Published in final edited form as:
Cell Rep. 2016 October 4; 17(2): 399–412. doi:10.1016/j.celrep.2016.09.015.

Type I Interferons Regulate Immune Responses in Humans with Blood-Stage *Plasmodium falciparum* Infection

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Summary

The development of immunoregulatory networks is important to prevent disease. However, these same networks allow pathogens to persist and reduce vaccine efficacy. Here, we identify type I interferons (IFNs) as important regulators in developing anti-parasitic immunity in healthy volunteers infected for the first time with *Plasmodium falciparum*. Type I IFNs suppressed innate immune cell function and parasitic-specific $CD4^+$ T cell IFNγ production, and they promoted the development of parasitic-specific IL-10-producing Th1 (Tr1) cells. Type I IFN-dependent,

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M.M.O., J.S.M., and C.R.E. designed the experiments and wrote the paper. M.M.O., R.K., F.L.R., F.H.A., M.S., R.J.F., P.T.B., S.E.B., L.B., S.S.N., and C.L.E. performed the experiments. L.B., G.M.B., A.H., R.N.P., N.M.A., J.R.L., J.B., and D.L.D. helped analyze data, provided samples or reagents, and edited the manuscript.

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parasite-specific IL-10 production was also observed in *P. falciparum* malaria patients in the field following chemoprophylaxis. Parasite-induced IL-10 suppressed inflammatory cytokine production, and IL-10 levels after drug treatment were positively associated with parasite burdens before anti-parasitic drug administration. These findings have important implications for understanding the development of host immune responses following blood-stage *P. falciparum* infection, and they identify type I IFNs and related signaling pathways as potential targets for therapies or vaccine efficacy improvement.

Introduction

Eliminating pathogens typically requires the generation of robust host immune responses. However, these responses need to be tightly controlled so that they do not damage host tissues. Therefore, humans have developed potent immunoregulatory networks to control inflammation and prevent disease (Chaudhry and Rudensky, 2013; Engwerda et al., 2014; Sakaguchi et al., 2008, 2013). Many pathogens take advantage of these immunoregulatory mechanisms to persist in their host (Belkaid and Rouse, 2005). In the case of parasites that cause diseases such as malaria, toxoplasmosis, and leishmaniasis, persistent infection can also maintain concomitant immunity, which may be especially important in protecting against new infections with pathogenic parasite strains in disease-endemic areas (Sacks, 2014). A better understanding of how immunoregulatory networks develop and are maintained following infection is needed if they are to be manipulated for therapeutic advantage or to improve vaccine efficiency.

Malaria remains a significant global health problem, with more than 250 million cases and 500,000 deaths annually (WHO, 2014). *Plasmodium falciparum* is responsible for most of this morbidity and mortality, with young children being most affected (WHO, 2014). Results with the RTS,S/AS01 vaccine show that despite having approximately 50% vaccine efficacy in healthy volunteers participating in controlled human malaria infection (CHMI) studies (Kester et al., 2009; Ockenhouse et al., 2015), efficacy fell when tested in healthy adults living in a high malaria transmission region (Polhemus et al., 2009) and provided similar, relatively modest protection in children living in malaria-endemic areas (Rts, 2015). The reason for this difference is not clear, but this phenomenon has also been observed with other vaccines, such as those developed to protect against tuberculosis (Pitt et al., 2013; Skeiky and Sadoff, 2006), respiratory syncytial virus (RSV) (Christiaansen et al., 2014), and HIV (Boussiotis et al., 2000; Migueles and Connors, 2015; Rodríguez-García et al., 2011). One possible explanation is that early exposure to pathogens promotes the development of immunoregulatory networks that impede the generation of efficient vaccine-induced immunity.

A number of regulatory molecules and cell populations have been identified in pre-clinical models of malaria, as well as in malaria patients. These include cytokines such as interleukin (IL) 10 (Couper et al., 2008; Plebanski et al., 1999) and transforming growth factor β (TGF-β) (Omer and Riley, 1998; Walther et al., 2005), as well as immune checkpoint molecules such as CTLA-4 (Jacobs et al., 2002; Schlotmann et al., 2000), LAG-3 (Butler et al., 2011; Illingworth et al., 2013), PD-1 (Butler et al., 2011; Hafalla et al., 2012), and TIM-3 (Costa et

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al., 2015; Huang et al., 2013). Specialized sub-populations of $CD4^+$ T cells have emerged as major regulators of inflammation during parasitic diseases (Belkaid and Rouse, 2005; Engwerda et al., 2014). These regulatory T (Treg) cells can be broadly divided into two types. First, natural Treg cells are produced in the thymus and express the transcription factor FoxP3 that is critical for their suppressive functions (Sakaguchi et al., 2013). Treg cells with increased suppressive function have been reported in adults with malaria (Minigo et al., 2009; Walther et al., 2005). However, studies in African children showed that neither Treg cell number nor Treg cell function differs between patients with uncomplicated malaria and those with severe malaria (Walther et al., 2009). A study of Ugandan children from areas of different malaria exposure indicated that burden of disease may have an important impact on number and function of Treg cells (Boyle et al., 2015). Furthermore, a study in malaria patients from the Peruvian Amazon showed that neither Treg cell frequency nor Treg cell number was associated with the risk of malaria-related symptoms (Torres et al., 2014), suggesting that alternative mechanisms of immune regulation may be important for controlling inflammation and thus preventing disease.

A second type of Treg cell consists of inducible Treg cells, which emerge from the thymus as conventional $CD4^+$ T cells but develop regulatory functions in the periphery following exposure to appropriate inflammatory stimulation. These include IL-10-producing T helper 1 (Th1) or type I regulatory (Tr1) cells (O'Garra et al., 2004). Tr1 cells, but not Treg cells, were shown to be more prevalent in Gambian children with uncomplicated malaria compared to those with severe disease (Walther et al., 2009). Furthermore, Tr1 cells emerge relatively quickly in children living in malaria-endemic areas (Jagannathan et al., 2014; Portugal et al., 2014), but their role in suppressing either inflammation or anti-parasitic immunity remains unclear.

Type I interferons (IFNs) have emerged as important immune regulators in infectious disease (McNab et al., 2015). They are required for anti-viral defenses but can also promote pathogen survival by suppressing immunity during infections, such as those caused by lymphocytic choriomeningitis virus (Teijaro et al., 2013; Wilson et al., 2013), influenza (Arimori et al., 2013), *Mycobacteria* (Antonelli et al., 2010; Berry et al., 2010; Desvignes et al., 2012; Stanley et al., 2007; Teles et al., 2013), *Listeria* (Auerbuch et al., 2004; Carrero et al., 2004), *Staphylococcus* (Parker and Prince, 2012), *Leishmania* (Xin et al., 2010), and *Plasmodium* (Haque et al., 2011; Sharma et al., 2011). In mice infected with *Mycobacteria tuberculosis*, type I IFNs suppressed inflammatory cytokine production by stimulating the production of IL-10 and IL-1 receptor antagonists (Mayer-Barber et al., 2011; McNab et al., 2014). We reported that type I IFNs inhibited the ability of dendritic cells (DCs) to activate $CD4^+$ T cells in experimental murine malaria models, and this was associated with changes in co-stimulatory molecule expression, as well as the promotion of DC IL-10 production (Haque et al., 2011, 2014).

To understand better how and when regulatory mechanisms develop during malaria, we examined peripheral blood from human volunteers participating in CHMI studies with *P. falciparum* 3D7. We identified type I IFNs as major immunoregulatory molecules during blood-stage malaria. We show that they suppressed innate and adaptive immunity, as well as promoting the generation of Tr1 cells following resolution of infection with an anti-parasitic

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drug. We also show that IL-10 production by peripheral blood mononuclear cells (PBMCs) from *P. falciparum* malaria patients collected in the field were regulated by type I IFNs. These results provide insights into the development of immunity during malaria and provide a potential explanation for why vaccines administered in disease-endemic areas may function sub-optimally.

Results

CD4$^+$ T Cell Responses during Blood-Stage *P. falciparum* Infection

To understand how CD4$^+$ T cells respond following first exposure to *P. falciparum*, we isolated PBMCs at various times throughout CHMI studies involving multiple cohorts in the context of various clinical trials of new anti-malaria drugs. Infection in healthy volunteers was established by intravenous injection of 1,800 *P. falciparum* 3D7 parasitized red blood cells (pRBCs), with drug treatment initiated when blood parasitemia, determined by qPCR (Rockett et al., 2011), was greater than 1,000 parasites/mL (Figure 1A). This occurred on either day 7 or day 8 post-infection (p.i.), at which time most volunteers showed no clinical signs of malaria.

First, we measured the ability of PBMCs to produce the pro-inflammatory cytokine IFNγ in response to parasite antigen throughout the study period (Figures 1A and 1B). As reported by others (Good, 1994; Ndungu et al., 2006), we observed some IFNγ production by PBMCs from volunteers before infection, presumably by memory T cells generated in response to cross-reactive antigens from other pathogens (Currier et al., 1995). IFNγ production in response to parasite antigen was also significantly increased relative to controls (normal red blood cells [nRBC]) 7 days after infection and further increased 7 days after the commencement of drug treatment and maintained until at least 28 days after infection, albeit at decreased levels at the latter time point (Figure 1B).

