Antigen-Presenting Phagocytic Cells Ingest Malaria Parasites and Increase HIV Replication in a Tumor Necrosis Factor α-Dependent Manner

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Background. Plasmodium falciparum infection induces human immunodeficiency virus (HIV) replication and accelerates a decline in CD4+ T-cell count. The mechanisms contributing to these interactions have not been fully elucidated.

Methods. We infected peripheral blood mononuclear cells (PBMCs) with HIV type 1 (HIV-1) and then cocultured them with P. falciparum–infected red blood cells (iRBCs) or uninfected RBCs (uRBCs). Levels of HIV-1 p24 antigen and activation-associated cytokines were measured in culture supernatants. T-cell surface activation was assessed by flow cytometry.

Results. It has been reported that iRBCs increase HIV replication, compared with uRBCs; that neutralizing tumor necrosis factor α (TNF-α) abrogates this increase; and that hemozoin enhances HIV production. In this study, we confirmed that TNF-α plays an important role in this interaction. We show that iRBCs increased CD4+ T-cell expression of HLA-DR+/CD38+ (P = .001), that monocyte/macrophage depletion reduced HIV production by 40%–50% (P < .001), and that hemozoin-laden monocytes/macrophages that were preincubated with iRBCs also stimulated HIV production.

Conclusions. iRBCs activate CD4+ T cells and stimulate HIV replication in a TNF-α–dependent manner following malarial antigen processing by monocytes/macrophages. These results suggest that the persistent elevation of HIV replication during and after acute bouts of P. falciparum malaria may be due, at least in part, to ongoing stimulation of CD4+ T cells by hemozoin-loaded antigen-presenting cells within lymphoid tissues.

Keywords. HIV; P. falciparum; interaction; mechanism of increased viral load; malaria; co-infection.

Plasmodium falciparum causes an increase in human immunodeficiency virus (HIV) replication both in vitro [1, 2] and in vivo [3–5], but the mechanism(s) responsible for the increase in HIV production have not been fully elucidated. During an episode of clinical malaria, elevated plasma levels of both proinflammatory cytokines (including tumor necrosis factor α [TNF-α] and interferon γ [IFN-γ]) and antiinflammatory cytokines (such as interleukin 10 [IL-10]) are secreted by the innate and adaptive cells of the immune system in an attempt to control the infection [6]. Monocytes, macrophages, and dendritic cells ingest parasite-infected red blood cells (iRBCs), present malarial antigens, and activate T cells and B cells during the course of a vigorous immune response to an acute infection with P. falciparum. It has been proposed that HIV replication in latently infected cells is activated in conjunction with cellular activation and that plasma levels of HIV RNA rise, at least in part, as T-cell populations respond to the infection [5]. Plasma HIV RNA levels remain
elevated well after the parasitemia has cleared, and thus it is likely that there are other factors at play.

Xiao et al. showed that in the presence of *P. falciparum* antigen, TNF-α secretion caused an increase in HIV replication through downstream signaling resulting in stimulation of the HIV long-terminal repeat sequence [2]. More recently, Diou et al. showed that hemozoin, a byproduct of *P. falciparum* heme breakdown, caused enhanced dendritic cell-mediated transfer of HIV between CD4⁺ T cells, leading to increased HIV production in vitro [7]. Using an in vitro coculture system that we developed and described previously [1], we examined which cytokines and cell types were involved in the interaction and further evaluated the potential role of hemozoin-loaded macrophages in the immunopathogenesis of *P. falciparum* infection in persons with HIV coinfection.

**METHODS**

**Peripheral Blood Mononuclear Cell Collection and Isolation**

PBMCs were isolated by Histopaque 1077 centrifugation and cultured overnight at 37°C in 5% CO₂ in interleukin 2/phytohemagglutinin-free R20 medium (20% fetal bovine serum [FBS] and 1% Pen-Strep in Roswell Park Memorial Institute [RPMI] 1640 medium) and used the following day in cocultures. PBMCs were obtained from malaria-naïve donors enrolled in the blood draw program (informed consent was obtained per protocol HS103) at the Seattle Biomedical Research Institute, which was approved by the Western Institutional Review Board.

