Low-Level *Plasmodium falciparum* Blood-Stage Infection Causes Dendritic Cell Apoptosis and Dysfunction in Healthy Volunteers

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**Background.** Dendritic cells (DCs) are highly specialized antigen-presenting cells that are crucial for initiation of immune responses. During naturally acquired malaria, DC number and function is reduced.

**Methods.** The timing of, parasitemia threshold of, and contribution of apoptosis to DC loss were prospectively evaluated in 10 men after experimental challenge with approximately 1800 *Plasmodium falciparum*–parasitized red blood cells (pRBCs) and after drug cure initiated at a parasite level of $\geq1000$ parasites/mL.

**Results.** The nadir levels of total, myeloid, and plasmacytoid DCs occurred 8 days after infection. DC loss was partially attributable to apoptosis, which was first detected on day 5 (median parasite level, 238 parasites/mL) and maximal at day 7. Remaining DCs exhibited a reduced ability to uptake particulate antigen. DC numbers recovered approximately 60 hours after antimalarial drug administration. There was no loss of DC number or function before or after drug cure in 5 men inoculated with $<180$ pRBCs and treated on day 6, when their parasite level was approximately 200 parasites/mL.

**Conclusions.** *Plasmodium* causes DC loss in vivo, which is at least partially explained by apoptosis in response to blood-stage parasites. In primary infection, loss of DC number and function occurs early during the prepatent period and before or with onset of clinical symptoms. These findings may explain in part the inadequate development of immunity to blood-stage malaria infection.

Malaria is caused by *Plasmodium* parasites and affects 350–550 million people each year [1]. In regions where *Plasmodium falciparum* is endemic, sterile immunity to blood-stage parasites rarely occurs [2, 3]. Dendritic cells (DCs) are highly specialized antigen-presenting cells that are crucial for the initiation of effective immune responses, including cellular and antibody responses to *P. falciparum* [4, 5]. Studies using in vitro–generated DCs show that *P. falciparum* can impair DC function [6–8]. However, there is a paucity of data on DC function and number in controlled clinical settings. Reduced DC expression of HLA-DR in children [9, 10] and of CD86 in adults [11] with malaria implies impaired DC function or maturation. Among individuals with malaria, the number of circulating blood DCs has been reported to be reduced in adults [12], including pregnant women [13], yet unchanged in children [14]. Differences in age, genetic background, disease severity, and study methods confound interpretation of these findings. Nevertheless, most studies examining circulating DCs have shown a reduced DC number, while few have directly assessed DC function.

The mechanisms underlying reduced DC numbers in human malaria and the relationships with function and parasite biomass in vivo are not clear. Previous
in vitro studies have demonstrated that human monocyte-derived DCs undergo apoptosis after exposure to high but not low doses of P. falciparum-parasitized red blood cells (pRBCs) [8], but the physiological significance of apoptosis at high-dose remains uncertain. DC apoptosis has been reported in Plasmodium chabaudi AS infection in rodent models [15]. However, whether P. falciparum induces DC apoptosis in humans in vivo, and whether this occurs directly in response to blood-stage infection alone, has not been determined.

We used a novel human blood-stage experimental infection protocol in which malaria-naïve human volunteers were experimentally infected with low doses of pRBCs, with drug cure administered once parasitemia reached ≥1000 parasites/mL [16]. During a small pilot study to establish safety, volunteers received an ultra-low-dose inoculation of <180 pRBCs. While all volunteers in this cohort became polymerase chain reaction (PCR) positive for P. falciparum, none reached the target parasitemia of ≥1000 parasites/mL before the time point when the protocol required that antimalarial treatment commence. The use of the designated parasite inoculum (approximately 1800 pRBCs) in subsequent cohorts afforded the opportunity for direct comparisons of inoculation size and subsequent parasite expansion on DC numbers and function between the low and ultra-low doses. Hence, the aim of this study was to use these controlled experimental challenges to determine whether blood-stage P. falciparum causes reduced DC numbers and function at low parasitemia, to determine whether this was associated with apoptosis, and to establish the kinetics of such changes in relation to parasite biomass and subsequent drug treatment.

