectional and longitudinal assessments of all circulating DC subsets during uncomplicated *Pf* and *Pv* including convalescent *Pf* infection in 67 Papuan adults. Results were compared with a cohort of 17 malaria-exposed uninfected and asymptomatic Papuan adults. Given the low frequency of blood DC and restricted volumes that could be taken from patients, subcohorts were used for the various analyses. Our results demonstrate that the marked loss of functional DC in the peripheral circulation of patients with *Pf* or *Pv* malaria is associated with significant levels of spontaneous

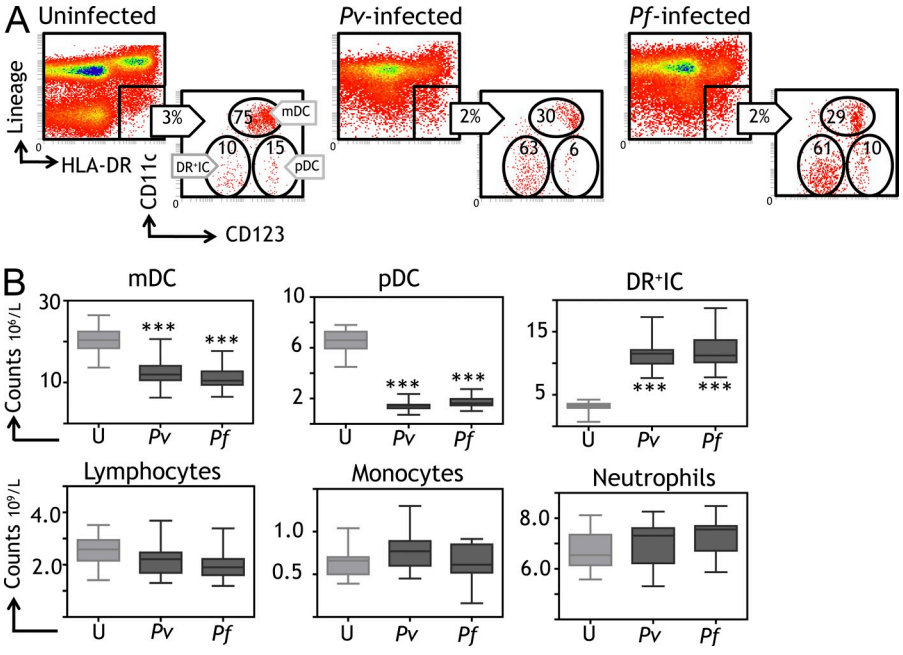

Figure 1. Altered blood DC subset distribution in malaria patients. (A) Peripheral blood DCs were identified as Lin⁻HLA-DR⁺ cells and analyzed for the expression of CD11c (y axis) and CD123 (x axis) by flow cytometry. The gating strategy for mDC, pDC, and a minor population of HLA-DR⁺ immature cells (DR⁺IC) is shown. Representative dot plots of the blood DC subset distribution in uninfected control and *Pf* or *Pv* patients are shown. Numbers indicate the percentage of cells within each gate. (B) Blood leukocyte counts including lymphocyte, monocyte, neutrophil, mDC, pDC, and DR⁺IC were estimated in a cohort of 45 patients with malaria (*Pv*, $n = 19$; *Pf*, $n = 26$) and compared with uninfected controls (U, $n = 12$). Absolute mDC, pDC, and DR⁺IC counts are expressed as 10^6/liter and all other counts are expressed as 10^9/liter. Box plots include means, standard deviations, and ranges. Significant differences compared with uninfected controls are indicated as ***, $P < 0.001$.

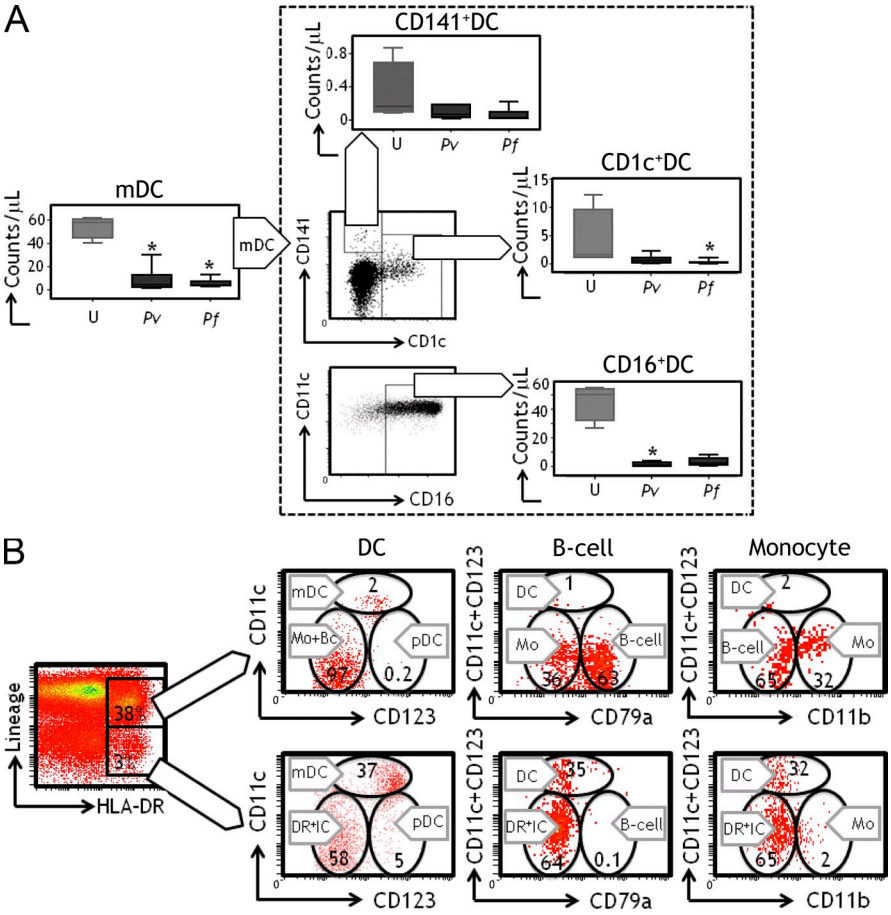

Figure 2. Altered mDC subset distribution in malaria patients. (A) mDCs (mDC, CD11c$^+$ CD123$^+$) were analyzed by flow cytometry for expression of CD141, CD1c, and CD16. DCs were gated as shown, and the populations were enumerated in the blood of 12 patients with malaria (*Pv*, $n = 6$; *Pf*, $n = 6$) and uninfected controls (U, $n = 5$). Data are expressed as cells/µl. Box plots include means, standard deviations, and ranges. Significant differences compared with uninfected controls are indicated as *, $P < 0.05$. (B) In a cohort of *Pv* ($n = 5$) and uninfected ($n = 5$) individuals, lineage-positive HLA-DR$^+$ cells were analyzed for expression of CD11c and CD123 (DC), CD79a (B-cells), and CD11b (monocytes). Representative FACS plot from one *Pv* patient shown.

DC apoptosis. Impaired viability and numerical changes also correlate with functional impairment in the ability of DCs to up-regulate costimulatory and MHC-class II molecules as well as antigen uptake and presentation to CD4 T cells. Interestingly, elevated and sustained levels of plasma IL-10, as observed in patients with *Pf* or *Pv*, were implicated in the induction of DC apoptosis. As such, DC apoptosis was reversed upon IL-10 blockade and DC function recovered completely after completion of antimalarial treatment and normalization of IL-10 levels by convalescence. Our data provide key information on the mechanisms behind blood DC suppression during acute malaria and will assist in developing better strategies to harness DC's immunotherapeutic potential and improve vaccination outcomes.

RESULTS

Study cohort

42 adult Papuans with acute uncomplicated *Pf* malaria, 25 adult Papuans with acute *Pv* malaria, and 17 uninfected malaria-exposed Papuan adults were included in the study. 10 of the 42 patients with *Pf* malaria were also assessed at days 7 and 28 after antimalarial treatment. There was no significant difference in the age between groups although there were fewer female volunteers in the uninfected group (Table 1). Median peripheral parasitemia was similar in acute *Pf* and *Pv* malaria patients, but only *Pf* patients had detectable plasma histidine-rich protein II (HRP2). No peripheral parasitemia or HRP2 was detected in any *Pf* patient at days 7 or 28 after treatment.