**P. falciparum Culture**

*P. falciparum* NF54 parasites were grown in type O human RBCs in RPMI 1640 medium (Invitrogen) with 5 g/L albumax (Invitrogen), 2 g/L dextrose (Fisher), 50 mg/L hypoxanthine (Sigma), 2.25 g/L sodium bicarbonate (Sigma), 11 mg/L gentamicin (Invitrogen), and 5% pooled human AB serum (Valley Biomedical). Parasite chambers were gassed with 5% O₂/5% CO₂/90% N₂ and incubated at 37°C. Parasite cultures were maintained continuously and split 1–2 days before setting up cocultures. iRBCs were used once 6%–7% of the RBCs in the culture were parasitized, as assessed by light microscopy. Cultures were routinely monitored for mycoplasma contamination by polymerase chain reaction (Takara) and shown to be mycoplasma free.

**Cocultures**

The day following isolation, PBMCs were placed in 96-well plates (2 × 10⁵ cells/well/200 µL) and infected with HIV (multiplicity of infection, 25) without exogenous mitogens/cytokines in R20 medium. iRBCs or uninfected RBCs (uRBCs) were added at the time of HIV infection to selected wells in a 10:1 ratio of RBCs to PBMCs (2 × 10⁶ RBCs/well/200 µL). All conditions were run in triplicate. After 22 hours, the entire 200 µL of medium was collected and replaced with new medium. A total of 100 µL of the culture supernatants was collected at days 4, 6, 8, and 10 and replaced with fresh medium. These supernatants were frozen at −80°C and later used to determine HIV p24 antigen and cytokine levels. Viral production was quantified at the University of California–San Diego Center for AIDS Research Translational Virology Core by determining the amount of p24 antigen in the culture supernatants, using a p24 antigen-capture enzyme-linked immunosorbent assay (Perkin Elmer). Malaria parasites were observed daily by means of thin smears in the iRBC/PBMC cocultures and were found to continue maturing and invading RBCs for up to 4 days (data not shown).

For the monocyte/macrophage-depletion experiments, PBMCs were isolated as described above and cultured overnight in tissue culture (TC)-treated flasks. The next day, only the cells that did not attach to the plastic were collected and used for experiments. For the CD3⁺/CD4⁺ T-cell-enrichment experiments, PBMCs were isolated as described above and allowed to rest overnight, and then either the CD3⁺ T-cell enrichment kit or the CD4⁺ T-cell enrichment kit (StemCell Technologies) was used to isolate only CD3⁺ T cells or only CD4⁺ T cells, respectively, from recovered cells. Unfractionated PBMC cocultures were always set up in parallel as a control. For the TNF-α and IFN-γ neutralizing antibody coculture experiments, cocultures were set up as described above, and monoclonal antibodies to either TNF-α (500 pg/ml) or IFN-γ (300 pg/ml) were added at the time of set up (keeping the volume at 200 µL). The same concentration of antibody was added 22 hours later when the medium was changed, and half of the concentration of antibody was added at days 4, 6, and 8 when 100 µL of the supernatant was collected and replaced with fresh medium.

For the pretreatment experiments, PBMCs were isolated (day-3) as described above and allowed to rest overnight in TC-treated culture flasks. The next day (day-2), all cells that did not attach were discarded, and only those that attached to the flask after 2 washes with phosphate-buffered saline (PBS) remained (primarily monocytes and macrophages, approximately 20%–30% CD4⁺ T cells, as determined by flow cytometry; data not shown). The cells that attached were replated in 12-well plates (3 × 10⁵ cells/well in 1 mL of medium), and RBCs were added in a 40:1 ratio of RBCs to attached cells (1.2 × 10⁷ RBCs/well) and cocultured for 48 hours. On day-1, whole PBMCs were isolated from the same donor(s) as on day-3 and allowed to rest overnight. On day 0, whole PBMCs were plated in 96-well plates (2 × 10⁵, as described above) in triplicate. Either RBCs (2 × 10⁶) or the pretreated attached cells (2 × 10⁴) were added to the plated PBMCs. All 200 µL of medium was changed on day 1, and 100 µL of supernatant was collected and replaced with 100 µL of fresh medium on days 4, 6, 8, and 10 after initiation of cocultures.