**MATERIALS AND METHODS**

**Study Cohort**
The conduct of the clinical study (ClinicalTrials.gov identifier: NCT01055002) [16] and the PCR method used to quantify parasitemia [17] are described in detail elsewhere. Ten of 13 healthy men aged 19–43 years (median age, 27 years [inter-quartile range {IQR}, 23–32]) who received approximately 1800 pRBCs (low-dose pRBC group) via intravenous injection in 2.0 mL of saline volunteered to participate in this substudy. Because logistical constraints limited our ability to evaluate all participants, 10 men were randomly selected. Antimalarial drug treatment was given 7–9 days later, when a treatment threshold of ≥1000 parasites/mL, confirmed by PCR, was achieved. Eight participants (80%) were treated during the evening of day 7, and 1 each was treated on day 8 or day 9 after challenge. Antimalarial treatment was randomly assigned and consisted of 4 artemether-lumefantrine tablets (Riamet, Novartis; 5 recipients) with food twice daily for 3 days or 4 atovaquone-proguanil tablets (Malarone, GSK; 5 recipients) with food daily for 3 days. Five men (median age, 24 years [IQR, 22–27]) received an ultra-low-dose challenge (<180 pRBCs), and their infection was terminated by drug cure with the same drug regimen 6 days after infection, when parasitemia was <200 parasites/mL. The study was approved by the Human Research Ethics Committees of Queensland Institute of Medical Research and Menzies School of Health Research.

**Blood Collection**
Blood samples anticoagulated with lithium heparin were collected before inoculation and at the same time each morning on days 5–10. On day 9, we were unable to collect blood from 2 donors, resulting in fewer data points for that day. All functional and flow cytometric assays were undertaken using fresh whole blood processed within 2 hours of collection. Plasma was cryopreserved within 30 minutes of collection.

**Flow Cytometric Analysis**
DCs were enumerated from 100 μL of whole blood, using lineage markers (CD3, CD14, CD19, CD20, CD56, and CD34, all PE), CD123 FITC, HLA-DR PerCP, and CD11c APC (all from BD Biosciences, San Jose, CA). Red blood cells were lysed with FACS lysing solution (BD Biosciences), and the cells were fixed with 1% paraformaldehyde in phosphate-buffered saline and acquired on a FACScalibur (BD Biosciences). CD3+ and CD4+ T cells were enumerated from an additional 100 μL of whole blood, using anti-CD3 and CD4 antibodies (BD Biosciences).

The absolute number of DCs was determined by adding the automated cell counter–derived lymphocyte and monocyte counts (10⁹ cells/L), dividing the sum by 100, multiplying the quotient by the percentage of DCs (gated according to Figure 1), and multiplying the product by 1000 to give the cell count per microliter.

Annexin V staining was used to assess DC apoptosis in whole blood according to the protocol described by Hodge et al [18]; 2 separate whole-blood-staining procedures were undertaken for each subject, and the mean value was calculated. Whole-blood DC function was assessed using the uptake of 1 mg/mL FITC-dextran (Sigma, Australia) after 1 hour at 37°C or on ice as a control. Two separate whole-blood-staining procedures were undertaken for each subject, and the median fluorescence intensity (MFI) was determined. The data were expressed as AMFI (calculated as [MFI for cells incubated at 37°C]–[MFI for control cells incubated on ice]). Flow cytometry data were analyzed using FlowJo software (TreeStar, Ashland, OR).

The cytometric bead array (BD Biosciences) was used according to the manufacturer’s instructions to measure the concentration of tumor necrosis factor (TNF), interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 12 (IL-12) in plasma.

**Parasite Biomass**
Parasitemia was monitored daily from 3 days after infection, using quantitative PCR (limit of detection, 64 parasites per
500 μL of packed RBCs, approximating a count of 64 parasites/mL [17]) and microscopy. Parasitemia was monitored retrospectively on plasma, using a histidine-rich protein 2 (HRP2) enzyme-linked immunosorbent assay [ELISA], as previously described [19]. Recombinant PfHRP2 used to derive a standard curve was a gift from Dr D. Sullivan (Johns Hopkins Malaria Institute, Baltimore, MD).