Subset distribution of DC in patients with malaria

The blood DC compartment can be identified by flow cytometry as Lin$^-$HLA-DR$^+$ cells (Thomas et al., 1993). This population includes two different subsets distinguished into myeloid DC (mDC) and plasmacytoid DC (pDC) lineages (Robinson et al., 1999). Because previous studies suggest dissimilar changes in the frequency of these subsets in acute malaria (Urban et al., 2006; Diallo et al., 2008; Jangpatarapongsa et al., 2008), we set out to carefully analyze the blood DC compartment in a cohort of 45 patients with acute *Pf* or *Pv* malaria (Fig. 1 A). Our data demonstrate a significant reduction in absolute counts of mDC (CD11c$^+$) and pDC (CD123$^+$) concurrent with the marked accumulation of HLA-DR$^+$ immature cells (DR$^+$IC) in patients with acute malaria (Fig. 1 B). These immature cells lacked expression of CD11c or CD123 markers yet expressed high levels of the HLA-DR antigen (Pinzon-Charry et al., 2005). Interestingly, this immature population represented only 16.8 ± 2.0% of the blood DC compartment in uninfected donors, while representing a much larger proportion in patients with *Pv* (38.6 ± 3.4%) or *Pf* (46.7 ± 1.8%) infection. The reduction in DC counts was,

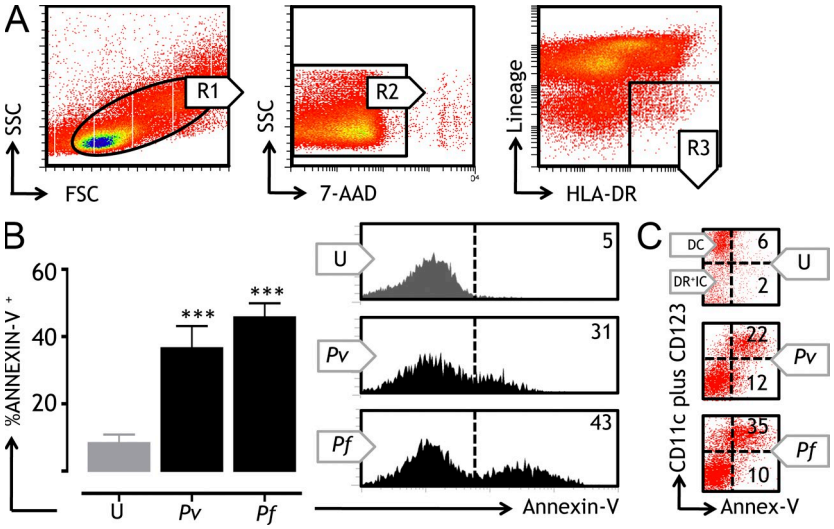

Figure 3. Spontaneous apoptosis of blood DC in malaria patients. Blood DC from patients with malaria (*Pv*, $n = 19$; *Pf*, $n = 26$) and uninfected matched controls (U, $n = 12$) were analyzed for apoptosis by flow cytometry. (A) Cells were gated on viable mononuclear cells (R1), which were further gated on 7-AAD–negative cells (R2). Blood DCs were identified as $Lin^-HLA\text{-}DR^+$ cells (R3). Representative dot plots are shown. (B) Apoptosis in blood DC from uninfected donors (gray) and patients with malaria (black) was determined using Annexin-V binding assays. In all experiments, each patient was tested in parallel with at least one uninfected donor. Representative histograms and summary of apoptosis data (mean ± SEM) are shown. (C) DC (mDC plus pDC) and DR^+IC in uninfected donors and malaria patients were evaluated for apoptosis by Annexin-V staining. Numbers indicate the percentage of cells that are positive for Annexin-V. Statistically significant differences between uninfected controls and patients are shown. ***, $P < 0.001$.

however, not generalized to other leucocytes. As such, lymphocyte counts were only mildly (not significantly) reduced, monocyte counts were comparable, and neutrophil counts were only marginally increased (not significantly) compared with uninfected donors (Fig. 1 B).

Given that mDCs can be further subdivided into three subtypes based on their respective expression of CD141, CD16, and CD1c antigens (Piccioli et al., 2007; Jongbloed et al., 2010), numerical characterization of these mDC subsets was also undertaken. Our data confirmed reduced counts of $CD141^+$DC, $CD1c^+$DC, and $CD16^+$DC in patients with acute *Pf* and *Pv* malaria (Fig. 2 A). Although individual analyses revealed only significant reductions of $CD1c^+$DC in *Pf* and $CD16^+$DC in *Pv*, composite analyses of all malaria patients (*Pv* plus *Pf*) confirmed significantly reduced counts ($P < 0.05$) for all mDC subtypes, suggesting a sample size effect.

To confirm that other DC subpopulations induced during inflammatory settings were not excluded by confining our analyses to the $Lin^-HLA\text{-}DR^+$ population, characterization of the $Lin^+HLA\text{-}DR^+$ population was undertaken in five *Pv* patients (Fig. 2 B) compared with five uninfected controls. This confirmed the minimal proportion of DCs (<2%) and the rather large proportion of B cells ($CD79a^+$ cells) and monocytes ($CD11b^+$ cells) within this gate in malaria patients and uninfected donors (*Pv*: B cells, 59 ± 18%; monocytes, 30 ± 5% vs. uninfected: B cells, 68 ± 10%; monocytes, 35 ± 8%). These results confirm that in patients with acute malaria, the marked reduction in $Lin^-HLA\text{-}DR^+$ cells in the peripheral circulation reflects significant reductions in all DC numbers (mDC subsets and pDC) and the concurrent accumulation of immature cells (DR^+IC).

Spontaneous apoptosis of DC in patients with malaria

To determine whether reduced viability accounted for some of the changes observed in the DC compartment, we assessed spontaneous apoptosis in blood DC ex vivo. To include all cells undergoing apoptosis while eliminating cellular debris, gating was set as described in Fig. 3 A. Blood DCs were identified as $Lin^-HLA\text{-}DR^+$ cells and apoptosis was estimated using Annexin-V. As shown in Fig. 3 B, the minimal proportion of spontaneously apoptotic blood DC in uninfected donors was significantly increased in patients with *Pv* or *Pf* infection. Interestingly, apoptosis was mostly evident in DCs (mDC and pDC) as opposed to immature cells (DR^+IC), partly explaining the large accumulation of DR^+IC in patients with acute malaria (Fig. 3 C). Other antigen-presenting cells, such as B cells ($CD19^+$) or monocytes ($CD14^+$), displayed no significant apoptosis in patients with *Pv* ($CD19^+AnnexinV^+$: 6.4 ± 2.1%; and $CD14^+AnnexinV^+$: 7.3 ± 3.5%) or *Pf* ($CD19^+AnnexinV^+$: 5.4 ± 3.1%; and $CD14^+AnnexinV^+$: 8.3 ± 2.3%) compared with uninfected controls ($CD19^+AnnexinV^+$: 5.7 ± 5.2%; and $CD14^+AnnexinV^+$: 8.3 ± 3.4%). These data suggest that in acute malaria, a process of specific DC suppression occurs whereby functional DCs are induced to undergo apoptosis while circulating in the blood.

Impaired phenotype and function of DC in patients with malaria

Next, we set out to determine if the reduced viability of blood DC was associated with phenotypic or functional abnormalities. Because expression of MHC II and costimulatory molecules has been correlated to DC's immunostimulatory capacity, first we evaluated the expression of CD83, CD86, and HLA-DR in patients with acute *Pf* or *Pv* malaria. As shown in Fig. 4 A, ex vivo expression (gray histograms) of CD83, CD86, and HLA-DR in blood DCs from *Pv* or *Pf* patients was comparable to uninfected controls. However, upon overnight incubation (black histograms), blood DCs from *Pv* or *Pf* patients showed markedly reduced ability to up-regulate expression of costimulatory and HLA-DR molecules (Fig. 4, A and B). Functional assessment also demonstrated significant DC impairment in malaria patients. Here, blood DCs from *Pv* or *Pf* patients had a markedly reduced ability to take-up particulate