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Table 1. Summary of Interactions Between Human Immunodeficiency Virus (HIV) and Plasmodium falciparum

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Culture Condition</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p24 Ratio (95% CI)</td>
<td>Effect Size (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>A. Effect of malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+ T cells only</td>
<td>1.7 (1.5–1.9)</td>
<td>2.4 (1.8–2.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CD4+ T cells only</td>
<td>1.7 (1.5–1.9)</td>
<td>2.4 (1.8–2.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Macrophage depleted</td>
<td>1.7 (1.5–2.0)</td>
<td>1.8 (1.3–2.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Neutralized TNF-α</td>
<td>1.7 (1.5–1.9)</td>
<td>2.3 (1.9–2.8)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Neutralized IFN-γ</td>
<td>1.7 (1.5–1.9)</td>
<td>2.4 (1.9–2.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Macrophage pretreatment</td>
<td>1.7 (1.5–1.9)</td>
<td>2.3 (1.9–2.8)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

B. Effect of culture condition

| p24 Ratio (95% CI) | Effect Size (95% CI) | P value | p24 Ratio (95% CI) | Effect Size (95% CI) | P value |
|-------------------------------|------------------------------------------------------------------------|---------|------------------------------------------------------------------------|---------|
| A. Malaria Parasites Present (iRBC) |                                                                       |         |                                                                        |         |
| CD3+ T cells only             | 0.1 (.1–2.)               | −8.7 (−9.8 to −7.8)          | <.001   | 0.2 (.2–3.)          | −6.4 (−7.5 to −5.3)   | <.001   | .0028   |
| CD4+ T cells only             | 0.1 (.1–2)                | −8.9 (−10.0 to −7.6)         | <.001   | 0.2 (.2–3)           | −6.9 (−8 to −5.8)     | <.001   | .0092   |
| Macrophage depleted           | 0.6 (.5–8.)               | −1.5 (−2.4 to −0.7)          | <.001   | 0.5 (.4–6)           | −2.6 (−3.4 to −1.7)   | <.001   | .067    |
| Neutralized TNF-α             | 0.6 (.5–8.)               | −2 (−2.8 to −1.2)            | <.001   | 1.0 (.8–1.2)         | −0.1 (−0.9 to 0.7)    | .79     | .0013   |
| Neutralized IFN-γ             | 1.0 (.8–1.3)              | 0 (−1 to 1)                  | .98     | 1.1 (.9–1.4)         | 0.4 (−0.6 to 1.4)     | .44     | .53     |
| Macrophage pretreatment       | 1.1 (.9–1.5)              | 0.5 (−0.6 to 1.6)            | .33     | 1.4 (1.1–1.8)        | 1.5 (1–2.7)           | .0095   | .14     |

A. This table demonstrates the effect that malaria parasites have on HIV replication by comparing the amount of HIV produced in the iRBC cocultures to the amount produced in uRBC cocultures in the control condition (unfractionated PBMCs, run in parallel for each culture condition) and the culture conditions listed in the left column. B. The p24 ratio and effect size were calculated for each culture condition, compared with the control, in the presence of iRBCs and of uRBCs. Abbreviations: CI, confidence interval; IFN-γ, interferon-γ.