T cells were a major source of IFNγ measured in this assay, although natural killer (NK) cells and other lymphocytes also produced IFNγ, as indicated by analysis of the frequency of IFNγ-producing cells in PBMC cultures isolated during the study period (Figures S1A and S1B). However, CD4$^+$ T cells accounted for substantial amounts of cytokine in most volunteers, because when major histocompatibility complex (MHC) class II antigen presentation was blocked in PBMC cultures at day 7 p.i., the amount of IFNγ produced was significantly diminished (Figure 1C). The frequency of CD4$^+$ or CD8$^+$ T cells in the blood of volunteers at this time point was relatively unchanged (Figure 1D). Altogether, these data show that parasite-reactive CD4$^+$ T cell responses could be detected throughout the course of infection and were greatest 7 days after commencement of drug treatment.

Type I IFNs Produced in Response to *P. falciparum* Suppress CD4$^+$ T Cell IFNγ Production

Previously, we reported that type I IFNs suppressed anti-parasitic Th1 cell responses in experimental models of malaria (Haque et al., 2011, 2014). Parasite-stimulated IFNα2 was produced in response to *P. falciparum* antigen throughout the course of infection and was significantly elevated at day 7 p.i. (Figure 2A). We next investigated the cellular sources of type I IFNs at day 7 p.i. and found that although plasmacytoid dendritic cells (pDCs; HLA-

DR$^+$ CD123$^+$ CD304$^+$) were a major source of this cytokine in response to CpG, other cell types were also capable of type I IFN production (Figure S2). Furthermore, multiple cell types were able to produce IFNα species when cultured with nRBC or pRBC (Figure 2B). We also found that multiple type I IFNs were expressed by different PBMC populations isolated from PBMCs at 7 days p.i. (Figure S3). Thus, increased type I IFN production in response to *P. falciparum* infection comes from different cell sources and results from increased expression of multiple type I IFN species.

When type I IFN signaling was blocked with an antibody reactive against type I IFN receptor, antigen-specific IFNγ production was significantly increased at day 7 p.i. (Figure 3A), but this effect was not observed before infection or after commencement of drug treatment (data not shown). Although the frequencies of IFNγ-producing cell populations were relatively unchanged after type I IFN blockade (Figures S1B and S1C), the increased IFNγ production by PBMCs at day 7 p.i., following type I IFN blockade, appeared to be largely MHC class II antigen dependent (Figure 3B). We next examined the relationship between parasite-specific IFNγ levels at day 7 p.i. and parasite burdens in volunteers over the interval before drug treatment, as assessed by the area under the curve (AUC) of blood parasitemia. This revealed a significant inverse correlation between IFNγ levels and parasite burden (Figure 3C). In addition, analysis of IFNγ mean fluorescence intensity (MFI) showed that type I IFN blockade significantly increased IFNγ expression by CD4$^+$ T cells but not by other IFNγ-producing cell populations (Figure 3D). Hence, type I IFNs produced during first exposure to *P. falciparum* suppressed the ability of parasite-specific CD4$^+$ T cells to produce IFNγ. We observed similar effects on IL-17, IL-1β, and IL-6 production but not on tumor necrosis factor (TNF) production (Figure 4), suggesting some level of specificity for the suppressive effects of type I IFNs.

Type I IFNs Inhibit Parasite-Mediated IL-6 Production by Monocytes

The effect of type I IFN blockade on IL-6 production following exposure of PBMCs to parasite antigen was especially pronounced before infection, with a greater than 100-fold increase in IL-6 production upon type I IFN blockade (Figure 4A). This suggested that parasite-induced type I IFNs may target innate cell populations. fluorescence-activated cell sorting (FACS) analysis (Figure 5A) revealed that most of this IL-6 came from DCs (Figures 5B and 5C) and monocytes, with the latter cell population producing most of this cytokine on an individual cell basis (Figure 5D). Strikingly, IL-6 production by monocytes, but not by DC subsets, was significantly affected by type I IFN blockade, indicating a selective effect of type I IFNs on monocytes following exposure to *P. falciparum*. Thus, in addition to regulating CD4$^+$ T cell responses during sub-clinical malaria, type I IFNs suppressed early inflammatory responses by innate immune cells following exposure to parasites.

Type I IFNs Promote the Development of Immunosuppressive Antigen-Specific Tr1 Cells after Anti-parasitic Drug Treatment

Tr1 cells have emerged as a dominant CD4$^+$ T cell response in highly exposed children living in malaria-endemic regions (Jagannathan et al., 2014; Portugal et al., 2014; Walther et al., 2009). We found significant levels of IL-10 production by PBMCs in response to parasite antigen 7 days after drug treatment was initiated (14 days p.i.) (Figure 6A). This

was not related to the type of drug used to treat the *P. falciparum* infection, because the same response was observed when different drugs were used (Figures 6A–6C, 7A, and 7B; Table 1), as was the case for all immune parameters examined. The increased parasite-specific IL-10 production was associated with a significant increase in the frequency of Tr1 cells at this time point (Figures 6B and 6C). $CD4^+$ T cells were an important source of IL-10 in these cell cultures, because MHC class II blockade significantly reduced both frequency of Tr1 cells and IL-10 levels in culture supernatants (Figures 6C and 6D). Blockade of type I IFN signaling had a similar effect (Figures 7A and 7B), consistent with the idea that parasite-induced type I IFNs promoted the generation of Tr1 cells and/or IL-10 production by these cells. Hence, type I IFNs not only suppressed parasite-specific IFNγ production by $CD4^+$ T cells during sub-microscopic, pre-symptomatic *Plasmodium* infection but also contributed to the generation of Tr1 cells following anti-parasitic drug treatment.

To explore the functional consequences of increased IL-10 production, we examined the relationship between IL-10 levels and parasite burdens. A significant, positive correlation between the amount of IL-10 produced after drug treatment and the parasite burdens in volunteers over the interval before treatment, as assessed by the AUC of blood parasitemia, was found (Figure 7C). However, we cannot determine whether increased IL-10 at this time point indicates earlier immune suppression that allowed increased parasite growth or increased parasite burdens promoted the development of more Tr1 cells and/or IL-10 production. Nevertheless, IL-10 produced in response to parasite antigen 7 days after drug treatment suppressed parasite-specific inflammation, as indicated by increased parasite-specific IFNγ and TNF production when IL-10 was blocked in PBMC cultures (Figures 7D and 7E). Finally, to test whether type I IFNs promoted IL-10 production in clinical malaria in endemic settings, we cultured PBMC from Indonesian patients naturally infected with *P. falciparum* (Table 2). Cells were isolated at the time of admission and 7 days after commencement of drug treatment. Parasite-specific IL-10 was detected at both time points, and blockade of type I IFN signaling resulted in reduced IL-10 levels 7 days after commencement of drug treatment (Figure 7F), as previously observed in healthy volunteers (Figure 7A). Hence, type I IFN-dependent, parasite-specific IL-10 production was a feature of anti-parasitic immunity after administration of anti-parasitic drug, and altogether, these data show that type I IFN-dependent IL-10 production by $CD4^+$ T cells has important functional consequences for developing anti-parasitic immunity.

Discussion

In this study, we identified three distinct phases of immune regulation mediated by type I IFNs during blood-stage *P. falciparum* infection of humans involving innate immune cells, effector $CD4^+$ T cells, and anti-inflammatory Tr1 cells. In each of these phases, type I IFNs suppressed infection-induced inflammation. This included inhibiting production of the pro-inflammatory cytokines IL-6, IL-1β, IL-17, and IFNγ but not of TNF. Surprisingly, we found no correlation between parasite-specific type I IFN production and either parasite burden or IFNγ or IL-10 production in our studies, suggesting complex regulatory networks being mediated by this family of cytokines. We were unable to detect any cytokines directly in serum before drug treatment and were thus limited in our examination of relationships between type I IFNs and pro-inflammatory cytokine production. Type I IFNs have been

shown to inhibit the activity of various innate immune cells, including macrophages, monocytes, and neutrophils (Auerbuch et al., 2004; Berry et al., 2010; Carrero et al., 2004; Desvignes et al., 2012; Parker and Prince, 2012; Stanley et al., 2007; Xin et al., 2010). Previous studies in mouse models of malaria showed direct suppressive effects of type I IFNs on tissue resident DC subsets, associated with changes in co-stimulatory molecule expression and increased DC IL-10 production (Haque et al., 2014). Although we were unable to examine tissue resident DCs in the CHMI studies undertaken here, we found that type I IFNs suppressed IL-6 production by blood monocytes, suggesting these cells are an important target during blood-stage malaria. Monocytes play protective roles during malaria through phagocytosis of pRBC, antibody-dependent cell inhibition, and production of pro-inflammatory cytokines (Chua et al., 2013; Sheel and Engwerda, 2012). Furthermore, IL-6, along with TGF-β, is important for IL-17 production (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), and the suppression of both IL-6 and IL-17 identifies a major immunological axis regulated by type I IFNs during blood-stage *P. falciparum* infection.