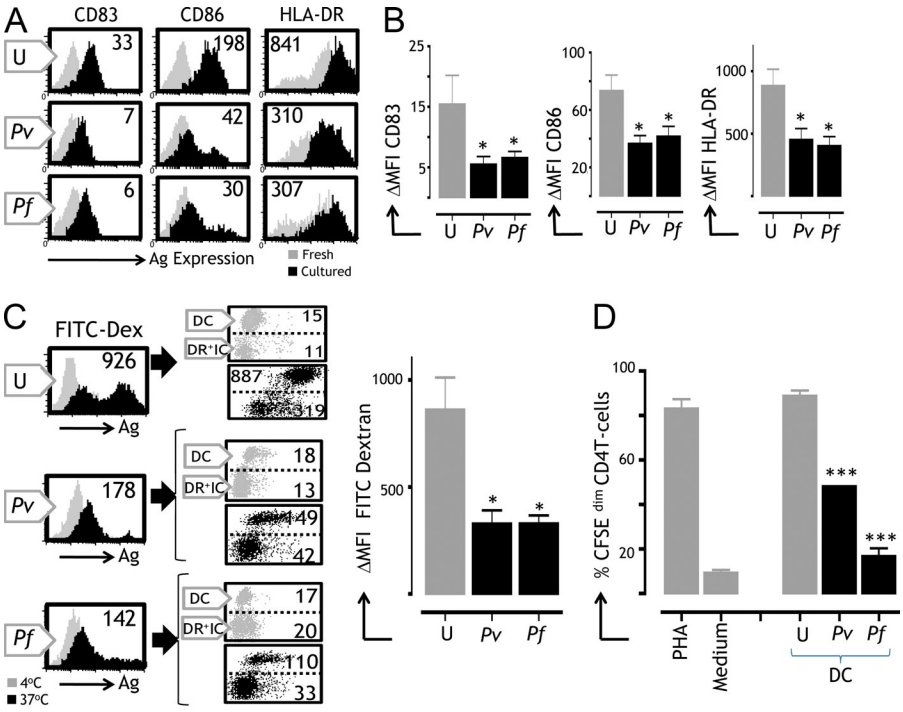

Figure 4. Impaired phenotype, antigen uptake, and stimulatory capacity in DC from malaria patients. (A) Expression of the costimulatory molecules CD83, CD86, and HLA-DR was analyzed on blood DC in a cohort of 45 patients with malaria (*Pv*, $n = 19$; *Pf*, $n = 26$) compared with uninfected controls (U, $n = 12$) directly ex vivo (gray histograms) or after overnight incubation (black histograms) without exogenous cytokines. Representative histograms are shown. (B) Summary of phenotypic analysis (mean ± SEM) for each antigen expressed as ΔMFI (*y* axis) between cultured and fresh samples. (C) Blood DCs from uninfected donors (U, $n = 12$) or patients with malaria (*Pv*, $n = 19$; *Pf*, $n = 26$) were incubated with FITC-dextran at 4°C (gray) or 37°C (black), and uptake was measured by flow cytometry. Graph shows summary of antigen uptake data (mean ± SEM) presented as ΔMFI (y axis) between test and control for all patients. (D) Blood DC from patients (*Pf*, $n = 5$; *Pv*, $n = 5$) and uninfected volunteers (U, $n = 5$) was tested against allogeneic CD4 T cells from a panel of healthy Australian donors ($n = 5$). CD4 T cells and blood DCs were co-cultured at a 30:1 T/DC ratio. Cells were harvested after 96 h of culture and CD4 T cell proliferation estimated by CFSE dilution. Bars indicate the percentage of $CFSE^{dim}$ CD4 T cells for each stimulatory condition. In all experiments, each patient was tested in parallel with at least one uninfected donor. Results are representative of five separate experiments performed. Statistically significant differences compared with uninfected controls are indicated. *, $P < 0.05$; ***, $P < 0.001$.

antigens (Fig. 4 C) and were poor stimulators of allogeneic CD4 T cell proliferation (Fig. 4 D) and Th1 cytokine secretion (see Fig. 8 B). As shown in Fig. 4 C, impaired antigen uptake was evident in DC (mDC and pDC) as well as in immature cells (DR^+IC), although less markedly, explaining the overall dysfunction of all Lin^-DR^+ populations in patients with malaria. These data demonstrate that the impairment of blood DC in acute malaria affects key aspects of DC function, including maturation (up-regulation of costimulatory and MHC class II molecules), ability to capture antigen, and adequate stimulation of allogeneic CD4 T cell proliferation and cytokine secretion.

Central role for IL-10 in induction of DC apoptosis during malaria

We also set out to assess possible mechanisms responsible for the marked levels of blood DC apoptosis in patients with malaria. Given that no significant apoptosis was observed in uninfected donors and a direct cytopathic effect of the parasite on DC has been excluded (Urban et al., 1999; Skorokhod et al., 2004; Elliott et al., 2007), we hypothesized that apoptosis related to the inflammatory response during infection rather than to the parasite itself. To examine this hypothesis, first we evaluated levels of proinflammatory cytokines in plasma of all patients with acute malaria. We found significantly elevated levels of inflammatory cytokines, including IL-2, IL-4, IFN-γ, TNF, IL-6, and IL-10 in all patients with acute *Pv* or *Pf* infection (Fig. 5 A). Interestingly, IL-2, IL-4, and IFN-γ levels corrected to baseline levels by day 7 (at which time parasites had cleared), whereas elevation of TNF, IL-6, and particularly IL-10 persisted beyond this point. Moreover, levels of plasma IL-10 (but not IL-6 or TNF) correlated with the extent of blood DC apoptosis (Fig. 5 B) yet no significant correlation was found between parasitemia or plasma HRP2 and either DC numbers or apoptosis (not depicted). This evidence suggested a key role for IL-10 in blood DC apoptosis during acute infection.

Interestingly, sustained exposure of DC to high levels of IL-10 during their maturation has been associated with induction of apoptosis (Chang et al., 2007). Therefore, we set out to directly evaluate the role of plasma IL-10 from malaria patients on DC survival in vitro. For this purpose, healthy DCs

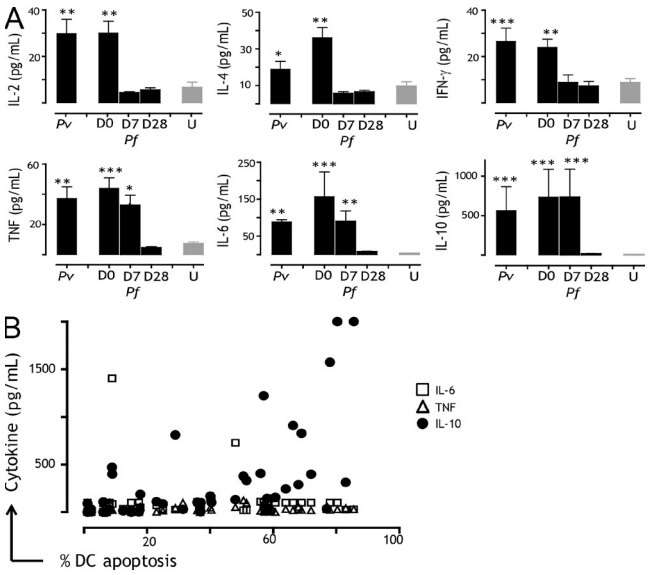

Figure 5. Profile of cytokines in plasma of patients with malaria. (A) The indicated plasma cytokines were measured by CBA assay in samples from *Pf* patients ($n = 10$) at days 0 (D0), 7 (D7), and 28 (D28) after antimalarial drug treatment and compared with patients with acute *Pv* ($n = 19$) or uninfected donors (U, $n = 12$). Summary of cytokine levels are presented as mean ± SEM for each cytokine. Significant differences compared with uninfected controls are indicated as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. (B) Correlation between the percentage of blood DC undergoing apoptosis (x axis) and cytokine levels for IL-6, TNF, and IL-10 (y axis) in 45 patients with acute malaria (*Pf*, $n = 26$ and *Pv*, $n = 19$). Spearman's Rank Test, $R = 0.60$, $P < 0.0001$ for IL-10; $R = -0.14$, $P = 0.35$ for IL-6; and $R = -0.07$, $P = 0.6$ for TNF.

were induced to mature with LPS and exposed to recombinant human IL-10 or plasma samples from either uninfected donors containing low levels of IL-10 (mean 58.6 pg/ml, $n = 3$) or from patients with *Pf* ($n = 3$) or *Pv* ($n = 3$) containing high levels of IL-10 (*Pf*: mean 602.6 pg/ml; *Pv*: mean 353.8 pg/ml). First, we confirmed the proapoptotic effect of high and sustained levels of rhIL-10 on healthy maturing blood DC (Fig. 6 A) as previously reported (Chang et al., 2007). More importantly, we found that exposure of healthy maturing DC ($n = 5$) to plasma from *Pf* or *Pv* patients resulted in a significant proportion of DC apoptosis (Fig. 6 A). To further confirm the role of IL-10 in this process, specific IL-10 blocking was undertaken. As shown individually in Fig. 6 A, and summarized in Fig. 6 B, blockade of rhIL-10 or infected plasma IL-10 prevented a comparably large proportion (mean rhIL-10: 63% vs. *Pf* plasma: 53% vs. *Pv* plasma: 52%) of blood DC from undergoing apoptosis. These data confirmed the central role of IL-10 rather than other plasma cytokines in affecting DC survival and indicate that elevated levels of plasma IL-10 to the extent observed in patients with *Pf* or *Pv* can induce apoptosis in a significant proportion of blood DC.