Cytokine Quantification

Cytokine levels were measured in culture supernatants, using a BioPlex platform (BioRad). A 5-plex kit containing IL-10, interleukin 6 (IL-6), TNF-α, IFN-γ, and macrophage inflammatory protein 1α (MIP-1α) or interleukin 4 (IL-4), IL-6, TNF-α, IFN-γ, and MIP-1α was used according to the manufacturer’s protocol (BioRad), with 1 alteration: for the last step, the beads were resuspended in 1% formaldehyde in assay buffer and after a 30-second shake the plate was read on a Bioplex200 (BioRad). An 8-point standard curve was used to determine cytokine concentrations, using a 5-parameter logistic regression curve. Detection limits for cytokines are as follows: IL-4, 0.7 pg/mL; IL-6, 2.6 pg/mL; IL-10, 0.3 pg/mL; IFN-γ, 6.4 pg/mL; TNF-α, 6.0 pg/mL; and MIP-1α, 1.6 pg/mL.

Flow Cytometry

PBMCs were isolated as described above and the next day were plated (2 × 10⁶ PBMCs/well) in a 96-well plate and cocultured with CD3/CD28 antibodies, iRBCs, uRBCs (2 × 10⁶ RBCs/well), or medium only in triplicate. CD3 antibodies (BD) were used at a concentration of 1 µg/mL, and CD28 antibodies (BD) were used at a concentration of 2 µg/mL. Cultures were incubated at 37°C in 95% O₂ for 48 or 72 hours. At the indicated time point, all 3 wells for a given condition were combined into a single fluorescence-active cell sorter (FACS) tube. Cells were washed once in FACS buffer (PBS plus 2% FBS), resuspended in 50 µL of Live/dead–Aqua stain (Invitrogen), and stained with either CD3-fluorescein isothiocyanate (FITC; eBioscience), CD4-Pacific Blue (eBioscience), CD8-allophycocyanin (APC; BD Pharmingen), HLA-DR–peridinin chlorophyll protein (PerCP; Biolegend), and CD38-phycocerythrin (PE)-Cy5.5 (BD Pharmingen) or CD3-FITC, CD4-Pacific Blue, CD8-APC, CD45RO-PE, CD69-PerCP-Cy7 (eBioscience), and CD25-PE-Cy5.5 (eBioscience). Cells were stained at room temperature for 20 minutes and washed twice with FACS buffer. The RBCs were then lysed using 120 µL of BD FacsLyse at room temperature for 10–15 minutes, washed once with PBS, and resuspended in 130 µL of 2% formaldehyde in distilled H₂O. Samples were subjected to flow cytometric analysis within 18 hours of fixing on the BD LSRII. All data were analyzed using FlowJo (Treestar).

Statistical Methods

For the flow cytometry experiments, we fit a mixed-effects linear model to the log₁₀-transformed biomarker response as a function of the 4 conditions. The within-subject correlations are modeled via a normal random effect term (random intercept). We computed point estimates, 95% confidence intervals, and P values for the 2 contrasts of interest: iRBC versus uRBC,
and CD3/CD28 versus iRBC. A significantly positive difference for both contrasts shows a significant ranking of responses as uRBC < iRBC < CD3/CD28. The \( P \) values are based on the Wald test. The \( P \) values were not corrected for multiple comparisons; \( P \) values of <.05 are considered statistically significant. All models were fit using the nlmef package in the R statistical program [8]. For the area under the curve (AUC) calculations, total log-transformed HIV p24Ag and cytokine production were integrated over the 10 days in culture, using a trapezoidal method (AUC) and were computed and analyzed for 7 different types of biomarkers (conditions): control (no enrichment), CD3+ T cells only (T-cell enrichment), CD4+ T cells only (CD4+ T-cell enrichment), monocyte/macrophage depletion, TNF-\( \alpha \) neutralizing antibody, IFN-\( \gamma \) neutralizing antibody, and the monocyte/macrophage-pretreatment condition. For each biomarker, the AUC was computed under 4 conditions—iRBC, uRBC, iRBC plus biomarker, uRBC plus biomarker—in a 2 \( \times \) 2, factorial, repeated-measures design (4 data points per subject). For each biomarker, we fit the AUCs by using a mixed-effects linear model. The within-subject correlations were modeled via a subject-specific normal random effect (random intercept). In each case, we examined (1) the effect of malaria (ie, the difference between the iRBC and uRBC groups) separately for the controls (no biomarker) and in the presence of the biomarker (culture conditions), (2) the effect of the biomarker condition (ie, the difference in response between the biomarker condition and the control) separately for the iRBC and uRBC groups, and (3) the interaction between the presence of the biomarker and the malaria infection status, examining whether the effect of malaria infection is different in the biomarker cell types than in the controls (or, conversely, whether the biomarker effect is different in the iRBC and uRBC groups). The results were reported by back transformation on the original p24 scale, in terms of the p24 ratio between groups. The \( P \) values were computed by means of the Wald test and were not adjusted for multiple comparisons; \( P \) values of <.05 were deemed statistically significant. Table 1 summarizes the interactions between HIV and \( P. falciparum \). HIV production was quantified as above. This data were log_{10} transformed and the AUC levels were computed over the 10 day follow-up period. To calculate the p24 ratio, the mean AUC difference