Statistical Analysis
All statistical analyses were performed using GraphPad Prism 5 (Graphpad Software, La Jolla, CA). The Wilcoxon matched-pairs test was used for comparison of longitudinal data. The Mann–Whitney U test was used for comparisons of low-dose and ultra-low-dose time points.

RESULTS

Parasite Detection
Parasites were detectable by PCR in all subjects from day 3 after the low-dose challenge (approximately 1800 pRBCs), as previously reported [20], and from day 5 after the ultra-low-dose challenge (<180 pRBCs) [16]. At no point were parasites detected by microscopy, nor was PfHRP2 antigen detected in plasma by ELISA. As described elsewhere [16], all individuals receiving the low dose of parasites developed symptoms consistent with the early clinical features of malaria, including malaise, chills, headache, muscle ache, and/or nausea, commencing 7 days after challenge; 2 subjects experienced fever 7 days after challenge. No clinical symptoms attributable to infection were observed in the subjects who received the ultra-low-dose challenge, in which infection was terminated by drug cure with the same drug regimens on day 6, when parasitemia was <200 parasites/mL. All individuals recovered fully following antimalaria drug treatment.

Decline in Circulating Peripheral Blood DCs After Low-Dose Challenge
Blood DCs from the same volunteers were evaluated before and after inoculation. DCs were characterized as lineage-negative, HLA-DR-positive cells (Figure 1), and myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) were distinguished by expression of CD11c and CD123, respectively. After the low-dose challenge, the median absolute DC number declined at the time when parasitemia most increased (47 DCs/µL [IQR, 20–56] on day 7 vs 60 DCs/µL [IQR, 37–76] on day 0; Figure 2). The median DC number continued to decline for a further 2 days after the peak of parasitemia (27 DCs/µL [IQR, 13–34] on day 9) and recovered to a level approximating that found before infection after a 2-day lag following antiparasitic treatment (41 DCs/µL [IQR, 38–89] on day 10; Figure 2). No significant change in the median DC number was observed in the group challenged with the ultra-low dose (50 DCs/µL [IQR, 44–64] on day 6 vs 61 DCs/µL [IQR, 36–71] on day 0; Figure 2).

Following low-dose challenge, the absolute number of mDC and pDC subsets dropped, with the largest and most significant reduction on day 8 (median parasite level, 299 parasites/mL), at the time of clinical symptoms (Figure 3). On day 8, the median mDC number had declined to 48% of the baseline level (23 mDCs/µL [IQR, 12–31] vs 48 mDCs/µL [IQR, 27–61]), and the median pDC count had declined to 30% of the baseline level (1.9 pDCs/µL [IQR, 1.5–3.2] vs 6.5 pDCs/µL [IQR, 5.4–8.3]; Figure 3). The decline in DC number was not related to subject age, with no significant difference in the absolute DC count in people 19–43 years of age [21]. No change in mDC and pDC number was observed following the ultra-low-dose challenge. The absence of DC decline in the ultra-low-dose cohort despite receipt of identical drug treatment regimens indicates that the DC loss following low-dose challenge was not due to an antimalarial drug effect.

Ten days after the low-dose challenge and approximately 60 hours after evening initiation of antimalaria drug therapy.
The median parasite count (0 parasites/mL), the median total DC number recovered to a level approximating that identified at baseline (41 DCs/µL [IQR, 38–89] on day 10 vs 60 DCs/µL [IQR, 37–76] on day 0; Figure 2). The median mDC count was 75% recovered within 60 hours of antimalaria drug treatment (36 DCs/µL [IQR, 29–80] on day 10 vs 48 DCs/µL [IQR, 27–61] on day 0; Figure 3). In contrast, pDC recovery appeared incomplete, with 47% of the median baseline number detected approximately 60 hours after drug treatment (2.8 DCs/µL [IQR, 2.4–4.1] on day 10 vs 6 DCs/µL [IQR, 4.7–7.7] on day 0; Figure 3), although the difference was not statistically significant.