Effect of antimalarial treatment on DC phenotype and function

Finally, to determine whether antimalarial treatment could reverse the impaired phenotype and viability of blood DC, we performed a longitudinal assessment in a cohort of 10 patients with *Pf* infection. In these patients, blood DCs were analyzed during acute malaria at day 0 and also at days 7 and 28 after successful antimalarial drug treatment. We have demonstrated elsewhere that artemisinin combination therapy drugs do not affect blood DC phenotype or function (Woodberry et al., 2012). Here, blood DCs were analyzed for their subset

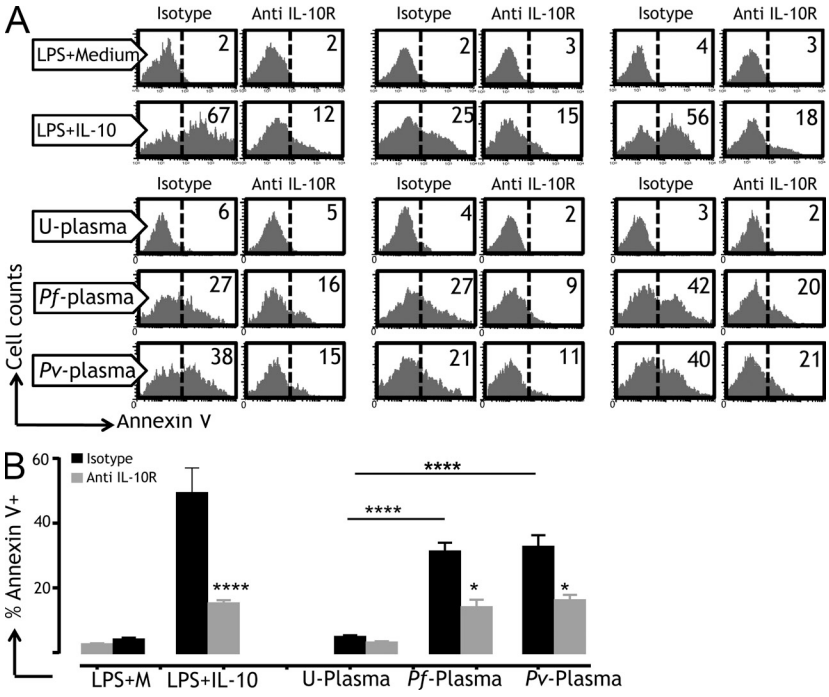

Figure 6. IL-10 induces apoptosis of maturing blood DC. (A) Blood DCs from healthy Australian donors ($n = 5$) were induced to mature by 24 h incubation with LPS and subsequently exposed to either 500 pg/ml of exogenous recombinant human IL-10 (LPS + IL-10) or 50% (vol/vol) plasma samples from uninfected volunteers containing low levels of IL-10 ($n = 3$, mean 58.6 pg/ml, U Plasma) or high levels of IL-10 from *Pf* ($n = 3$; mean 602.6 pg/ml, Pf plasma) or *Pv* ($n = 3$; mean 353.8 pg/ml, Pv plasma) patients with or without anti–IL-10R antibodies. Representative histograms from three donors are shown. Numbers indicate the percentage of apoptotic (Annexin-V$^+$) blood DC in each culture. Results are representative of three separate experiments. (B) Graph shows summary data of all donors. Statistically significant differences between samples exposed to blocking (anti–IL-10R) and nonblocking (Isotype) antibodies as well as between U plasma and *Pf* or *Pv* plasma are indicated. Error bars show SEM. *, $P < 0.05$; ****, $P < 0.0001$.

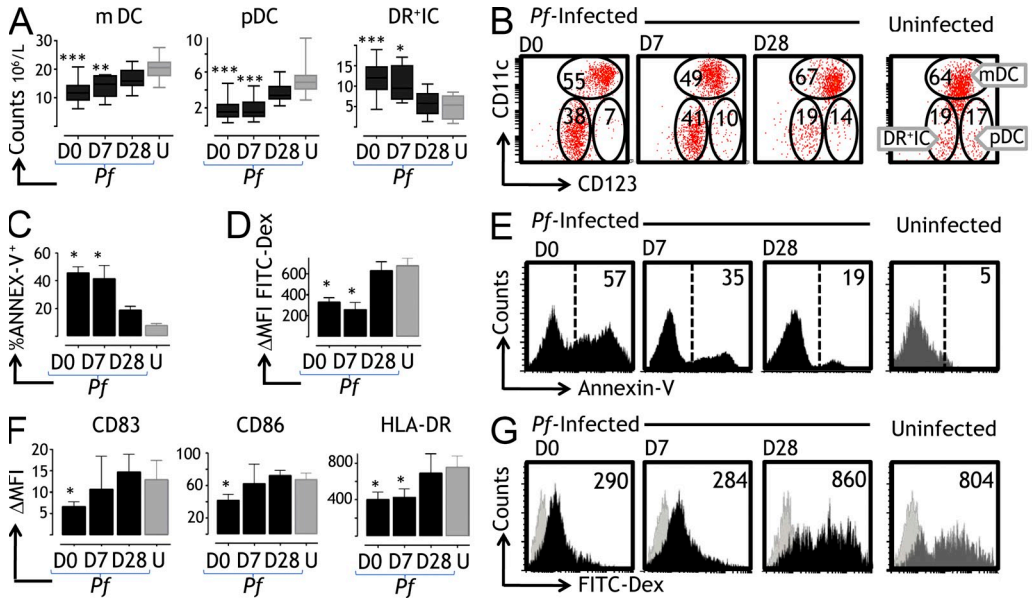

Figure 7. Reversal of DC dysfunction after antimalarial treatment. (A and B) Blood DCs were analyzed during acute *Pf* malaria at day 0 (D0) and also at days 7 (D7) and 28 (D28) after antimalarial treatment in a cohort of 10 patients and compared with uninfected volunteers (U, $n = 12$). In all experiments, each patient was tested in parallel with at least one uninfected volunteer. The blood DC compartment was analyzed for absolute counts of mDC, pDC, and DR$^+$IC (10^6/liter; A) and blood DC subset distribution. (B) Representative dot plots of one uninfected control as well as one patient assessed at days 0 (D0), 7 (D7), and 28 (D28) are shown. Values indicate percentage of cells within respective gates. (C) Summary of blood DC apoptosis data (mean ± SEM). (D) Summary of antigen uptake (FITC-Dextran) data assessed as the difference between uptake at 37°C (test) or 4°C (control) expressed as ΔMFI (mean ± SEM). (E) Representative histograms of apoptosis from one patient at days 0 (D0), 7 (D7), and 28 (D28) compared with one uninfected control. Values indicate percentage of Annexin-V–positive cells. (F) Summary of phenotypic maturation estimated by up-regulation in expression of CD83, CD86, and HLA-DR expressed as ΔMFI (mean ± SEM). (G) Representative histograms of antigen uptake from one patient at days 0 (D0), 7 (D7), and 28 (D28) compared with one uninfected control. Gray histograms indicate uptake at 4°C (control) and black histograms represent uptake at 37°C (test). Values indicate uptake as ΔMFI between cultured and fresh samples. Statistically significant differences compared with uninfected volunteers are indicated as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

distribution, viability, phenotype, and ability to take up antigen and stimulate allogeneic CD4 T cells. We found that patients with *Pf* infection exhibited a delayed correction in the numbers of mDC and pDC. In fact, mDC, pDC, and DR$^+$IC counts only corrected to levels comparable to uninfected donors by day 28 (Fig. 7, A and B). Similarly, the proportion of blood DC undergoing apoptosis remained significantly elevated by day 7 and only returned to baseline levels equivalent to uninfected individuals by day 28 (Fig. 7, C and E). Antigen uptake also demonstrated a delayed improvement with efficient uptake comparable to uninfected donors only achieved by day 28 (Fig. 7, D and G). Up-regulation of the costimulatory phenotype followed a more rapid trend, with adequate expression of CD83 and CD86 achieved by day 7 yet efficient up-regulation in the expression of HLA-DR being delayed until day 28 (Fig. 7 F).

In keeping with these findings, characterization of the function and phenotype of T cells induced by stimulation with malaria DC revealed impaired allogeneic CD4 T cell proliferation (Fig. 8 A) and inadequate induction of Th1 cytokines (Fig. 8 B). Interestingly, no significant differences in the expression of T cell activation markers CD25 (*Pf*: 48 ± 12% vs. U: 55 ± 12%), CD27 (*Pf*: 70 ± 8% vs. U: 73 ± 12%), or CD69 (*Pf*: 7 ± 4% vs. U: 9 ± 5%) was evident in cultured T cells, suggesting that malaria DCs were able to activate but not properly stimulate Th1 cell function in vitro. Reversal of such DC impairment, however, was evident in DCs collected by day 28 after treatment (Figs. 7 and 8). Interestingly, plasma IL-10 showed parallel kinetics to DC dysfunction with low IL-10 levels comparable to uninfected donors attained by day 28 after treatment (Fig. 5). Although these results suggest a protracted period of DC impairment, they confirm that full recovery of functional and competent blood DC is only achievable after antimalarial drug treatment and normalization of IL-10 levels.