In addition to suppressive effects on monocytes, we observed a profound impact of type I IFNs on antigen-specific IFNγ production by $CD4^+$ T cells in pre-symptomatic *P. falciparum*-infected volunteers. This finding is in line with our previous work in mouse models of malaria, in which we observed type I IFN-dependent suppression of anti-parasitic $CD4^+$ T cell responses (Haque et al., 2011, 2014). In the absence of type I IFN signaling blockade, parasite-specific IFNγ production 7 days after inoculation was no different from background levels detected before infection, consistent with little detectable inflammatory signature in subjects with sub-microscopic, pre-symptomatic *P. falciparum* infection at this time point. However, parasite-induced type I IFNs were readily detected at this time, and blockade of type I IFN signaling caused greatly enhanced MHC class II-restricted IFNγ production, as well as production of several other potent inflammatory cytokines, with the exception of TNF. IFNγ production in response to parasite antigen was highly variable among cohorts at day 7 p.i., and we attribute this to the varied timing of blood sampling in relation to the parasite life cycle and, in particular schizogony and RBC rupture, when we anticipate a spike in inflammation. Nevertheless, our data indicate that type I IFNs play an important role in suppressing inflammation during sub-microscopic, pre-symptomatic *P. falciparum* infection, and given the reported importance of IFNγ production for control of blood-stage malaria parasites (McCall et al., 2010; Meding et al., 1990; Shear et al., 1989; Stevenson et al., 1990; Su and Stevenson, 2000; van der Heyde et al., 1997; Yoneto et al., 1999), these findings identify a potent mechanism for the early suppression of anti-parasitic immunity.

An emerging paradigm in the evolution of anti-pathogen cellular immunity is the development of self-regulating T cell subsets during established infection (Belkaid and Rouse, 2005; Engwerda et al., 2014; Jankovic et al., 2010; O'Garra et al., 2004). Tr1 cells are one such cell population that arises from anti-parasitic Th1 cell responses, and their production of IL-10 appears to be a major mechanism of immune regulation (Anderson et al., 2007; Couper et al., 2008; Jankovic et al., 2007; Nylén et al., 2007). IL-10 acts on multiple levels of cellular immune responses, including antigen processing, production of cytokines, and various anti-microbial products (Saraiva and O'Garra, 2010), as well as the expression of inhibitory receptors such as PD-1, TIM-3, and LAG-3 (Camisaschi et al.,

2010; Ha et al., 2008; Jin et al., 2010). These actions of IL-10 can favor persistence of pathogens, but IL-10 also protects against tissue damage and associated disease (Jankovic et al., 2007; Montes de Oca et al., 2016; O'Garra et al., 2004). Tr1 cells are prevalent in children living in malaria-endemic regions (Jagannathan et al., 2014; Portugal et al., 2014; Walther et al., 2009), and results presented here not only demonstrate the emergence of Tr1 cells after resolution of first blood-stage *P. falciparum* infection but also show that the IL-10 produced by these cells potently suppressed parasite-specific, pro-inflammatory cytokine production. Thus, we identify type I IFNs as important regulators of IL-10 production both in infected study subjects and in patients living in malaria-endemic areas.

One finding in our study was the development of readily detected, parasite-specific Tr1 cells 7 days after commencement of drug treatment in subjects with their first *P. falciparum* infection. This occurred alongside the development of a robust Th1 cell response, presumably as a result of available parasite antigen caused by drug-mediated killing. The rapid development of immunoregulatory networks following a single exposure to the parasite highlights the potential of these regulatory networks to influence subsequent immunity, particularly to vaccines. Hence, our findings indicate that incorporating inhibitors of specific immune checkpoints into vaccine formulations may be one way to transiently reduce immune suppression and thus allow generation of robust vaccine-mediated, anti-parasitic immunity. However, given potential differences in the immunobiology resulting from intravenous challenge with parasites, compared with mosquito-transmitted parasites, and in particular changes in parasite virulence (Spence et al., 2013), it will be important to evaluate these strategies in the context of natural infection.

In summary, we have identified type I IFNs as powerful inhibitors of innate and adaptive immune responses following first exposure to *P. falciparum*. Our results support the idea that the early establishment of immunoregulatory networks following infection may act to suppress subsequent immune responses to new infections. These findings not only provide insights into how immune responses develop and are regulated during malaria but also help identify strategies to improve vaccines and other therapies aimed at preventing, and ultimately eradicating, one of the most important parasitic diseases.

Experimental Procedures

CHMI Studies

The work described here comprises studies performed nested within a series of clinical trials designed to test the anti-malarial activity of various newly discovered anti-malarial drugs (Table 1) against early blood-stage *P. falciparum* infection in healthy individuals. The studies were undertaken at Q-Pharm under the approval of the QIMR Berghofer Human Research Ethics Committee (QIMR-HREC). All studies were registered with the Australian and New Zealand Clinical Trial Registration scheme (ANZCTR: ACTRN12612000323820, ACTRN12612000814875, ACTRN12613000565741, and ACTRN12613001040752) or the U.S. NIH ClinicalTrials.gov (NCT: NCT02281344, and NCT02389348). The studies involved the collection of blood (20–40 mL) at various time points, including days 0 (before challenge), 7, 14, and 28–35, to assess cellular immune responses. Participants were healthy males and females between 18 and 55 years of age with no prior exposure to malaria or

residence in malaria-endemic regions. All participants were infected intravenously with *P. falciparum* (clone 3D7, drug-sensitive strain), which results in induced blood-stage malaria (IBSM) challenge. Real-time qPCR, as described by Rockett et al. (2011), was used to monitor peripheral parasitemia twice daily beginning 4 days after inoculation. Drug was administered once parasitemia had exceeded ≥1,000 parasites/mL, and participants were monitored closely for 48 hr as in-patients. Although the aim was to minimize subject symptoms, some participants reported headaches, nausea, vomiting, and increased body temperature, all of which may have been related to symptoms of early *P. falciparum* infection.

Clinical Malaria

PBMCs were isolated from 12 adult patients attending primary health care clinics with uncomplicated *P. falciparum* malaria in Timika, Papua, Indonesia (Ratcliff et al., 2007), a region of unstable malaria transmission (Karyana et al., 2008). Patient clinical data for samples from Timika are shown in Table 2. Median age was 34 years (range 20–43 years). Nine patients (75%) were male, with half of Papuan Highlander ethnicity and half non-Papuan. Median parasitemia was 2,195 parasites/mL (interquartile range [IQR] 810.75–4640.25 parasites/μL), with 11 patients (92%) treated with artemisinin combination therapy and one patient (8%) treated with chloroquine plus sulfadoxine-pyrimethamine. PBMCs were cryopreserved on the day of presentation and 7 days after treatment.

Human PBMC Isolation

Human PBMCs were isolated by Ficoll-gradient separation from whole blood. Briefly, 20–40 ml of blood was collected into lithium heparin tubes (BD Biosciences). Whole blood was inverted gently a few times before centrifugation at 784 × *g* for 10 min at room temperature to remove 1–2 ml of plasma, which was stored at −20°C to −80°C until required. After plasma removal, 25 ml of sterile 1× PBS (QIMR Berghofer) was added, and the tubes were inverted gently a few times to ensure a homogenous mixture. Then, 13 ml of Ficoll-Paque Plus (GE Healthcare) was layered gently under the blood and centrifuged at 392 × *g* for 30 min at room temperature without brake. After centrifugation, the buffy coat was collected, and then PBMCs were washed with 50 ml of 1× PBS and centrifuged at 392 × *g* for 10 min at 4°C. The PBMC pellet was then re-suspended in 4 ml complete media. Cells were diluted 1:10 in 0.1% trypan blue in PBS (0.4%; MP Biomedicals) and counted on a hemocytometer (Blaubrand counting chamber; Neubauer).

PBMC Assay

PBMCs were isolated as described as earlier, counted on the hemocytometer, and then adjusted to a concentration of 2×10^6 cells/mL in complete media: 45 ml of RPMI 1640, 5 ml of fetal calf serum (FCS), and 20 μL of gentamicin. For cryopreserved samples (field samples collected from Timika, Indonesia) (Figure 3B), each vial underwent a quick thaw procedure (37°C for 5 min in the water bath). Once samples were thawed, complete media was added drop-wise (1 ml), followed by the addition of 9 ml of media. Cells were centrifuged for 600 × *g* for 7 min at room temperature. Cells were then counted, and the viability of the cells was determined on the hemocytometer. All samples were adjusted to a concentration of 2×10^6 cells/mL in complete media.

Both nRBCs and pRBCs were prepared as follows for use as antigen in the PBMC assay, which was set up in a 96-well U-bottom plate. Cryopreserved stocks of nRBCs and pRBCs were allowed to thaw on ice and then adjusted to a working concentration of 9×10^6 nRBCs or pRBCs/mL in complete media. Then, 25 μL of this working stock was added to each corresponding well (for a final concentration of 1×10^6 nRBCs or pRBCs/mL). As a positive control, 25 μL of a mitogen, phytohemagglutinin (PHA, final concentration 10 μg/mL; Sigma-Aldrich), was added into corresponding wells. After all antigens and mitogens had been plated out, 200 μL of PBMCs (final concentration of 4×10^5 cells/well) were added to each respective well. The following blocking antibody treatments were then added: human anti-IFNα/β receptor chain 2 antibody (clone MMHAR-2, final concentration of 5 μg/mL; Merck) or its mouse IgG2A isotype control (clone 20102, final concentration of 5 μg/mL; R&D Systems), human anti-IL-10 antibody (clone 25209, final concentration of 20 μg/mL; R&D Systems) or its mouse IgG2B isotype control (clone 20116, final concentration of 20 μg/mL; R&D Systems), and Ultra-LEAF (low endotoxin, azide free) purified anti-human HLA-DR antibody (clone L243, final concentration of 20 μg/mL; BioLegend) or its Ultra-LEAF purified mouse IgG2a, κ isotype control (clone MOPC-173, final concentration of 20 μg/mL; BioLegend). The plate was then placed in a humidified incubator at 37°C, 5% (v/v) CO_2 for 24–72 hr. Culture supernatants were harvested at 24 and 72 hr post-PBMC re-stimulation and stored at −20°C until required.