Figure 1. \( Plasmodium falciparum \)-infected red blood cells (iRBCs) activate CD4+ T cells but not CD8+ T cells in cocultures. Peripheral blood mononuclear cells (PBMCs) were cultured with iRBCs or uninfected RBCs (uRBCs), without human immunodeficiency virus, for 48 or 72 hours. Cells were stained with either CD3–fluorescein isothiocyanate (FITC), CD4-Pacific blue, CD8–allophycocyanin (APC), HLA-DR–peridinin chlorophyll protein (PerCP), and CD38–phycocerthythin (PE)-Cy5.5 or with CD3–FITC, CD4–Pacific Blue, CD8–APC, CD8–PerCP-Cy7, and CD25–PE-Cy5.5 and acquired using an LSR II. A, iRBCs increased activation of CD4+ T cells (CD38/HLA-DR double-positive cells) at 48 hours, compared with uRBCs, but activation was less than that due to CD3/CD28 antibodies. B, iRBC-induced activation decreased by 72 hours. C and D, iRBCs did not significantly activate CD8+ T cells, compared with uRBCs, but there was significant activation of CD8+ T cells due to CD3/CD28 antibodies.
(iRBC-uRBC) per day was transformed back to the original scale. A p24 ratio of 1.7 corresponds to a 70% increase in HIV production. Cohen’s d effect size (ES) measures the relative difference between the means of two populations; an ES of 2.4 signifies that the mean amount of HIV produced in the presence of iRBCs is 2.4 standard deviations greater than the mean amount of HIV produced in the presence of uRBCs, on the logarithmic scale. A significant interaction between the variables (presence of malaria and culture condition) was found if the p24 ratio under the iRBC and uRBC conditions differed between the control and culture condition groups.

RESULTS

iRBCs Activate CD4+ T Cells and Cause an Increase in HIV Production

We previously showed that when PBMCs are cocultured with iRBCs in the absence of any activating cytokines/mitogens, HIV production increases by 70%, compared with findings involving uRBC cocultures (P < .001; Table 1) [1]. To explore whether this increase in HIV production in the iRBC cocultures was associated with increased T-cell activation, surface expression of activation markers on CD4+ and CD8+ T cells was measured 48 and 72 hours after cocultures were initiated with iRBCs, uRBCs, or medium alone (Figure 1). We found that HLA-DR antigen expression and dual expression of HLA-DR/CD38 antigens was increased (P = .008 and P = .001, respectively) on CD4+ T cells 48 hours after cocultivation with iRBCs, compared with findings when uRBCs were used (Figure 1A). These changes were less apparent at 72 hours (Figure 1B). We saw no increase in expression of CD38 alone, CD25, or CD69 in the iRBC cocultures, compared with the uRBC cocultures. To assess the relative extent to which CD4+ T cells were activated by iRBCs, compared with when CD4+ T cells were more broadly activated, we also measured cell surface expression of markers of activation after ligation of CD3 and CD28 with monoclonal antibodies. The increased expression of all markers due to CD3/CD28 stimulatory antibodies was significantly greater, compared with iRBC stimulation (Figure 1A and 1B). In contrast, CD8+ T cells were broadly activated by CD3/CD28 ligation but not by iRBCs (Figure 1C and 1D). Thus, iRBC-mediated T-cell activation was restricted exclusively to the CD4+ T-cell subset.