DISCUSSION

The aim of this study was to evaluate the blood DC compartment in patients with *Pf* or *Pv* malaria to identify factors suitable for clinical implementation (i.e., antimalarial treatment) to improve their function. We focused on the blood DC compartment because this population directly reflects the natural biology of immune responses occurring in vivo. Therefore, detailed cross-sectional and longitudinal assessments in 67 patients with acute uncomplicated *Pf* or *Pv*, as well as convalescent *Pf* malaria, were performed to demonstrate significant and protracted impairment in number, function, and viability of blood DC during acute malaria. More importantly, our results

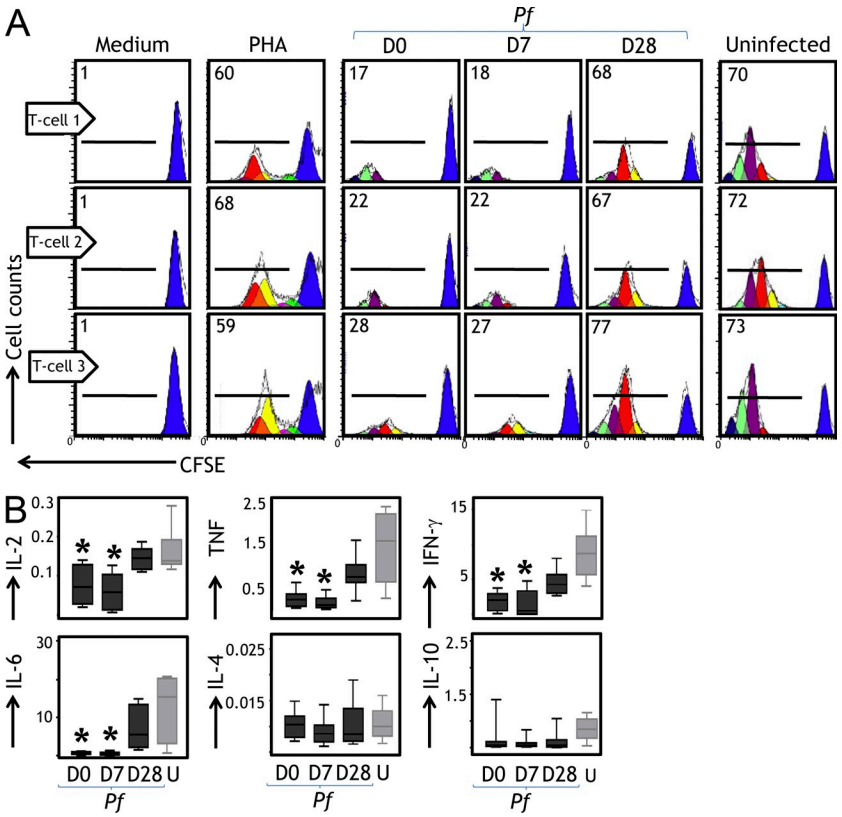

Figure 8. Recovery of DC allostimulatory capacity after antimalarial treatment. (A) Naive allogeneic CD4 T cells purified from a panel of healthy Australian donors ($n = 5$) were co-cultured with blood DC from malaria patients at day 0, 7, or 28 ($n = 8$) or uninfected controls at a 30:1 T/DC ratio ($n = 12$). Cells were harvested after 96 h of culture and CD4 T cell proliferation estimated by CFSE dilution (x axis). Representative histograms are shown with numbers indicating the percentage of $CFSE^{dim}$ CD4 T cells for each stimulatory condition. The representative pairs giving maximal responses for three different T cell donors are shown. In all experiments, each patient was tested in parallel with at least one uninfected volunteer. (B) Supernatants from cultures in A were harvested after 72 h and levels of IL-2, TNF, IFN-γ, IL-6, IL-4, and IL-10 estimated using CBA assay by FACS. Box plots include means, standard deviations, and ranges. Significant differences compared with uninfected controls are indicated as *, $P < 0.05$. Results are representative of five separate experiments performed.

demonstrate that full recovery of functional and competent blood DC is achievable only after adequate antimalarial drug treatment and normalization of IL-10 levels in patients with acute malaria.

Although previous studies have reported changes of blood DC in people with malaria, results have been contradictory and limited functional data are available. Children with severe *Pf* have been reported to exhibit increased numbers of mDC (Urban et al., 2006), whereas adults with *Pf* have reduced pDC during uncomplicated and severe disease (Pichyangkul et al., 2004). Adults with *Pv* also appear to have reduced mDC and pDC (Jangpatarapongsa et al., 2008; Gonçalves et al., 2010). Our data, examining adults with acute malaria demonstrate the significant and comparable reduction in mDC, including all mDC subsets ($CD141^+$DC, $CD1c^+$DC, and $CD16^+$DC) as well as pDC counts in patients with *Pf* or *Pv* infection. An immature population (DR^+IC) distinct from the myeloid (mDC) and plasmacytoid (pDC) subtypes also emerged as a significant proportion of the DC compartment. These immature cells are known to express high levels of HLA-DR (100%) and display variable expression of other DC markers like CD2, DC-SIGN, BDCA-4, CD4, or CD1c (3–20%). Similarly, a variable proportion of DR^+ICs express progenitor antigens like CD7, CD10, CD13, or CD33 (3–20%), myeloid antigens like MPO or CD15 (1–5%), lymphoid markers like CD79a (15–20%), or integrins like CD11b, CD62L, CD41, or CD61 (5–30%), altogether suggesting that multiple small subpopulations of progenitors, as well as immature DCs, coexist in the circulation (Pinzon-Charry et al., 2005). Other reports also indicate increased numbers of immature DCs during uncomplicated malaria in pregnancy (Aldebert et al., 2007; Diallo et al., 2008) and in children (Urban et al., 2001a). Although the reduction in functional DC and the accumulation of immature cells had been proposed to contribute to immune dysfunction in malaria (Aldebert et al., 2007; Diallo et al., 2008), the mechanisms behind such changes and their functional consequences had not been investigated in the past. Recently, we demonstrated reduced counts and increased apoptosis of blood DC in healthy volunteers undergoing an experimental low-dose *P. falciparum* challenge (Woodberry et al., 2012). Here, we expand and complement our data by thoroughly characterizing blood DC phenotype, viability, and function in patients with clinical *Pf* and *Pv* malaria also establishing for the first time that IL-10 is implicated in the process.

Declining blood DC counts could be a direct consequence of increased migration to lymphoid tissues. In this regard, murine malaria models indicate rapid and vigorous DC migration to the spleen after infection (Rossi and Young, 2005). DC migration in human infection has been more difficult to assess as simultaneous data from blood and lymphoid organs are not available. However, in vitro models demonstrate that exposure of human DC to parasitized red cells increase their expression of chemokine receptors CCR7 and CXCR4 (Pichyangkul et al., 2004; Giusti et al., 2011), suggesting enhanced lymphoid organ migration during infection. Preliminary data from our laboratory also support this notion and show

increased expression of CCR7, CXCR4, and CCR5 in blood DC of patients with *Pf* or *Pv* malaria (unpublished data). Although increased migration to lymphoid organs is likely occurring, its effect on peripheral counts is unclear and a definitive answer will only arise from the simultaneous assessment of DC in blood and lymphoid tissues. Myeloid suppression or impaired production of DC progenitors could also be proposed to explain declining blood DC numbers in human malaria. However, our data on the accumulation of immature cells and similar reports of increased immature DC during maternal human infection (Aldebert et al., 2007; Diallo et al., 2008) or $CD34^+$ DC progenitors in blood of children with malaria (Urban et al., 2001a) suggest that myeloid suppression is unlikely to be of significance. Finally, impaired viability of blood DC could contribute to declining counts if a significant proportion of cells are programmed to die while in the circulation. Our results not only confirm the latter hypothesis but suggest that the cytokine milieu occurring in vivo is impacting vigorously on the longevity of all blood DC subpopulations during infection.

The physiological significance of this finding is threepronged. First, circulating DCs are essential for adequate immunity as they continually replenish the pool of tissue-residing DCs. In fact, most circulating DCs are en route from the bone marrow to peripheral and lymphoid tissues or from nonlymphoid tissues to the regional lymph nodes and spleen (de la Rosa et al., 2003). Given that apoptotic cells are rapidly cleared by the reticulo-endothelial system, increased turnover rate of blood DC is likely occurring in these patients. As infection progresses, continual efforts to replace the pool of blood DC from bone marrow imposes pressure on hematopoietic capacity, resulting in the paucity of functional DC (Urban et al., 2001a; Aldebert et al., 2007; Diallo et al., 2008; Jangpatarapongsa et al., 2008) and the concurrent accumulation of immature cells in the circulation (Aldebert et al., 2007; Diallo et al., 2008). Second, apoptotic DCs are ineffective at inducing immunity (Kitajima et al., 1996), and this can contribute to systemic immune dysregulation and parasite evasion. Third, apoptosis can relate to the immune response against the parasite rather than a direct cytopathic effect of the parasite, and hence it may be amenable to modification through normalization of the suppressive cytokine environment in which DCs are engaged.