Impaired DC Function
The ability of DCs from whole blood to take up particulate antigen was assessed in 6 subjects who received the low-dose challenge (approximately 1800 *Plasmodium falciparum*–parasitized red blood cells (pRBC); 10 recipients) and ultra-low-dose challenge (<180 pRBCs; 5 recipients) (bars) versus polymerase chain reaction (PCR) quantitation of parasitemia (lines). The Wilcoxon matched-pairs test yielded a P value of .004 for the difference between days 0 and 8 in the low-dose group and a P value of .77 for the difference between days 0 and 8 in the ultra-low-dose group. Graphs show median data for all participants, except for DC counts following receipt of the low dose, which are for 9 individuals on day 0, for 8 on day 6, for 3 on day 8, for 9 on day 9, and for 4 on day 10. In the ultra-low-dose group, DC numbers were measured on days 0, 6, and 8 only. The vertical bars indicate the interquartile range of PCR data, and the arrows show the time of antimalaria drug treatment.

Death of Circulating Peripheral Blood DCs After Low-Dose Challenge
By use of Annexin V staining, apoptotic and dead cells were identified on the basis of binding to phosphatidylserines that are exposed on the surface of cells as an early event in apoptosis [18]. Annexin V–positive DCs were detected in fresh whole blood 5 days after the low-dose challenge (median parasite count, 238 parasites/mL), when all volunteers were asymptomatic. Peak annexin V staining occurred on day 7 (median parasite count, 2835 parasites/mL), concomitant with the onset of symptoms and 1 day before the largest decline in DC numbers (Figure 4). No apoptosis was seen in DCs at 6 or 8 days after the ultra-low-dose challenge (Figure 4). In addition, no Annexin V staining was detected on lineage-positive, HLA-DR–negative cells (including T and natural killer cells) or granulocytes (based on forward-scatter and side-scatter gating) any day after either the low-dose or ultra-low-dose challenge.

Reduction in Lymphocytes but Not Monocytes Following Low-Dose Challenge
Automated differential blood count data showed a decline in lymphocyte numbers only among individuals who received the low-dose challenge (Table 1). Lymphocyte numbers declined significantly 8 days after infection and remained reduced until day 9. T-cell counts and, more specifically, CD4⁺ T-cell counts also declined after only the low-dose challenge. As in clinical malaria [22], T-cell counts recovered rapidly (within 3 days) after antimalaria drug treatment. There
were no changes in monocyte numbers following both the low-dose and ultra-low-dose challenge.

**Plasma Cytokines**

Testing at each time point following low-dose or ultra-low-dose pRBC challenge showed no detectable elevation in plasma concentration of the cytokines TNF, IL-6, IL-10, or IL-12 above day 0 concentrations (data not shown).

**DISCUSSION**

By using a controlled experimental system involving challenge in humans, we have shown that a low-dose blood-stage *P. falciparum* infection causes a reduction in the number and function of circulating DCs. Reduced circulating DC numbers were at least partially explained by in vivo DC apoptosis, with the nadir level of circulating DCs closely following the peak in DC apoptosis. Loss of circulating DC numbers and function occurred at low parasitemia that was below the detection threshold for microscopy, around the time of onset of clinical symptoms in the malaria-naive volunteers.

Clinical studies of *falciparum* malaria in adults have shown reduced numbers of circulating DCs in patients presenting with uncomplicated malaria [12, 13], suggesting that our findings are relevant to clinical illness in areas of endemicity. In these clinical studies, patients have had parasitemia readily detectable by microscopy at the time of evaluation, often with a high parasite biomass. Our study is the first to prospectively evaluate the onset of changes in DC number and function, its relationship to parasitemia, and the effect of a primary infection. We have shown that the decrease in the function and number of DCs is directly related to the onset of a blood-stage *Plasmodium* infection in malaria-naive individuals.

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**Figure 3.** Longitudinal decline in myeloid dendritic cell (mDC) counts and plasmacytoid dendritic cell (pDC) counts after the low-dose challenge, as demonstrated by absolute numbers of blood CD11c+ mDCs and CD123+ pDCs following low-dose challenge (approximately 1800 *Plasmodium falciparum*-parasitized red blood cells [pRBCs]; 10 recipients) and ultra-low-dose challenge (<180 pRBCs; 5 recipients). The Wilcoxon matched-pairs test yielded a *P* value of .008 for the difference in mDC and .003 for the difference in pDC counts between day 0 and day 8 in the low-dose group. Box plots show the minimum, maximum, median, and interquartile range for data from all subjects (except for the low-dose group, when data were available for 9 individuals on day 5, for 8 on day 6, for 9 on day 8, for 3 on day 9, and for 4 on day 10).