Flow Cytometry

Intracellular cytokine analysis for IFNγ and IL-10 was performed 72 hr after PBMC re-stimulation. Briefly, for detection of ex vivo IFNγ alone, 10 μg/mL of brefeldin A (BFA; Sigma-Aldrich) was prepared in complete media, with 10% fetal bovine serum (FBS), and added to each well for the remaining 3 hr (at 69 hr) of re-stimulation. To detect Tr1 cells ($IFN\gamma^+IL\text{-}10^+$), 10 μg/mL of BFA, 25 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and 500 ng/mL of ionomycin calcium salt (Sigma-Aldrich) were prepared in complete media (with 10% FBS) and added to each well for the remaining 3 hr of re-stimulation. Then, 72 hr after re-stimulation, PBMCs were washed with Hank's balanced salt solution (HBSS; Life Technologies) and centrifuged at 338 × *g* for 3 min at 4°C. Next, 1 μg/mL (in a final volume of 50 μL) of Zombie near infrared (NIR) fixable viability kit (BioLegend) was diluted in HBSS, added to each well, and incubated for 15–20 min at room temperature, protected from light. PBMCs were washed with HBSS and centrifuged at 338 × *g* for 3 min at 4°C. Then, 1–5 μg/mL (in a final volume of 50 μL) of surface antibodies were diluted in FACS buffer, added to each well, and incubated for 15–20 min on ice, protected from light. PBMCs were then washed twice with 150–200 μL of FACS buffer and centrifuged at 338 × *g* for 3 min at 4°C. Next, 100 μL of BD Cytofix buffer (BD Biosciences) was added to each well and incubated on ice for 20 min, protected from light. PBMCs were then washed twice with 100–200 μL of 1× BD Perm buffer (BD Biosciences) and then centrifuged at 338 × *g* for 3 min at 4°C. Then, 2 μg/mL (final volume of 50 μL) of intracellular antibody (IFNγ PeCy7 and IL-10 PE), diluted in BD Perm buffer was added per well and incubated on ice for 60 min, protected from light. PBMCs were then washed twice with 150–200 μL of 1× BD Perm buffer and centrifuged at 338 × *g* for 3 min at 4°C. PBMCs were re-suspended in a final volume of 100 μL of 1% paraformaldehyde (PFA; MP Biomedicals), stored at 4°C, and protected from light until acquisition on a laser (LSR)

Fortessa 5 (BD Biosciences). Samples were generally acquired within 12–24 hr post-staining. $CD4^+$ T cells were defined as $CD16^-$ $CD56^-$ $CD3^+$ $CD4^+$, $CD8^+$ T cells were defined as $CD16^-$ $CD56^-$ $CD3^+$ $CD8^+$, NK cells were defined as $CD16^-$ $CD56^+$ $CD3^-$, pDCs were defined as $CD3^-$ $CD19^-$ $CD56^-$ $CD14^-$ $CD16^-$ HLA-DR^+ $CD123^+$ $CD11c^-$, myeloid derived dendritic cells (mDCs) were defined as $CD3^-$ $CD19^-$ $CD56^-$ $CD14^-$ $CD16^-$ HLA-DR^+ $CD123^-$ $CD11c^+$, monocytes were defined as $CD56^-$ $CD3^-$ $CD19^-$ HLA-DR^+ $CD14^+$ or $CD16^+$, and B cells were defined as $CD56^-$ $CD3^-$ $CD19^+$.

Detection of IFNα by Intracellular Cytokine Staining

For detection of IFNα, PBMCs were stimulated with nRBC, pRBC, or 50 μg/mL of CpG (ODN2216; InvivoGen) for 2 hr, and BFA was added for the remaining 4 hr of stimulation. Then, 6 hr after re-stimulation, PBMCs were washed with HBSS and centrifuged at 338 × *g* for 3 min at 4°C. Next, 1 μg/mL (in a final volume of 50 μL) of Zombie NIR fixable viability kit was diluted in HBSS, added to each well, and incubated for 15–20 min at room temperature, protected from light. PBMCs were washed with HBSS and centrifuged at 338 × *g* for 3 min at 4°C. Then, 1–5 μg/mL (in a final volume of 50 μL) of surface antibodies were diluted in FACS buffer and Brilliant stain buffer (Cat# 563794; BD Biosciences), added to each well, and incubated for 30 min at 37°C, protected from light. PBMCs were then washed twice with 150–200 μL of FACS buffer and centrifuged at 338 × *g* for 3 min at 4°C. Next, 100 μL of BD Cytofix buffer was added to each well and incubated on ice for 20 min, protected from light. PBMCs were then washed twice with 100–200 μL of 1× BD Perm buffer and centrifuged at 338 × *g* for 3 min at 4°C. Then, 5 μg/mL (final volume of 50 μL) of intracellular antibody (IFNα PE), diluted in BD Perm buffer, was added per well and incubated at room temperature for 45–60 min, protected from light. PBMCs were then washed twice with 150–200 μL of 1× BD Perm buffer and centrifuged at 338 × *g* for 3 min at 4°C. PBMCs were re-suspended in a final volume of 100 μL of 1% PFA, stored at 4°C, and protected from light until acquisition on a LSR Fortessa 5.

PerCP/Cy5.5-conjugated anti-BDCA-1 (L161, 1:50), anti-CD8 (SK1, 1:80), PerCP/Cy5.5-conjugated anti-CD56 (HCD56, 1:25), phycoerythrin-dazzle 594-conjugated anti-CD123 (6H6, 1:50), brilliant violet 421-conjugated anti-CD1c (BDCA-1) (L161, 1:50), brilliant violet 785-conjugated anti-CD303 (BDCA-2) (201A, 1:25), phycoerythrin-Cy7-conjugated anti-CD304 (BDCA-4) (12C2, 1:25), brilliant violet 650-conjugated anti-CD16 (3G8, 1:25), allophycocyanin-conjugated anti-CD141 (M80, 1:25), Alexa Fluor 700-conjugated anti-CD3 (SK7, 1:50), anti-CD16 (3G8, 1:50), fluorescein isothiocyanate-conjugated anti-CD3 (SK7, 1:200), brilliant violet 605-conjugated anti-CD19 (H1B19, 1:20), phycoerythrin-Cy7-conjugated anti-CD123 (6H6, 1:50), and allophycocyanin-conjugated PD1 (EH12.2H7, 1:20) were purchased from BioLegend. Allophycocyanin-Cy7-conjugated anti-CD4 (RPA-T4, 1:25), anti-HLA-DR (L243, 1:30), Horizon V500-conjugated anti-CD4 (RPA-T4, 1:30), Alexa Fluor 700-conjugated anti-CD11c (B-ly6, 1:50), fluorescein isothiocyanate-conjugated anti-CD19 (SJ25C1, 1:20), phycoerythrin-Cy7-conjugated anti-CD45RA (HI100, 1:20), anti-IFNγ (4S.B3, 1:100), phycoerythrin-conjugated anti-IL-6 MQ2-13A5, 1:20), anti-IL-10 (JES3-19F1, 1:100), brilliant violet 421-conjugated anti-CD56 (NCAM16.2, 1:20), anti-CXCR3 (1C6/CXCR3, 1:20), and anti-CXCR5 (RF8B2, 1:20) were purchased from BD Biosciences. Qdot 605-conjugated anti-CD14 (TüK4, 1:100) was

purchased from Invitrogen (Molecular Probes). Phycoerythrin-conjugated anti-IFNα (LT27:295, 1:10) was purchased from Miltenyi Biotec. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Near Infra-Red Stain, (Invitrogen, Molecular Probes), according to the manufacturer's instructions.

MACS Purification of CD4$^+$, CD56$^+$, and CD14$^+$ Cells

After isolation of PBMCs, the cells were washed in 10 ml of sterile Dulbecco's PBS (Life Technologies) and centrifuged at 392 × *g* for 10 min at 4°C to remove any FBS carryover from the complete media. Magnetic-activated cell sorting (MACS) Miltenyi bead kits (Miltenyi Biotec) were used for positive selection of CD4$^+$, CD56$^+$, and CD14$^+$ cells per manufacturer's instructions. For the positive selection of CD4$^+$ cells, after the first elution, the sample was loaded onto a fresh column and then underwent a second round of elution to increase the purity of selected population. Purity was ≥95%–99%. Cells were washed with 9 ml of Dulbecco's PBS and centrifuged at 392 × *g* for 10 min at 4°C, and then the pellet was gently re-suspended in 500 μL of RNA-later (Sigma-Aldrich). Samples were transferred to 4°C overnight and then to −80°C for long-term storage until RNA was extracted.

Real-Time qPCR

RNA was extracted from CD4$^+$, CD56$^+$, and CD14$^+$ MACS-purified cells from days 0, 7, and 10 p.i., using the RNeasy Plus Mini kit per manufacturer's instructions (QIAGEN). RNA concentration and quality were determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription kit per manufacturer's instructions (Applied Biosystems). Primers for the following genes were purchased from Quantitect (QIAGEN), *ifnα1*, *ifnα2*, *ifnα4*, *ifnα5*, *ifnα6*, and *ifnβ*. qPCRs were performed in duplicate, using the GoTaq qPCR kit for SYBR green (A6001; Promega) on a CFX384 Touch Real-Time PCR Detection system (Bio-Rad) per manufacturer's instructions. Values for each transcript were normalized to expression levels of the GeoMean of two house-keeping genes: B2M (beta-2-microglobulin) and actin. Fold change was then determined for each cell type and normalized to its corresponding day 0, using the $2^{-\Delta\Delta Ct}$ method.