In this regard, cytokines that regulate the inflammatory response, and particularly IL-10, have been suggested to affect blood DC during acute malaria (Urban et al., 2006; Diallo et al., 2008). Maturing DCs undergo apoptosis after exposure to high levels of IL-10 (Chang et al., 2007), and a correlation between high IL-10 and reduced DC counts has been suggested for human *Pf* (Urban et al., 2006; Diallo et al., 2008) and *Pv* infections (Jangpatarapongsa et al., 2008; Gonçalves et al., 2010). Here, we demonstrate for the first time a direct causal relationship between increased levels of plasma IL-10 and blood DC apoptosis in patients with clinical *Pf* or *Pv* infections. In malaria, IL-10 is an important cytokine and immune modulator that strikes a balance between immune protection and immunopathology (Couper et al., 2008). However, sustained exposure of DC to IL-10 can suppress the induction of antiapoptotic genes like *bcl-2*, *bcl-x*, and *bfl-1* at a time of increased sensitivity to viability signals, i.e., during DC maturation (Chang et al., 2007). Additionally, exocrine IL-10 can drive secretion of autocrine IL-10 by DC and other cell types further impacting on cell survival (Corinti et al., 2001). In human malaria for example, in vitro–derived DCs exposed to infected erythrocytes fail to secrete IL-12 yet produce increasing levels of IL-10 (Urban et al., 1999). Similarly, in rodent malaria, DCs progressively increase their ability to produce IL-10 and induce an IL-10–dominated T cell response (Perry et al., 2005). Therefore, while controlling immunopathology, the scale of IL-10 exposure throughout the maturation process appears to determine the lifespan of blood DC via mechanisms involving cytokine regulation and antiapoptotic gene modulation.

Although apoptosis and high levels of IL-10 appeared central to DC impairment in the present study, other mechanisms have also been proposed. Earlier studies using in vitro–derived DC indicate that *Plasmodium* can compromise DC via a mechanism involving direct contact through *Pf*EMP1 (Urban et al., 2001b). However, more recent evidence using a similar in vitro approach suggests that modulation is independent of parasite contact and rather depends on parasite dose (Elliott et al., 2007). Because *Pv*-infected red cells do not express *pf*EMP1 and are minimally cytoadherent, our finding of comparable DC impairment with either parasite would support the contact-independent nature of DC modulation. Other reports also implicate hemozoin in the modulation of DC through TLR-dependent binding (Coban et al., 2005; Parroche et al., 2007) and suggest that induction of TLR tolerance is central to DC dysregulation (Perry et al., 2005). In our study, sample limitation precluded direct assessment of blood DC–parasite interactions ex vivo. However, the observations that DC apoptosis and dysfunction persisted beyond parasite clearance (day 7), was in part IL-10 dependent, and normalized by day 28 upon resolution of the cytokine response suggested that DC impairment during clinical disease was primarily related to the immune response against the parasite rather than to the parasite itself. The lack of correlation between baseline parasitemia or plasma HRP2 and either DC numbers or apoptosis also indicated that by the time of presentation with clinical malaria, DC impairment was cytokine driven rather than parasite driven.

Whereas an alteration in the numerical and maturational phenotype of blood DC in acute malaria had been previously described (Jangpatarapongsa et al., 2008; Gonçalves et al., 2010), there had only been few reports on the function of blood DC in patients with clinical malaria (Arama et al., 2011) and no longitudinal studies in clinical disease had been described to date. Our cross-sectional assessment in 67 Papuan adults with acute malaria allowed us to demonstrate that the marked changes in blood DC viability were associated with significant impairment in all aspects of DC function (maturation, antigen uptake, and presentation) in patients with either type of parasite. In fact, we show that blood DC from *Pf* and *Pv* patients display impaired ability to up-regulate costimulatory

and MHC class II molecules as well reduced antigen uptake and ability to stimulate CD4 T cell proliferation and cytokine secretion. Moreover, upon longitudinal follow up, we demonstrate that parasite clearance and normalization of the cytokine milieu after antimalarial treatment facilitate recovery of fully competent blood DC in patients with malaria.

Therefore, our findings point out that impairment of blood DC number and function, as well as induction of apoptosis, is not species-specific and occurs with the two *Plasmodium* species causing the majority of global malaria. Consequently, our results have significant implications not only for understanding immune dysfunction during malaria but also for the development of effective interventions to improve vaccination outcomes against this disease. In view of the remarkable diversity of immunosuppressive pathways in malaria, any clinical response achieved by vaccination is already considered an achievement. However, to improve responses to any of the current subunit or whole parasite vaccine formulations (Pinzon-Charry and Good, 2008), vaccines will need to be combined with other strategies that can offset the suppressive environment in which DCs are primed. Given the protracted nature of the DC dysfunction described here in patients with acute infection and the observation that parasite clearance and normalization of the cytokine milieu after antimalarial treatment facilitate DC recovery, combination strategies will likely be required. Based on the evidence presented here, we propose that vaccination in the context of iterative preventive therapies, pre-vaccination antimalarial treatment, immunization during nontransmission season, or the use of approaches which vigorously stimulate DC and cellular immunity (Pinzon-Charry et al., 2010) should be considered to improve vaccination outcomes for malaria.

MATERIALS AND METHODS

Study subjects and samples. 84 highland Papuans were recruited in Timika, a lowland region of Papua, Indonesia, with perennial unstable malaria transmission of both *Pf* and *Pv* (Karyana et al., 2008). Subjects were enrolled in trials of chloroquine and sulphadoxine-pyrimethamine or artemisinin combination therapy after providing informed consent (Ratcliff et al., 2007a,b). Individuals with acute uncomplicated malaria—as defined by acute onset of fever within 48 h of enrolment—with no alternative cause identified and parasitemia by microscopically identified *Pf* ($n = 42$) or *Pv* ($n = 25$) were included in the study. No patients with mixed infection were included. For all patients, venous blood was collected at the time of presentation. Additionally, in 10 *Pf* patients with clinical and parasitological cure, venous blood was also collected at 7 and 28 d after successful antimalarial drug treatment with chloroquine plus sulfoadioxine-pyrimethamine or artemisinin combination therapy (ACT). Malaria-exposed uninfected subjects ($n = 17$) served as controls; these were resident in the Timika district for at least 2 yr and had no fever or symptoms of malaria within the preceding 2 wk as previously described (Randall et al., 2010). The studies were approved by the Ethics Committees of the National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia; the Menzies School of Health Research, Darwin, Australia; and the Queensland Institute of Medical Research, Brisbane, Australia.

Antibodies and reagents. The following mAbs were used in this study: CD3, CD14, CD19, CD20, CD56, CD34, HLA-DR, CD86, CD25, CD27, CD69, CD79a, and CD11b and IgG1, IgG2a, and IgG2b isotype controls (BD); and HLA-DR, CD83, CD19, and IgG1 isotype control (Beckman Coulter). All antibodies were used as FITC, PE, biotin, APC, or PE-Cy5 conjugated. Complete media included RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 25 mM Hepes, and nonessential amino acids (Gibco). 10 ng/ml LPS and 1 mg/ml FITC-Dextran were purchased from Sigma-Aldrich.

Flow cytometry, antigen uptake, and mixed leukocyte reaction. PBMCs were stained with the lineage mixture (CD3, CD14, CD19, CD20, CD56, and CD34) and HLA-DR. Flow cytometry (FACS) was used to evaluate DC numbers, phenotype, antigen uptake, and function. Blood DCs were defined as $Lin^{-}HLA\text{-}DR^{+}$ cells. Blood DC subsets were identified using CD11c (mDC) and CD123 (pDC) antibodies. mDC subsets were further characterized using CD141, CD1c, and CD16 antigens. Antigen uptake was assessed after cells were incubated (10^7 cells/ml) for 60 min with FITC-Dextran at either 37°C (test) or 4°C (control). Antigen uptake was calculated as the difference in mean fluorescence intensity (ΔMFI) between the test and control. To assess allostimulatory capacity, PBMCs from patients or uninfected volunteers were individually tested against naive allogeneic CD4 T cells obtained from buffy coats from healthy Australian volunteers ($n = 5$, Australian Red Cross). Naive allogeneic CD4 T cells (>95% CD4) were purified by negative immunoselection using the naive CD4 T cell isolation kit (Miltenyi Biotec) and labeled using the vibrant CFDA-SE Cell Tracer Kit (Molecular Probes). CD4 T cells were adjusted at 10^5/well and cultured with DCs from malaria patients or uninfected controls at a 30:1 T/DC ratio in PBMC cultures. In all cases, DC numbers were adjusted based on absolute counts estimated by FACS before culture. Cells were harvested after 96 h of culture and CD4 T cell proliferation estimated by CFSE dilution using MODFIT proliferation software (Verifit Software). Similar results were found for all T/DC ratios. In all experiments, cells were analyzed within 1 h of staining and $5–10 \times 10^5$ events were collected within the mononuclear cell gate. Absolute DC counts were calculated from the number of PBMCs/liter of blood as determined by the automated cell counter multiplied by the percentage of DCs determined by FACS. Lymphocyte, monocyte, and neutrophil counts were determined by automated cell counter. Serum and supernatant cytokines were measured using the cytometric bead array (CBA) for human Th1/Th2 cytokine kit (BD). Data were acquired on a FACSCalibur equipped with CellQuestPro and CBA software (BD) according to the manufacturer's instructions.