Figure 2. Increase in tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and macrophage inflammatory protein 1 α (MIP-1α) secretion in Plasmodium falciparum–infected red blood cell (iRBC) cocultures. Peripheral blood mononuclear cells from malaria-naïve, healthy donors were infected with human immunodeficiency virus and cocultured with iRBCs or uninfected RBCs (uRBCs). Cytokine production in the iRBC (red lines) and uRBC (black lines) cocultures was measured at days 1, 4, 6, and 8 after initiation of the cocultures. Each point represents the average cytokine secretion from 5 donors; the error bars represent the standard error of the mean. iRBCs stimulated increased secretion of the proinflammatory cytokines TNF-α (A), IFN-γ (B), and MIP-1α (C), and peak levels were reached at days 4–6. D. Levels of interleukin 6 (IL-6), another proinflammatory cytokine, were not increased in the iRBC cocultures, compared with the uRBC cocultures.
The Increase in HIV Production Due to iRBC Stimulation is TNF-\(\alpha\) Dependent

Previously we reported that TNF-\(\alpha\), IFN-\(\gamma\), and MIP-1\(\alpha\) production by PBMCs is increased in the presence of iRBCs, compared with uRBCs, and that levels of these cytokines and of HIV replication increase further when experiments are conducted using PBMCs from individuals who are in the convalescent phase of an in vivo experimental challenge with \(P. falciparum\) [1]. Here, we demonstrated that TNF-\(\alpha\), IFN-\(\gamma\), and MIP-1\(\alpha\) production peak during days 4–6 when PBMCs are cocultured with iRBCs, while levels in the uRBC cocultures remained close to the lower level of detection (Figure 2A–2C). To further probe the potential roles of the proinflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) in stimulation of HIV production, neutralizing antibodies to TNF-\(\alpha\) or IFN-\(\gamma\) were added to cocultures at the time they were initiated (Figure 3). We found that neutralizing antibodies to TNF-\(\alpha\) reduced \(P. falciparum\)–stimulated HIV production by 40\% (\(P < .001\)), making it equivalent to levels of HIV production in the uRBC cocultures (\(P = .79\); Figure 3A and 3B). Addition of the IFN-\(\gamma\) neutralizing antibody did not change HIV production in either the iRBC or uRBC cocultures (\(P = .53\); Figure 3A and 3C).

HIV Production is Dependent on Monocytes and Macrophages

To determine which cell types contribute to iRBC-stimulated HIV production, we conducted a series of experiments with enriched subpopulations of T cells or monocytes. No HIV production was detected when iRBCs were cocultivated with purified populations of CD3\(^+\) or CD4\(^+\) T cells (Figure 4A and 4C). Next, we partially depleted monocytes and macrophages, antigen-presenting leukocytes, from the cocultures, resulting in a 40\%–50\% depletion of the CD14\(^+\)/CD11c\(^+\) monocyte/macrophage population (data not shown). While there was still significantly more HIV produced from the
monocyte/macrophage-depleted PBMCs when cocultured with iRBCs, compared with uRBCs ($P < .001$; Figure 4D), the depletion resulted in a 40%–50% reduction of HIV production in all cocultures, compared with the nondepleted coculture controls ($P < .001$ Figure 4D). These results suggest that when monocytes and macrophages are reduced by as little as 40%–50%, there is a significant decrease in HIV p24 antigen production by PBMCs.