**Figure 4.** Early dendritic cell (DC) apoptosis after the low-dose challenge. *Top graph,* Percentage of total blood DC staining with Annexin V after the low-dose challenge (approximately 1800 *Plasmodium falciparum*-parasitized red blood cells [pRBCs]; 10 recipients). The bottom graph shows comparative Annexin V staining on day 6 and day 8 after the low-dose challenge (filled bars) and ultra-low-dose challenge (<180 pRBCs; 5 recipients; empty bars). Significantly more apoptosis was detected on both days following the low-dose challenge (*P* = .02 on day 6 and *P* = .03 on day 8, by the Mann–Whitney U test). Box plots show the minimum, maximum, median, and interquartile range for data from all subjects (except for the low-dose group, when data were available for 4 individuals on day 6, for 7 on day 9, and for 2 on day 10). * * *P* < .05.
We found that human DCs undergo apoptosis in vivo in response to subpatent *P. falciparum* infection. Previous in vitro studies have demonstrated that human monocyte-derived DCs undergo apoptosis in a PfEMP1-independent manner after exposure to high but not low doses of pRBCs [8]. The physiological significance of these findings is unclear. In murine models, the level of DC apoptosis appears to be related to parasite load in response to *P. chabaudi* AS infection [15], and our data indicate that DC apoptosis occurs early in *P. falciparum* blood-stage infection in vivo at a low parasite biomass (median parasitemia, 238 parasites/mL). The number of blood-stage parasites used in the low-dose challenge in this study was at least 20 times lower than the estimated inoculum of merozoites released into the blood from a single infected hepatocyte following natural mosquito-borne sporozoite infection, in which approximately 30,000 parasites are released [16]. Thus, we hypothesize that DC apoptosis may occur earlier and to a greater degree in the prepatent, preclinical phase of blood-stage infection following natural exposure.

The mechanism(s) underlying *P. falciparum*-induced apoptosis are not clear. Although early in vitro studies suggested this may be PfEMP1 dependent [7], more recent studies show that apoptosis in vitro is contact independent and does not require PfEMP1 [8]. Exposure of DCs to IL-10 can cause DC apoptosis in vitro [23], and we recently found that functional impairment in blood DCs during clinical malaria in areas of endemicity appears to involve IL-10 (Pinzon-Charry, unpublished data). Because the present study focused on the assessment of blood DCs before onset of clinical symptoms (ie, during subpatent infection), systemic inflammatory responses were, as expected, of limited magnitude, with IL-10 being undetectable in plasma. Other factors in the microenvironment in which DCs are being engaged may be important. In this regard, autocrine IL-10 secretion by DCs and T cells locally produced during priming in secondary lymphoid organs could be of significance in determining early DC apoptosis [24, 25]. This process could be further amplified upon progressive parasitemia and immune activation, resulting in sustained and elevated IL-10 production during clinical infection, with ensuing immune dysfunction.

**Figure 5.** Impairment in the uptake of particulate antigen by dendritic cells (DCs) following low-dose challenge, as demonstrated in representative histograms showing whole-blood DC uptake of FITC-dextran in 3 individuals on days 5, 8, and 9 after the low-dose challenge. Black lines show the uptake at 37°C, and gray shading shows the staining for control cells incubated on ice. The summary bar graph indicates the ΔMFI of FITC-dextran uptake (calculated as [MFI for cells incubated at 37°C]−[MFI of control cells incubated on ice]) for all 6 subjects (except for day 9, when data were available for 3 subjects). Box plots show the minimum, maximum, median, and interquartile range for data from all subjects.

**Figure 6.** Intensity of HLA-DR expression on plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) in whole blood before (day 0) and 8 days after the low-dose challenge (10 recipients). The y-axis shows the median fluorescence intensity of HLA-DR.
Another potential mechanism accounting for the decreased number of circulating DCs is the relocation of DCs to organs and locations away from the blood. Indeed, we have demonstrated that DC mobilization can be achieved very quickly in response to acute changes, such as those associated with surgery [26]. Such studies are difficult in human cases of malaria, including those due to experimental challenge, because simultaneous measurements in blood and lymphoid organs are not available. While declining DC counts may, in part, be a consequence of increased migration to organs, the number of DCs circulating in the blood is particularly important because they replenish tissue-residing DCs.