Apoptosis and IL-10 assay. To determine the proportion of apoptotic cells, Annexin-V binding assays were performed according to the manufacturer's instructions (Annexin-V kit; BD). Briefly, PBMCs were adjusted to 10^6 cells/ml and stained with lineage markers and HLA-DR as above. Cells were washed and resuspended in binding buffer before incubating with Annexin-V and 7-AAD. 7-AAD was added as a viability indicator except when co-labeling for CD11c plus CD123 expression. In all experiments, DC apoptosis was analyzed by FACS within 1 h of staining. The proapoptotic effects of IL-10 on DC were tested as previously described (Chang et al., 2007). In brief, PBMC cultures (10^7 cells/ml) from healthy adult Australian volunteers ($n = 5$) were incubated in complete medium with 10 ng/ml LPS and either 500 pg/ml rhIL-10 (R&D Systems) or 50% (vol/vol) plasma from patients with *Pf* ($n = 3$; mean IL-10 concentration 602.6 pg/ml) or *Pv* ($n = 3$; mean IL-10 concentration 353.8 pg/ml) for 24 h. Blood DC apoptosis was estimated by Annexin-V staining. Cultures incubated with LPS alone or serum from uninfected volunteers ($n = 3$; mean IL-10 concentration 58.6 pg/ml) served as controls. To neutralize IL-10, 5 µg/ml anti–IL-10R1 mAb or the respective IgG1 isotype control (R&D Systems) was added to cultures as indicated. Doses and incubation times were optimized in preliminary experiments. In all experiments, $5–10 \times 10^5$ events were collected within the mononuclear cell gate. Data were acquired on a FACSCalibur and analyzed using CellQuest 3.1 or Summit (Cytomation) software.

Statistical analysis. All statistical analyses used Prism 5 (GraphPad Software Inc.). Comparisons of samples to establish statistical significance were determined by the two tailed Student's *t* test or one way analysis of variance

(ANOVA), followed by Bonferroni's test. Correlation analyses were performed using Spearman's Rank test. Results were considered to be statistically significant when p-value was <0.05.

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Author contributions: A. Pinzon-Charry , J.A. López, and T. Woodberry conceived the study. A. Pinzon-Charry, T. Woodberry, C. Engwerda, J.A. López, N.M. Anstey, and M.F. Good designed the study. A. Pinzon-Charry, V. Kienzle, V. McPhun, T. Woodberry, and G. Minigo performed the laboratory studies. D.A. Lampah and E. Kenangalem enrolled the patients. A. Pinzon-Charry, T. Woodberry, G. Minigo, and J.A. López analyzed the data. All authors contributed to writing the paper.

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REFERENCES

Aldebert, D., M. Diallo, M. Niang, D. Sarr, C. Cisse, J.C. Moreau, and R. Jambou. 2007. Differences in circulating dendritic cell subtypes in peripheral, placental and cord blood in African pregnant women. *J. Reprod. Immunol.* 73:11–19. http://dx.doi.org/10.1016/j.jri.2006.05.002

Arama, C., P. Giusti, S. Boström, V. Dara, B. Traore, A. Dolo, O. Doumbo, S. Varani, and M. Troye-Blomberg. 2011. Interethnic differences in antigen-presenting cell activation and TLR responses in Malian children during *Plasmodium falciparum* malaria. *PLoS ONE.* 6:e18319. http://dx.doi.org/10.1371/journal.pone.0018319

Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767–811. http://dx.doi.org/10.1146/annurev.immunol.18.1.767

Bettiol, E., D. Carapau, C. Galan-Rodriguez, C. Ocaña-Morgner, and A. Rodriguez. 2010. Dual effect of *Plasmodium*-infected erythrocytes on dendritic cell maturation. *Malar. J.* 9:64. http://dx.doi.org/10.1186/1475-2875-9-64

Bhattarai, A., A.S. Ali, S.P. Kachur, A. Mårtensson, A.K. Abbas, R. Khatib, A.W. Al-Mafazy, M. Ramsan, G. Rotllant, J.F. Gerstenmaier, et al. 2007. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. *PLoS Med.* 4:e309. http://dx.doi.org/10.1371/journal.pmed.0040309

Chang, W.L., N. Baumgarth, M.K. Eberhardt, C.Y. Lee, C.A. Baron, J.P. Gregg, and P.A. Barry. 2007. Exposure of myeloid dendritic cells to exogenous or endogenous IL-10 during maturation determines their longevity. *J. Immunol.* 178:7794–7804.

Coban, C., K.J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, et al. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201:19–25. http://dx.doi.org/10.1084/jem.20041836

Corinti, S., C. Albanesi, A. la Sala, S. Pastore, and G. Girolomoni. 2001. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol.* 166:4312–4318.

Couper, K.N., D.G. Blount, and E.M. Riley. 2008. IL-10: the master regulator of immunity to infection. *J. Immunol.* 180:5771–5777.

de la Rosa, G., N. Longo, J.L. Rodríguez-Fernández, A. Puig-Kroger, A. Pineda, A.L. Corbí, and P. Sánchez-Mateos. 2003. Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. *J. Leukoc. Biol.* 73:639–649. http://dx.doi.org/10.1189/jlb.1002516

Diallo, M., D. Aldebert, J.C. Moreau, M. Ndiaye, and R. Jambou. 2008. Decrease of lymphoid dendritic cells in blood from malaria-infected pregnant women. *Int. J. Parasitol.* 38:1557–1565. http://dx.doi.org/10.1016/j.ijpara.2008.04.013

Elliott, S.R., T.P. Spurck, J.M. Dodin, A.G. Maier, T.S. Voss, F. Yosaatmadja, P.D. Payne, G.I. McFadden, A.F. Cowman, S.J. Rogerson, et al. 2007. Inhibition of dendritic cell maturation by malaria is dose dependent and does not require *Plasmodium falciparum* erythrocyte membrane protein 1. *Infect. Immun.* 75:3621–3632. http://dx.doi.org/10.1128/IAI.00095-07

Giusti, P., B.C. Urban, G. Frascaroli, L. Albrecht, A. Tinti, M. Troye-Blomberg, and S. Varani. 2011. *Plasmodium falciparum*-infected erythrocytes and beta-hematin induce partial maturation of human dendritic cells and increase their migratory ability in response to lymphoid chemokines. *Infect. Immun.* 79:2727–2736. http://dx.doi.org/10.1128/IAI.00649-10

Gonçalves, R.M., K.C. Salmazi, B.A. Santos, M.S. Bastos, S.C. Rocha, S.B. Boscardin, A.M. Silber, E.G. Kallás, M.U. Ferreira, and K.K. Scopel. 2010. CD4+ CD25+ Foxp3+ regulatory T cells, dendritic cells, and circulating cytokines in uncomplicated malaria: do different parasite species elicit similar host responses? *Infect. Immun.* 78:4763–4772. http://dx.doi.org/10.1128/IAI.00578-10

Guerra, C.A., R.E. Howes, A.P. Patil, P.W. Gething, T.P. Van Boeckel, W.H. Temperley, C.W. Kabaria, A.J. Tatem, B.H. Manh, I.R. Elyazar, et al. 2010. The international limits and population at risk of *Plasmodium vivax* transmission in 2009. *PLoS Negl. Trop. Dis.* 4:e774. http://dx.doi.org/10.1371/journal.pntd.0000774

Jangpatarapongsa, K., P. Chootong, J. Sattabongkot, K. Chotivanich, J. Sirichaisinthop, S. Tungpradabkul, H. Hisaeda, M. Troye-Blomberg, L. Cui, and R. Udomsangpetch. 2008. *Plasmodium vivax* parasites alter the balance of myeloid and plasmacytoid dendritic cells and the induction of regulatory T cells. *Eur. J. Immunol.* 38:2697–2705. http://dx.doi.org/10.1002/eji.200838186

Jongbloed, S.L., A.J. Kassianos, K.J. McDonald, G.J. Clark, X. Ju, C.E. Angel, C.J. Chen, P.R. Dunbar, R.B. Wadley, V. Jeet, et al. 2010. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207:1247–1260. http://dx.doi.org/10.1084/jem.20092140

Karyana, M., L. Burdarm, S. Yeung, E. Kenangalem, N. Wariker, R. Maristela, K.G. Umana, R. Vemuri, M.J. Okoseray, P.M. Penttinen, et al. 2008. Malaria morbidity in Papua Indonesia, an area with multidrug resistant *Plasmodium vivax* and *Plasmodium falciparum*. *Malar. J.* 7:148. http://dx.doi.org/10.1186/1475-2875-7-148

Kitajima, T., K. Ariizumi, P.R. Bergstresser, and A. Takashima. 1996. Ultraviolet B radiation sensitizes a murine epidermal dendritic cell line (XS52) to undergo apoptosis upon antigen presentation to T cells. *J. Immunol.* 157:3312–3316.