HIV Production Can Be Induced by Incubating PBMCs With Monocytes/Macrophages That Have Been Pretreated With iRBCs and is Likely TNF-α Dependent

Next, we wanted to determine whether monocytes/macrophages that have ingested ruptured iRBC cell debris or whole iRBCs are sufficient for HIV production. In one experiment involving 2 different donors, monocytes/macrophages were pretreated with either iRBCs or uRBCs for 48 hours and then added to autologous PBMCs and infected with HIV (no RBCs were added to these cocultures). Parallel control cocultures with PBMCs from the same donor were set up with iRBCs/uRBCs. Giemsa smears of monocytes/macrophages after 48 hours of incubation with iRBCs demonstrated copious hemozoin crystals (Figure 5A). Hemozoin crystals were not observed after cocultivation with uRBCs (Figure 5B). The effect of malaria parasites (pretreatment with iRBCs vs uRBCs) on the amount of HIV produced from the cocultures in which only pretreated monocytes/macrophages were added was similar to the effect of malaria parasites (iRBC vs uRBC) on HIV production in the control cocultures ($P = .14$; Figure 5C and 5D). TNF-α production increased following exposure of iRBC-pretreated monocytes/macrophages...
to PBMCs (Figure 5E). In these experiments, the patterns of IFN-γ and MIP-1α secretion resembled those observed in uRBC cocultures (Figure 5F and 5G). Thus phagocytic, antigen-presenting cells are sufficient to induce HIV replication in the presence of malaria parasites, and this is likely dependent on TNF-α signaling.

### DISCUSSION

We have studied the pathogenesis of the complex interplay between HIV and *P. falciparum* in a model in vitro coculture system. We previously demonstrated that iRBCs stimulate production of HIV replication in unfractionated PBMCs and that iRBCs...
increase production of TNF-α, IFN-γ, and MIP-1α but not IL-6. In this study, we showed that iRBCs specifically activate CD4+ T cells and not CD8+ T cells and that antibody-mediated neutralization of TNF-α inhibits iRBC-driven HIV production, which is in agreement with previous studies showing that neutralizing TNF-α, but not IL-6, resulted in significantly decreased HIV production in the presence of malarial antigens [2]. We also demonstrated that monocytes and macrophages are required for this enhancement in HIV replication. iRBC-exposed monocytes and macrophages were as efficient as iRBCs in stimulating TNF-α production and HIV replication.

There are several limitations to this study. First, this is an in vitro system that cannot account for the complex interplay of immune cells within lymph nodes and affected tissues. Additionally, we studied only healthy volunteer blood donors without prior exposure to P. falciparum. We have previously demonstrated that iRBC-induced HIV production is further enhanced in PBMCs obtained from healthy volunteers after controlled experimental infection with P. falciparum [1]. Thus, we may have underestimated the extent of T-cell activation and HIV production that would be expected if PBMCs were isolated from individuals living in P. falciparum–endemic regions. In regions where HIV and malaria parasites are both highly endemic, evidence suggests that acute bouts of malaria drive higher plasma (and perhaps genital secretion) HIV RNA levels, which could contribute to increased HIV transmission rates [9]. Decline in the CD4+ T-cell count has been demonstrated to be accelerated by clinically apparent bouts of P. falciparum malaria [10]. Although clinical data have not yet been demonstrated, it is quite possible that subclinical parasitemia may also contribute to these phenomena. While constant low levels of malaria parasite replication do not cause the degree of inflammatory responses seen during episodes of symptomatic malaria, low levels of local inflammatory responses are highly likely. It is quite possible that monocytes and macrophages (and perhaps dendritic cells) that ingest infected red cells, merozoites, or iRBC debris would traffic to tissues and cause local activation of T cells and increased HIV replication, much like bacterial translocation in the gut of HIV-infected individuals causes local inflammatory responses in the gut-associated lymphoid...
tissue, which has also been correlated to increased rates of CD4+ T-cell count decline [11]. Last, the experiments looking at monocyte/macrophage depletion only resulted in about a 40%–50% reduction of the CD14+ and CD11c+ cell populations. In addition, with our monocyte-depletion technique, initially adherent dendritic cells were likely released into the nonadherent cell fraction during overnight cultures [12]. Thus, antigen-presenting, phagocytic cells were still present in the monocyte/macrophage-depleted cocultures. Despite these limitations in our monocyte-depletion technique, the similar reduction of monocytes/macrophages (40%–50% reduction in CD14+ and CD11c+ cells) and decrease in HIV production in depleted cultures (40%–50% decrease) supports an important role for monocytes and macrophages in the adverse interactions between HIV and *P. falciparum*.