Calculating the IFNα Index

To determine whether there were differences in IFNα production on a per cell basis, the IFNα index was calculated. Briefly, the frequency of IFNα$^+$ events was multiplied by the MFI of IFNα for each cell population.

Calculating the AUC

To determine whether there were associations between cytokines and parasite growth, parasitemia was expressed as the AUC to determine a more accurate indicator of parasite growth. Retrospective PCR parasitemia values were tested in duplicate, and on the day of treatment, parasitemia was tested in triplicate. AUC was only determined until drug treatment. The limit of detection (LOD) was assumed to be 64 parasites/mL (Rockett et al., 2011). After initial data checks for potential outliers, values below the LOD (between 2 and 63) were substituted with LOD/2 (32) and values that were not detected (ND) were set to 1.

All parasitemia values were log-10 transformed, and the mean of the log-10-transformed parasitemia was used as a summary measure per time point per participant. All regression analyses were performed using the mean log-10 parasitemia values and performed separately per participant. The parasitemia growth can be modeled as a sine-wave function, though it requires a minimum of six data points. Because most subjects (67%) had parasitemia data for fewer than six time points, the AUC was estimated using the trapezoid rule, using GraphPad Prism v.6.

Statistical Analysis

Statistical differences between groups were determined using the Wilcoxon matched-pairs signed rank test (day 0 versus day 7 or day 0 versus day 14), and the linear regression function was used to analyze associations between IL-10 or IFNγ levels and the AUC by GraphPad Prism v.6 for Windows; $p < 0.05$ was considered statistically significant. All data are presented as mean ± SEM unless otherwise stated.

Supplemental Information

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the staff at Q-Pharm, Brisbane, Australia, for help in collecting blood samples and volunteers participating in clinical trials for allowing us to collect these samples. We also thank Timika and Jakarta colleagues and staff for their contributions to the clinical studies in Timika, as well as Tonia Woodberry and Kim Piera for assistance shipping samples and scientific discussions. We thank Peter O'Rourke and Louise Marquart for advice on statistics. This work was made possible through Queensland State Government funding. This research was supported by grants and fellowships from the National Health and Medical Research Council of Australia, as well as an Australian Post-graduate Award through the School of Medicine, University of Queensland, and an INSPIRE Fellowship to R.K. provided by the Indian Department of Biotechnology. Thanks to Joerg Moehrle and Tim Wells and to Medicines for Malaria Venture (MMV) in Geneva for helpful discussions. Funding for the CHMI studies was provided by MMV from grants awarded by the Wellcome Trust and Bill and Melinda Gates Foundation.

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Highlights

- *Plasmodium falciparum*-induced type I IFNs suppress Th1 cell development
- Type I IFNs promote development of parasite-specific IL-10-producing Th1 (Tr1) cells
- Chemoprophylaxis stimulates type I IFN-dependent, parasite-specific IL-10 production
- Parasite-induced IL-10 suppresses inflammatory cytokine production

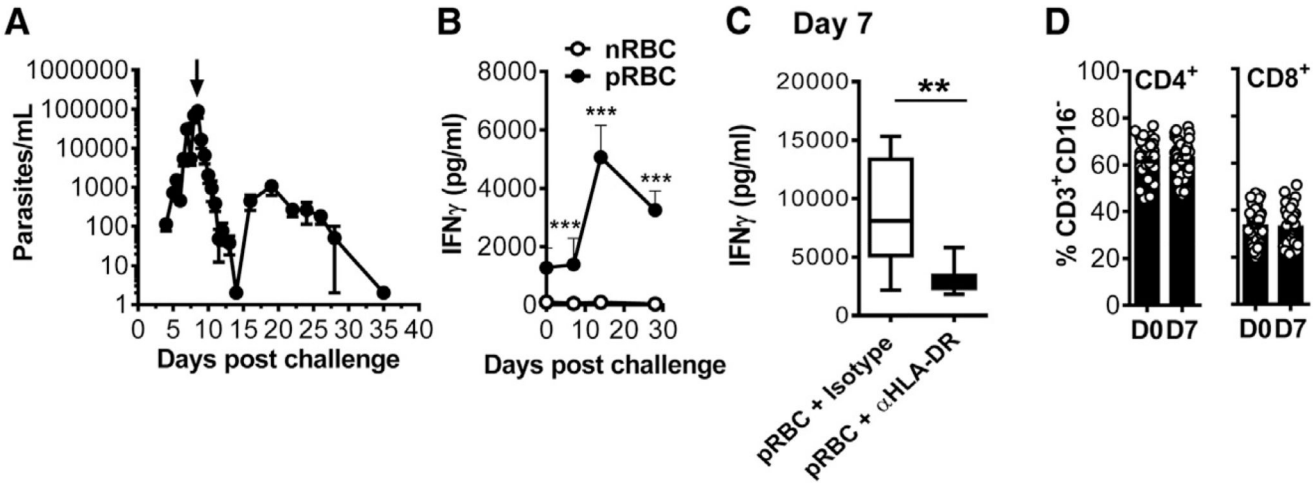

Figure 1. Blood-Stage *P. falciparum* Infection Induces Parasite-Specific CD4$^+$ T Cell Responses

(A) Volunteers were injected with 1,800 pRBCs/mL, and blood parasitemia was monitored by PCR beginning day 4 p.i. until day 35 p.i. (cohort 6; n = 7). Drug treatment commenced on day 7 p.i. (as shown by the arrow).

(B) PBMCs were isolated from participants throughout infection and cultured in the presence of nRBCs or pRBCs as indicated for 72 hr, and then cell culture supernatants were harvested and IFNγ levels were measured (cohorts 6–8; n = 16).

(C) PBMCs were isolated from participants 7 days p.i. and cultured in the presence of pRBCs and anti-HLADR antibody or its isotype control for 72 hr, and then cell culture supernatants were harvested and IFNγ levels were measured (cohorts 9 and 10; n = 13).

(D) The frequencies of CD4$^+$ and CD8$^+$ T cells in PBMCs isolated from volunteers at day 0 (D0) and day 7 (D7) p.i. were measured by flow cytometry (cohorts 1–5; n = 33).

Mean ± SEM (A, B, and D), median + min and max (C). ***p < 0.001, **p < 0.01, Wilcoxon matched-pairs signed rank test. See also Figure S1.

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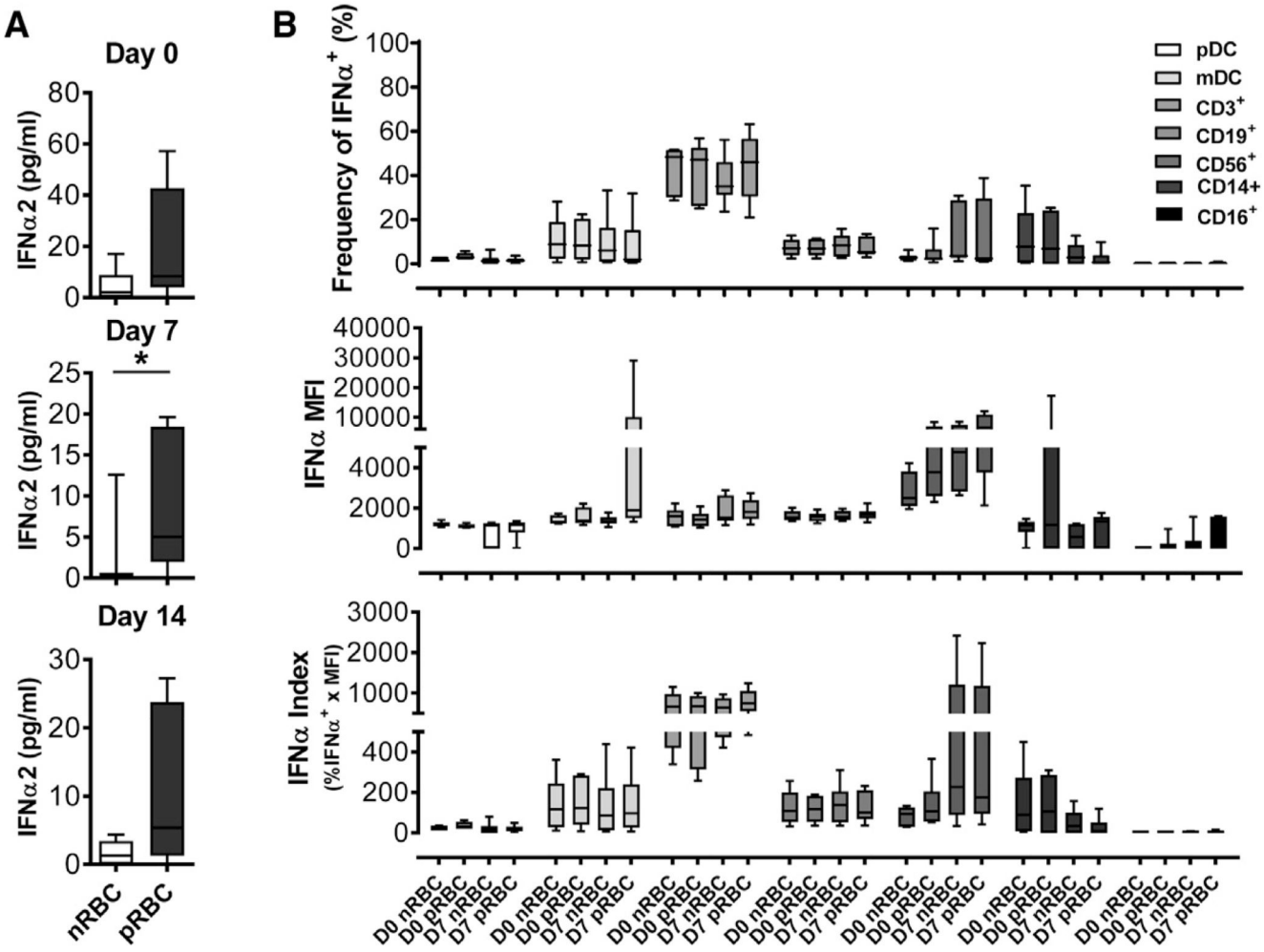