In assessing the effect on DCs of parasite clearance with antimalarial treatment, recovery of both DC number and function (based on HLA-DR expression) was slower for pDCs than mDCs, suggesting different kinetics of repopulation from bone marrow precursors. We were unable to follow DC kinetics beyond day 3 after treatment to characterize the recovery time for pDCs. The observation of stable monocyte counts after both the low-dose or ultra-low-dose challenge accords with the evidence showing independent regulation of monocyte and DC populations [26].

The absence of a change in DC number after the ultra-low-dose (<180-pRBC) challenge was notable and is consistent with the notion that ultra-low-dose whole parasite delivery may induce immunity [27, 28]. However, parasitemia in the 180-pRBC group was treated prior to reaching the threshold at which apoptosis occurred in the higher-dose group, so it was not possible to determine whether use of an ultra-low-dose inoculum prevents the induction of apoptosis. Studies to address this are being undertaken.

The experimental protocol for blood-stage infection of humans described in this study afforded a unique opportunity for prospective, detailed evaluation of the impact of *P. falciparum* on DC numbers and phagocytic function. We were able to evaluate an ultra-low-dose challenge group given identical doses of artemether-lumefantrine and atovaquone-proguanil and to show that there was no effect on DC numbers and HLA-DR expression, indicating reductions in DC numbers and function were independent of drug effects. Also, by infecting with blood-stage parasites only, we could be sure that the observed impairment in DC numbers and function reflected the immunomodulatory effects of blood-stage infection alone, independent of the immunomodulatory effects of skin- and liver-stage infections, which are inherent in both human mosquito experimental sporozoite challenge systems and natural sporozoite infection. The primary infection with a defined *P. falciparum* strain also avoided confounding effects inherent in studies in an area of endemicity, where past and/or recent or concurrent *Plasmodium* infection are difficult to exclude, as other *Plasmodium* species (e.g., *Plasmodium vivax*) are known to modulate immune responses. Finally, the infection of healthy, human immunodeficiency virus–negative adults from a temperate region with adequate nutrition and low incidence of confounding infections, such as helminth infection and tuberculosis, makes it unlikely that these factors had confounding immunomodulatory effects on our findings.

In summary, sterile immunity to blood-stage parasites rarely occurs in populations living in malaria-endemic regions [2, 3], and because of the central role of DCs in the initiation of cellular and antibody immune responses, parasite-induced DC apoptosis and impairment of function may well contribute to the inability to generate effective and/or sterilizing antiparasitic immune responses following natural infection with *P. falciparum*. The ability of a subpatent and subclinical *P. falciparum* infection to compromise DC numbers and function may be of concern for vaccination programs in malaria-endemic countries where asymptomatic parasitemia is common. In view of the renewed goal of malaria elimination and with malaria vaccines progressing to clinical trials, it is important that future research examines DC function in people with asymptomatic parasitemia, including children, to determine whether clearance of all parasitemia is required before immunization to ensure optimal vaccine-induced immunity.

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**Table 1. Absolute Cell Counts Following Challenge With Low or Ultra-Low Doses of *Plasmodium falciparum*-Infected Red Blood Cells**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low Dose (n = 10)</th>
<th>Ultra-Low Dose (n = 5)</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 8</td>
</tr>
<tr>
<td>Lymphocyte level</td>
<td>2.0 (1.7–2.5)</td>
<td>1.3 (1.0–1.6)</td>
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<tr>
<td>CD2+ T cell level</td>
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<tr>
<td>CD4+ T cell level</td>
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<tr>
<td>Monocyte level</td>
<td>0.49 (0.42–0.55)</td>
<td>0.46 (0.42–0.74)</td>
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</tbody>
</table>

Data are median cell count × 10⁶ cells/mL (interquartile range). Abbreviation: NA, not available.

* P < .05, compared with values on day 0 within the same group.
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