Kobbe, R., S. Adjei, C. Kreuzberg, B. Kreuels, B. Thompson, P.A. Thompson, F. Marks, W. Busch, M. Tosun, N. Schreiber, et al. 2007. Malaria incidence and efficacy of intermittent preventive treatment in infants (IPTi). *Malar. J.* 6:163. http://dx.doi.org/10.1186/1475-2875-6-163

Kolaczinski, K., J. Kolaczinski, A. Kilian, and S. Meek. 2007. Extension of indoor residual spraying for malaria control into high transmission settings in Africa. *Trans. R. Soc. Trop. Med. Hyg.* 101:852–853. http://dx.doi.org/10.1016/j.trstmh.2007.04.003

Nosten, F., M. van Vugt, R. Price, C. Luxemburger, K.L. Thway, A. Brockman, R. McGready, F. ter Kuile, S. Looareesuwan, and N.J. White. 2000. Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. *Lancet.* 356:297–302. http://dx.doi.org/10.1016/S0140-6736(00)02505-8

Oliveira-Ferreira, J., M.V. Lacerda, P. Brasil, J.L. Ladislau, P.L. Tauil, and C.T. Daniel-Ribeiro. 2010. Malaria in Brazil: an overview. *Malar. J.* 9:115. http://dx.doi.org/10.1186/1475-2875-9-115

Parroche, P., F.N. Lauw, N. Goutagny, E. Latz, B.G. Monks, A. Visintin, K.A. Halmen, M. Lamphier, M. Olivier, D.C. Bartholomeu, et al. 2007. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proc. Natl. Acad. Sci. USA.* 104:1919–1924. http://dx.doi.org/10.1073/pnas.0608745104

Perry, J.A., C.S. Olver, R.C. Burnett, and A.C. Avery. 2005. Cutting edge: the acquisition of TLR tolerance during malaria infection impacts T cell activation. *J. Immunol.* 174:5921–5925.

Piccioli, D., S. Tavarini, E. Borgogni, V. Steri, S. Nuti, C. Sammicheli, M. Bardelli, D. Montagna, F. Locatelli, and A. Wack. 2007. Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood.* 109:5371–5379. http://dx.doi.org/10.1182/blood-2006-08-038422

Pichyangkul, S., K. Yongvanitchit, U. Kum-arb, H. Hemmi, S. Akira, A.M. Krieg, D.G. Heppner, V.A. Stewart, H. Hasegawa, S. Looareesuwan, et al. 2004. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *J. Immunol.* 172:4926–4933.

Pinzon-Charry, A., and M.F. Good. 2008. Malaria vaccines: the case for a whole-organism approach. *Expert Opin. Biol. Ther.* 8:441–448. http://dx.doi.org/10.1517/14712598.8.4.441

Pinzon-Charry, A., C.S. Ho, R. Laherty, T. Maxwell, D. Walker, R.A. Gardiner, L. O'Connor, C. Pyke, C. Schmidt, C. Furnival, and J.A. López. 2005. A population of HLA-DR+ immature cells accumulates in the blood dendritic cell compartment of patients with different types of cancer. *Neoplasia.* 7:1112–1122. http://dx.doi.org/10.1593/neo.05442

Pinzon-Charry, A., V. McPhun, V. Kienzle, C. Hirunpetcharat, C. Engwerda, J. McCarthy, and M.F. Good. 2010. Low doses of killed parasite in CpG elicit vigorous CD4+T cell responses against blood-stage malaria in mice. *J. Clin. Invest.* 120:2967–2978. http://dx.doi.org/10.1172/JCI39222

Randall, L.M., E. Kenangalem, D.A. Lampah, E. Tjitra, E.D. Mwaikambo, T. Handojo, K.A. Piera, Z.Z. Zhao, F. de Labastida Rivera, Y. Zhou, et al. 2010. Age-related susceptibility to severe malaria associated with galectin-2 in highland Papuans. *J. Infect. Dis.* 202:117–124. http://dx.doi.org/10.1086/653125

Ratcliff, A., H. Siswantoro, E. Kenangalem, R. Maristela, R.M. Wuwung, F. Laihad, E.P. Ebsworth, N.M. Anstey, E. Tjitra, and R.N. Price. 2007a. Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: an open-label randomised comparison. *Lancet.* 369:757–765. http://dx.doi.org/10.1016/S0140-6736(07)60160-3

Ratcliff, A., H. Siswantoro, E. Kenangalem, M. Wuwung, A. Brockman, M.D. Edstein, F. Laihad, E.P. Ebsworth, N.M. Anstey, E. Tjitra, and R.N. Price. 2007b. Therapeutic response of multidrug-resistant *Plasmodium falciparum* and *P. vivax* to chloroquine and sulfadoxine-pyrimethamine in southern Papua, Indonesia. *Trans. R. Soc. Trop. Med. Hyg.* 101:351–359. http://dx.doi.org/10.1016/j.trstmh.2006.06.008

Robinson, S.P., S. Patterson, N. English, D. Davies, S.C. Knight, and C.D. Reid. 1999. Human peripheral blood contains two distinct lineages of dendritic cells. *Eur. J. Immunol.* 29:2769–2778. http://dx.doi.org/10.1002/(SICI)1521-4141(199909)29:09<2769::AID-IMMU2769>3.0.CO;2-2

Rossi, M., and J.W. Young. 2005. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *J. Immunol.* 175:1373–1381.

Savary, C.A., M.L. Grazziutti, B. Melichar, D. Przepiorka, R.S. Freedman, R.E. Cowart, D.M. Cohen, E.J. Anaissie, D.G. Woodside, B.W. McIntyre, et al. 1998. Multidimensional flow-cytometric analysis of dendritic cells in peripheral blood of normal donors and cancer patients. *Cancer Immunol. Immunother.* 45:234–240. http://dx.doi.org/10.1007/s002620050438

Skorokhod, O.A., M. Alessio, B. Mordmüller, P. Arese, and E. Schwarzer. 2004. Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-gamma-mediated effect. *J. Immunol.* 173:4066–4074.

Snow, R.W., C.A. Guerra, A.M. Noor, H.Y. Myint, and S.I. Hay. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature.* 434:214–217. http://dx.doi.org/10.1038/nature03342

Thomas, R., L.S. Davis, and P.E. Lipsky. 1993. Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol.* 150:821–834.

Urban, B.C., D.J.P. Ferguson, A. Pain, N. Willcox, M. Plebanski, J.M. Austyn, and D.J. Roberts. 1999. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature.* 400:73–77. http://dx.doi.org/10.1038/21900

Urban, B.C., T. Mwangi, A. Ross, S. Kinyanjui, M. Mosobo, O. Kai, B. Lowe, K. Marsh, and D.J. Roberts. 2001a. Peripheral blood dendritic cells in children with acute *Plasmodium falciparum* malaria. *Blood.* 98:2859–2861. http://dx.doi.org/10.1182/blood.V98.9.2859

Urban, B.C., N. Willcox, and D.J. Roberts. 2001b. A role for CD36 in the regulation of dendritic cell function. *Proc. Natl. Acad. Sci. USA.* 98:8750–8755. http://dx.doi.org/10.1073/pnas.151028698

Urban, B.C., D. Cordery, M.J. Shafi, P.C. Bull, C.I. Newbold, T.N. Williams, and K. Marsh. 2006. The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria. *Infect. Immun.* 74:6700–6706. http://dx.doi.org/10.1128/IAI.00861-06

Walsh, D.S., S. Looareesuwan, S. Vaniganonta, C. Viravan, and H.K. Webster. 1995. Cutaneous delayed-type hypersensitivity responsiveness in patients during and after *Plasmodium falciparum* and *Plasmodium vivax* infections. *Clin. Immunol. Immunopathol.* 77:89–94. http://dx.doi.org/10.1016/0090-1229(95)90141-8

WHO Expert Committee on Malaria. 2000. WHO Expert Committee on Malaria. *World Health Organ. Tech. Rep. Ser.* 892:i–v: 1–74.

Williamson, W.A., and B.M. Greenwood. 1978. Impairment of the immune response to vaccination after acute malaria. *Lancet.* 311:1328–1329. http://dx.doi.org/10.1016/S0140-6736(78)92403-0

Woodberry, T., G. Minigo, K.A. Piera, F.H. Amante, A. Pinzon-Charry, M.F. Good, J.A. Lopez, C.R. Engwerda, J.S. McCarthy, and N.M. Anstey. 2012. Low-level *Plasmodium falciparum* blood-stage infection causes dendritic cell apoptosis and dysfunction in healthy volunteers. *J. Infect. Dis.* 206:333–340. http://dx.doi.org/10.1093/infdis/jis366