Figure 2. Blood-Stage *P. falciparum* Induces IFNα Production upon First Exposure and Is Produced by Various Cell Types

(A) PBMCs from volunteers at days 0, 7, and 14 p.i. were cultured in the presence of nRBCs or pRBCs for 72 hr, and then cell culture supernatants were harvested and IFNα levels were measured (cohort 10; n = 7).

(B) Cryopreserved PBMCs from volunteers at day 0 (D0) or 7 (D7) p.i. were cultured in the presence of nRBCs or pRBCs for 6 hr (as indicated), and then cellular sources of IFNα was measured by intracellular cytokine staining (cohorts 9–10; n = 6). Total $IFN\alpha^+$ events were gated first, and then cell types were gated as shown in Figure S2, using CpG as a positive control for IFNα staining. Not all samples from these cohorts were used in this assay, because a limited number of samples were cryopreserved. Median + min and max. *$p < 0.05$, Wilcoxon matched-pairs signed rank test.

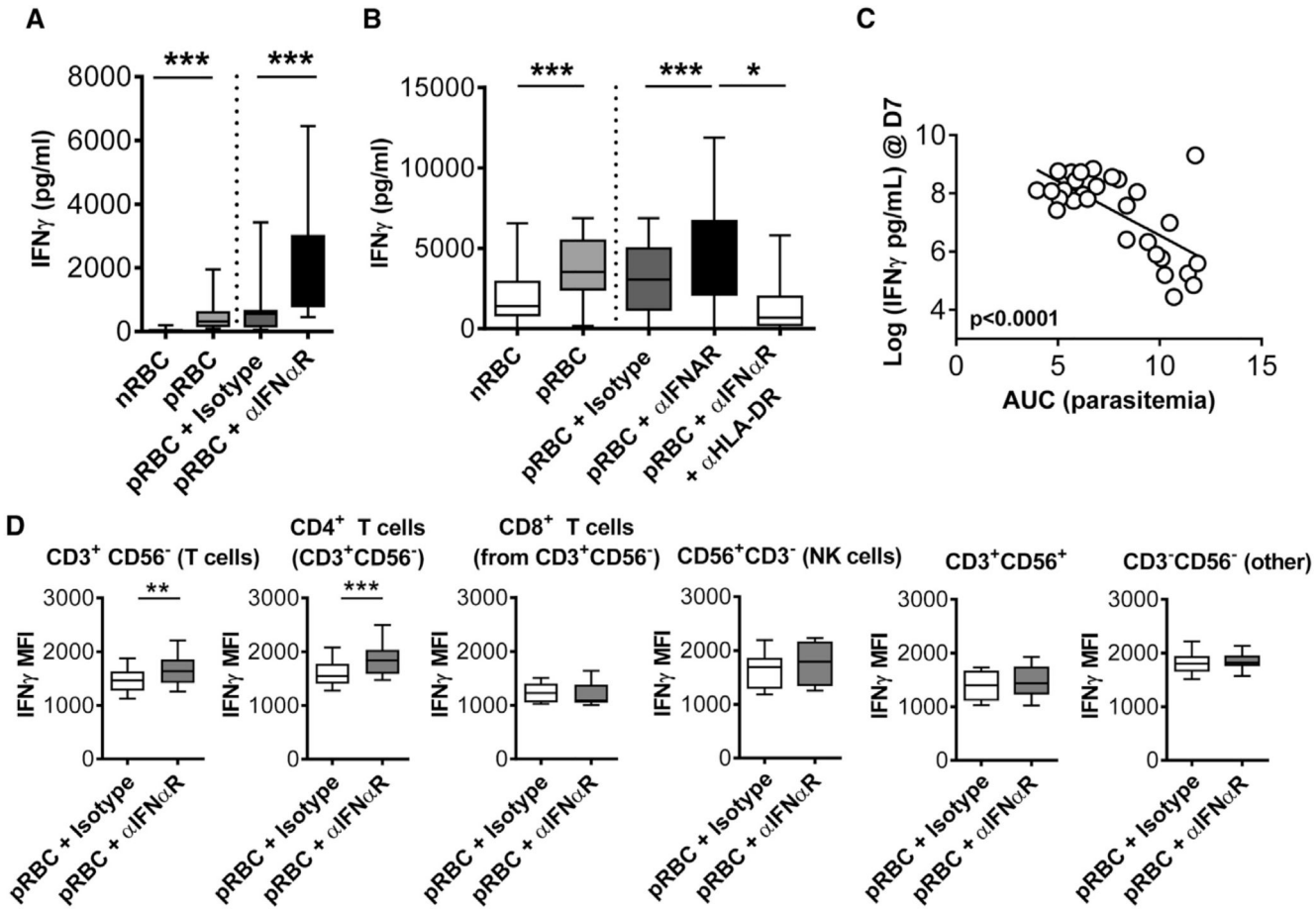

Figure 3. Blood-Stage *P. falciparum* Induces a Type I IFN Response that Suppresses IFNγ Production

(A) PBMCs were isolated from volunteers 7 days p.i. and cultured in the presence of nRBCs, pRBCs, or pRBCs + anti-IFNAR antibody or its isotype control for 72 hr, and then IFNγ levels were measured in cell culture supernatants (cohorts 6 and 7; n = 12).

(B) PBMCs were isolated from participants 7 days p.i. and cultured in the presence of nRBCs, pRBCs, pRBCs + anti-IFNAR, or pRBCs + anti-IFNAR + anti-HLA-DR antibody, as well as the relevant isotype controls, for 72 hr, and then IFNγ levels were measured (cohorts 6, 7, 9, and 10; n = 25).

(C) Linear regression analysis was performed to determine the correlation between log-transformed IFNγ levels and blood parasitemia using the AUC values from qPCR measurements of parasite numbers (cohorts 6–10; n = 29). D7, day 7. $p < 0.0001$, linear regression function (goodness of fit).

(D) PBMCs were isolated from volunteers 7 days p.i. and cultured in the presence of pRBCs + anti-IFNAR antibody or its isotype control for 72 hr, and then IFNγ levels measured by intracellular cytokine staining, in which the IFNγ MFI on IFNγ$^+$ CD4$^+$ and CD8$^+$ T cells, CD56$^+$ CD3$^-$ (NK) cells, CD56$^+$ CD3$^+$ cells, and CD3$^-$ CD56$^-$ (other) cells was determined using the GeoMean function in FlowJo (cohorts 7 and 10; n = 12).

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Median + min and max (A, B, and D). ***p < 0.001, **p < 0.01, *p < 0.05, Wilcoxon matched-pairs signed rank test.

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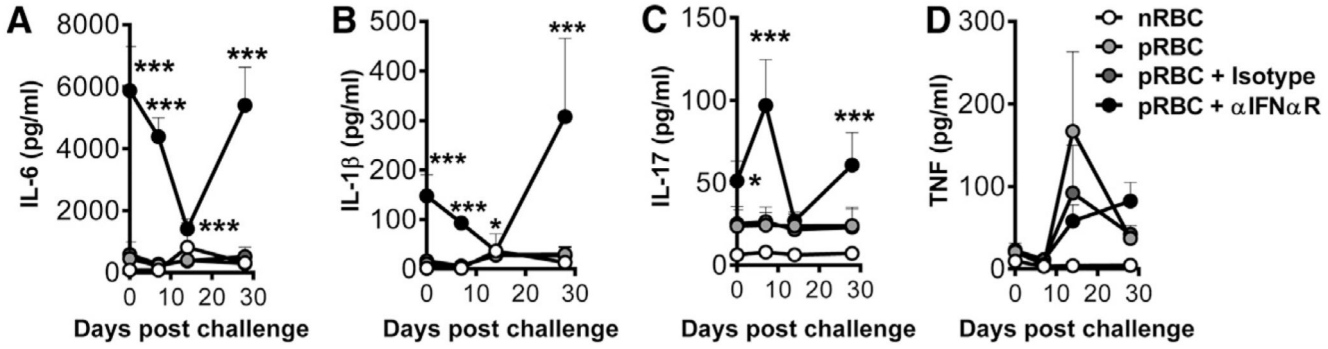

Figure 4. Type I IFNs Suppress IL-6, IL-1β, and IL-17, but Not TNF Production in Response to *P. falciparum*

(A–D) PBMCs were isolated from volunteers on days 0, 7, 14, and 28 p.i. and cultured in the presence of nRBCs, pRBCs, or pRBCs + anti-IFNAR antibody or its isotype control for 72 hr before measuring IL-6 (A), IL-1β (B), IL-17 (C), or TNF (D) levels.