Intracellular *P. falciparum* parasites produce hemozoin crystals following metabolism of hemoglobin, to avoid toxicity from accumulation of free heme. Hemozoin crystals are released with the escape of merozoites from erythrocytes. Phagocytic cells ingest free-floating hemozoin as well as iRBCs and accumulate cytoplasmic hemozoin deposits [13], which can persist in macrophages for several months following ingestion [14, 15]. It has been reported that heavily hemozoin-laden macrophages have impaired activation and function [16], that hemozoin crystals hinder monocyte maturation [7], and that hemozoin crystals reduce HIV replication in phagocytic cells [7, 17]; however, these effects may be more than offset by the ability of these cells to stimulate production of HIV in autologous CD4+ T cells. Hemozoin itself has been reported to enhance dendritic cell-mediated transfer of HIV to CD4+ T cells [7] and increase HIV production by CD4+ T cells that are in contact with hemozoin-loaded dendritic cells [7]. In pregnant women, TNF-α and IFN-γ production is increased in intervillous blood mononuclear cells isolated from HIV-seropositive women with increasing levels of hemozoin crystals, while in HIV-seronegative women, a suppressive effect of hemozoin was seen on the production of TNF-α, IFN-γ, and IL-10 [18]. In PBMCs isolated from simian immunodeficiency virus–infected animals during AIDS, hemozoin increased viral replication and TNF-α production [19]. In addition to hemozoin, other factors may contribute to the deleterious interaction between malaria parasites and HIV. *P. falciparum* glycosphingophosphatidylinositol (GPI) fragments activate macrophages to secrete TNF-α [20], and *Plasmodium* Cpg–containing DNA can activate dendritic cells to secrete IFN-γ and TNF-α [21].

Together, our studies and those of others suggest that the increase in plasma HIV RNA levels observed during an episode of clinical malaria involves a complex interplay of several factors. Phagocytic, antigen-presenting cells ingest free hemozoin, malaria-derived GPI fragments, Cpg-containing DNA, and/or iRBCs. These antigen-presenting cells traffic to tissues (macrophages) or lymph nodes (dendritic cells) and likely activate CD4+ T cells in a TNF-α–dependent fashion. In HIV/ *P. falciparum*–coinfected populations, plasma HIV RNA levels remain elevated from 4 weeks [22] to 9 weeks [5] after an episode of malaria has resolved. Prolonged hemozoin persistence in macrophages and dendritic cells may allow for prolonged stimulation of CD4+ T cells, driving them to produce HIV for weeks after all parasites have been eradicated from the peripheral blood. Our results showing increased HIV production from PBMCs cocultured with iRBC-pretreated monocytes and macrophages in the absence of iRBCs support this mechanism. While we did not focus on dendritic cells in this study, others have shown that dendritic cells loaded with hemozoin increase HIV replication and could also contribute to the sustained increases in HIV RNA levels in the presence of malaria parasites [7]. Based on this study and the contribution of others in this field, we believe that this complex interplay among the 2 pathogens and the multifaceted innate and adaptive immune response to *P. falciparum* (Figure 6) may all contribute to the enhanced morbidity and mortality observed in coinfected individuals.

**Notes**

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