Data from cohorts 6 and 7; n = 12. Mean ± SEM. ***$p < 0.001$, *$p < 0.05$, Wilcoxon matched-pairs signed rank test.

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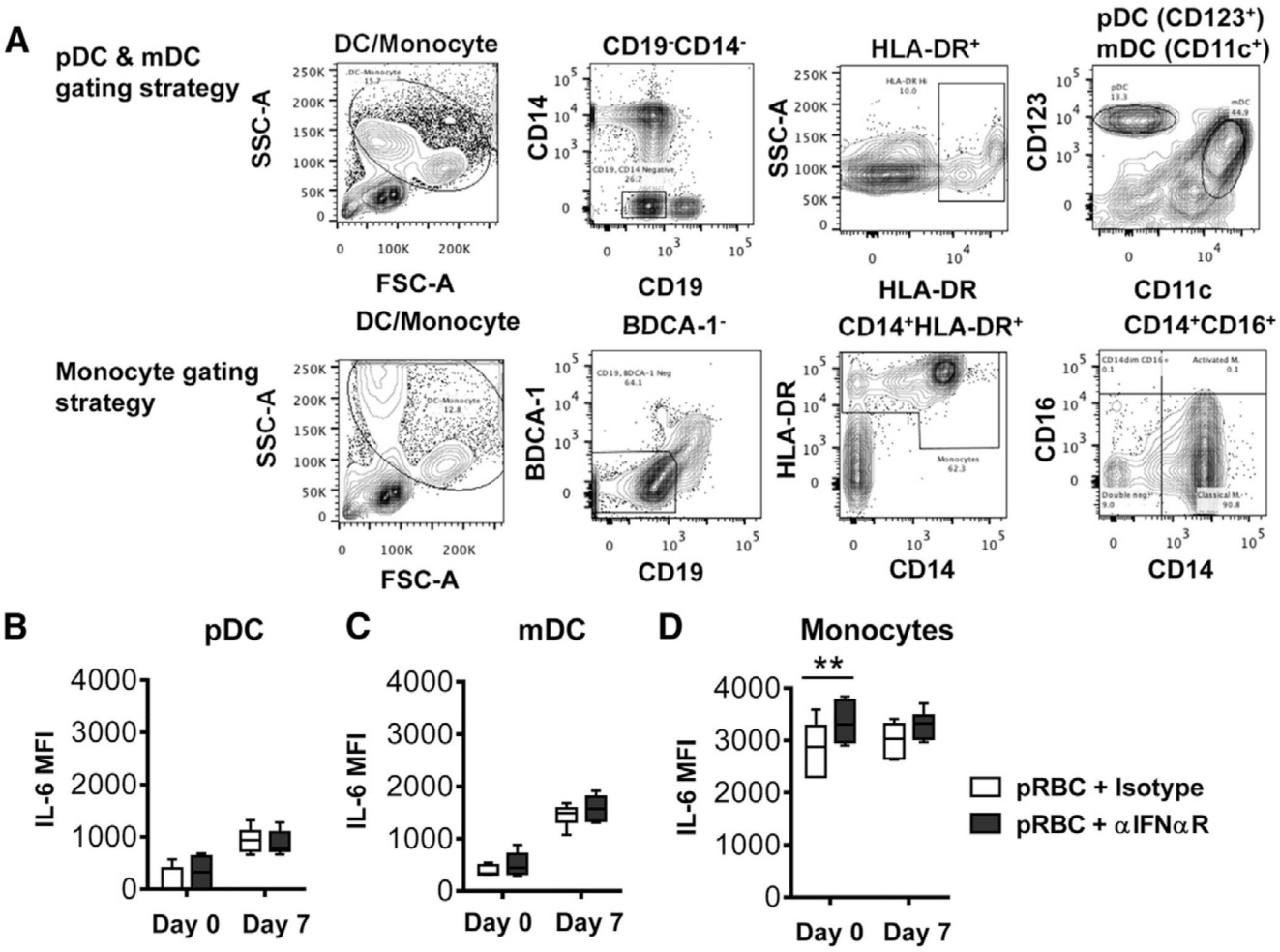

Figure 5. Type I IFNs Suppress Monocyte-Derived IL-6 Production in Response to *P. falciparum*

(A) Flow cytometry gating strategy is shown for pDCs and mDCs (top row) and for monocytes (bottom row) (cohort 6; n = 7).

(B–D) Isolated PBMCs were cultured in the presence of pRBCs + anti-IFNAR antibody or its isotype control for 24 hr, and then BFA added for the last 3 hr. IL-6 was measured by intracellular cytokine staining and flow cytometry. IL-6 MFI on pDCs (B), mDCs (C), and monocytes (D) at days 0 and 7 p.i. is shown (cohort 6; n = 7).

Median + min and max. **p < 0.01, Wilcoxon matched-pairs signed rank test.

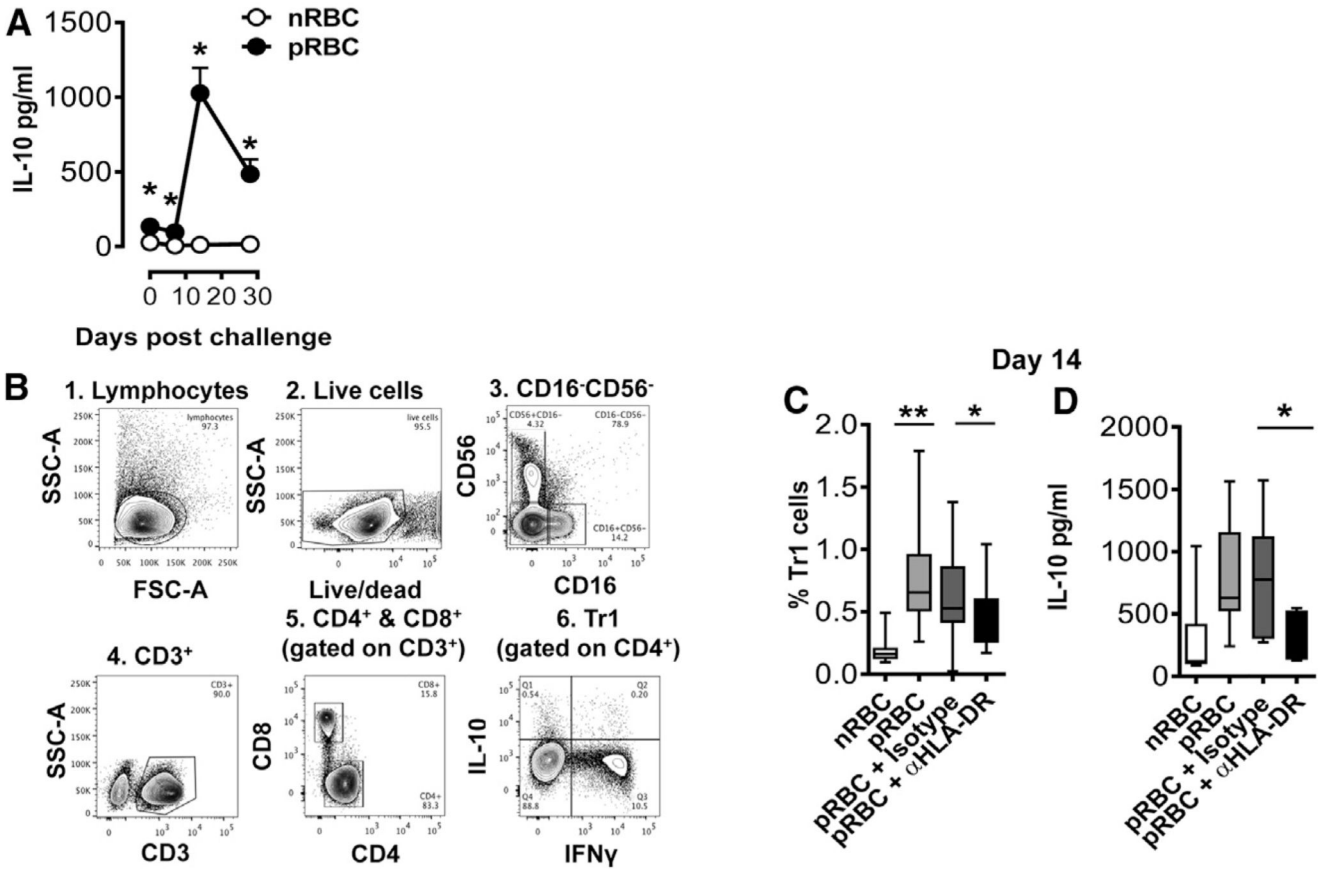

Figure 6. Blood-Stage *P. falciparum* Induces HLA-DR Restricted Tr1 Cells and IL-10 Production

(A) PBMCs were isolated from volunteers at days 0, 7, 14, and 28 p.i. and cultured in the presence of nRBCs or pRBCs for 72 hr, and then cell culture supernatants were harvested and IL-10 levels were measured (cohort 6; n = 7).

(B) PBMCs were isolated from volunteers 14 days p.i. (7 days after drug treatment) and cultured in the presence of nRBCs, pRBCs, or pRBCs + anti-HLADR antibody or its isotype control for 72 hr. BFA, PMA, and ionomycin were added for the remaining 3 hr of culture. Representative gating strategy from left to right shows lymphocytes, live or viable cells, $CD16^{-}$ $CD56^{-}$ (exclusion of NK and γδ T cells), $CD3^{+}$, $CD4^{+}$, and $IFN\gamma^{+}$ $IL\text{-}10^{+}$ (cohorts 10 and 11; n = 10).

(C and D) The frequency of Tr1 cells was measured by flow cytometry (C), and IL-10 levels were measured in cell culture supernatants (D) (cohorts 10 and 11; n = 10).

Mean ± SEM (A), median + min and max (C and D). **p < 0.01, *p < 0.05, Wilcoxon matched-pairs signed rank test.

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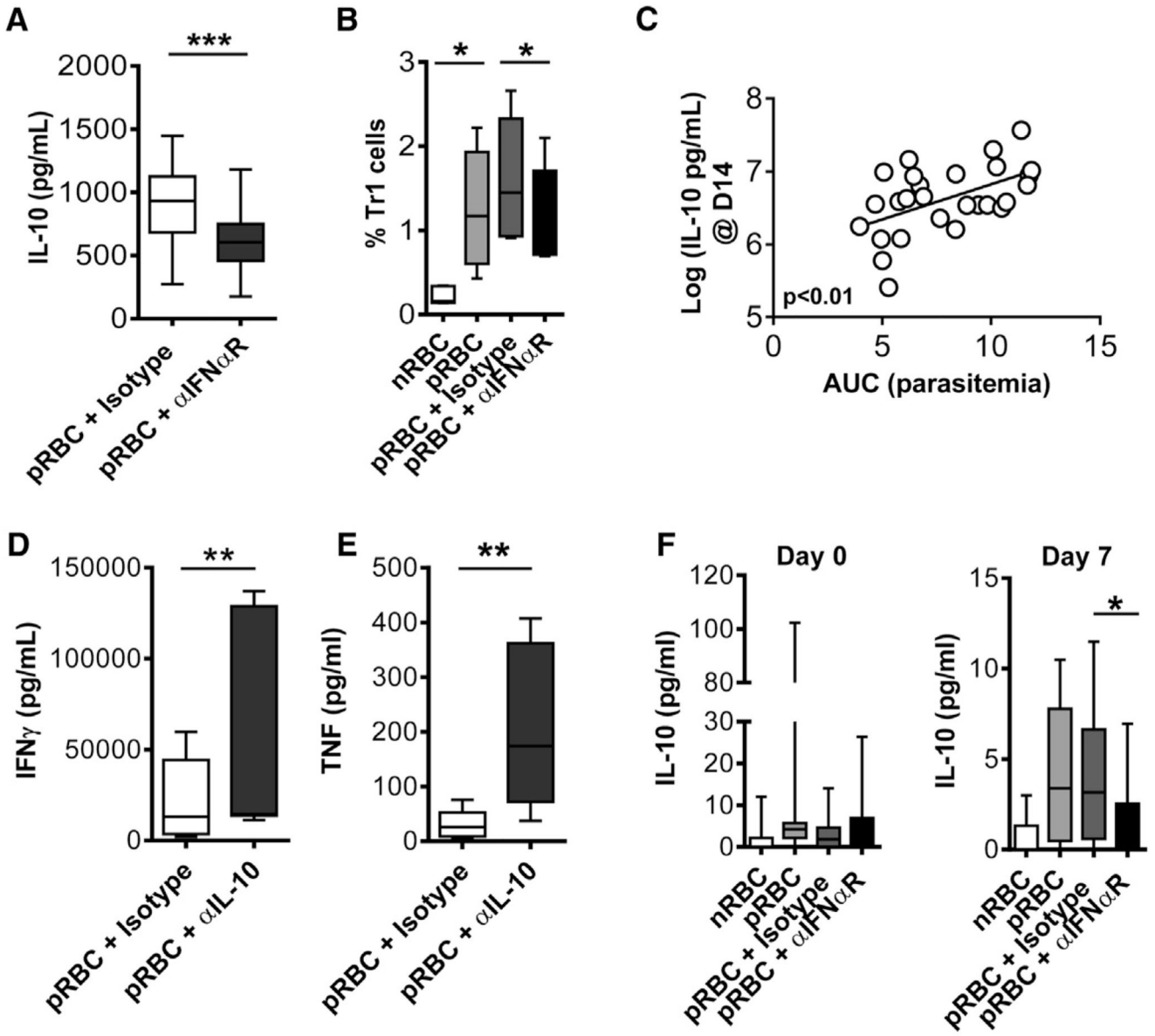

Figure 7. Type I IFNs Promote Tr1 Cell Development and IL-10 Production after Drug Treatment in Volunteers Infected with *P. falciparum* and Malaria Patients with Natural *P. falciparum* Exposure

(A) PBMCs were isolated from volunteers at day 14 p.i. (7 days after drug treatment) and cultured in the presence of pRBCs + anti-IFNAR antibody or its isotype control for 72 hr before measuring IL-10 levels (cohorts 6 and 7; n = 12).

(B) PBMCs were isolated from volunteers 14 days p.i. (7 days after drug treatment) and cultured in the presence of nRBCs, pRBCs, or pRBCs + anti-IFNAR antibody or its isotype control for 72 hr. BFA, PMA, and ionomycin were added for the remaining 3 hr of culture before frequencies of Tr1 cells were measured by flow cytometry (cohort 7; n = 5).

(C) Linear regression analysis was performed to determine the correlation between log-transformed IL-10 levels and blood parasitemia using the AUC values from qPCR

measurements of parasite numbers (cohorts 6–10; n = 29). D14, day 14. $p < 0.01$, linear regression function (goodness of fit).

(D and E) PBMCs were isolated from volunteers 14 days p.i. (7 days after drug treatment) and cultured in the presence of pRBCs + anti-IL-10 antibody or its isotype control for 72 hr, and then IFNγ levels (D) and TNF levels (E) were measured (cohorts 7 and 8; n = 9).

(F) Cryopreserved PBMCs from malaria patients infected with *P. falciparum* from Timika, Indonesia, taken at time of admission into the clinic (day 0) and 7 days after commencement of drug treatment (day 7) were thawed and cultured in the presence of nRBCs, pRBCs, or pRBCs + anti-IFNAR antibody or its isotype control for 72 hr, and then IL-10 levels were measured.

Median + min and max (A, B, and D–F). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Wilcoxon matched-pairs signed rank test.

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Table 1

Cohorts Used in These Studies

Study Cohort	Trial Registration	Year of Study	Trial Drug Study Cohort	Subject	Age (Years)	Gender
1	ACTRN12612000323820	2012	1	*S001*	31	M
				S006	23	M
				S011	26	M
2		2012	2	*S015*	20	F
				S016	27	M
				S017	22	F
				S018	24	F
				S019	20	F
				S020	24	F
				S022	23	M
				S025	25	M
3		2012	3	*S028*	28	F
				S032	27	F
				S033	28	M
				S035	25	M
				S036	25	F
				S038	37	M
				S041	25	F
				S042	34	M
4	ACTRN12612000814875	2012	1	*S005*	22	F
				S009	23	M
				S011	28	F
				S012	25	M
				S013	32	F
				S017	16	M
				S018	34	M

Study Cohort	Trial Registration	Year of Study	Trial Drug Study Cohort	Subject	Age (Years)	Gender
5		2012	2	*S024*	20	M
				S026	27	M
				S027	27	F
				S028	27	M
				S029	35	M
				S031	23	F
				S032	23	M
6	ACTRN12613001040752	2013	1	*S001*	25	M
				S002	22	M
				S003	24	F
				S007	22	F
				S008	27	M
				S009	41	F
				S010	26	F
7	ACTRN12613000565741	2014	3A	*S030*	25	M
				S033	21	F
				S035	23	M
				S036	26	F
				S037	21	F
8		2014	3B	*S045*	18	M
				S050	22	F
				S052	32	F
				S057	23	M
9	NCT02281344	2014	1	*S001*	29	M
				S002	28	M
				S005	29	M
				S006	24	M
				S008	23	M

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Study Cohort	Trial Registration	Year of Study	Trial Drug Study Cohort	Subject	Age (Years)	Gender
				S011	19	M
10	NCT02389348	2015	1	*S018*	37	M
				S019	55	M
				S020	22	M
				S017	25	M
				S011	21	F
				S021	19	F
				S007	24	F
11		2015	2	*S028*	27	F
				S029	23	M
				S022	24	F

n = 65. F, female; M, male.

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Table 2

Timika Patient Clinical Data

Age (Years)	Gender	Weight (kg)	Ethnicity	Treatment
43	F	55	non-Papuan	Artekin (dihydroartemisinin and piperaquine)
43	M	51	non-Papuan	Coart (artemether and lumefantrine)
30	M	45	Highland	Coart
25	M	46	non-Papuan	Coart
27	M	49	Highland	Coart
35	F	71	non-Papuan	Coart
37	M	55	non-Papuan	Artekin
20	M	59	Highland	Artekin
36	M	59	Highland	Coart
20	M	49	Highland	Coart
33	M	47	non-Papuan	Coart
40	F	55	Highland	Cq+SP (chloroquine and sulfadoxine-pyrimethamine)

All patients were *P. falciparum* positive. F, female; M, male.