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Hemozoin-Inducible Proinflammatory Events In Vivo: Potential Role in Malaria Infection

Maritza Jaramillo,* Isabelle Plante,* Nathalie Ouellet,* Karen Vandal,* Philippe A. Tessier,* and Martin Olivier‡†

During malaria infection, high levels of proinflammatory molecules (e.g., cytokines, chemokines) correlate with disease severity. Even if their role as activators of the host immune response has been studied, the direct contribution of hemozoin (HZ), a parasite metabolite, to such a strong induction is not fully understood. Previous in vitro studies demonstrated that both Plasmodium falciparum HZ and synthetic HZ (sHZ), β-hematin, induce macrophage/monocyte chemokine and proinflammatory cytokine secretion. In the present study, we investigated the proinflammatory properties of sHZ in vivo. To this end, increasing doses of sHZ were injected either i.v. or into an air pouch generated on the dorsum of BALB/c mice over a 24-h period. Our results showed that sHZ is a strong modulator of leukocyte recruitment and more specifically of neutrophil and monocyte populations. In addition, evaluation of chemokine and cytokine mRNA and protein expression revealed that sHZ induces the expression of chemokines, macrophage-inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and monocyte chemoattractant protein-1/CCL2; chemokine receptors, CCR1, CCR2, CCR5, CXCR2, and CXCR4; cytokines, IL-1β and IL-6; and myeloid-related proteins, S100A8, S100A9, and S100A8/A9, in the air pouch exudates. Of interest, chemokine and cytokine mRNA up-regulation were also detected in the liver of i.v. sHZ-injected mice. In conclusion, our study demonstrates that sHZ is a potent proinflammatory agent in vivo, which could contribute to the immunopathology related to malaria. The Journal of Immunology, 2004, 172: 3101–3110.

The human malarial parasite Plasmodium falciparum is responsible for 300–500 million clinical cases and 1–3 million deaths annually, mostly of children (1). Severe disease and high mortality associated with malaria mainly have been attributed to parasitic virulence factors (e.g., cytoadherence, rosetting, multiplication rate); however, different lines of evidence suggest that the host’s immunological response could also contribute to malaria pathophysiology (2). Elevated serum concentrations of IL-1, IL-6, IFN-γ, and TNF-α have been detected in P. falciparum malaria patients, and their levels correlated with disease severity (3–6). Even though these cytokines appear to play a pivotal role in parasite control, it has been proposed that an exacerbated inflammatory response could also participate in malaria pathogenesis, including severe anemia, cerebral malaria (CM),3 cytoadherence, and tissue cell damage (3, 7). In addition to proinflammatory cytokines, increased serum concentrations of strong leukocytes chemoattractants and activators; myeloid-related proteins (MRPs), S100A8, S100A9, and S100A8/A9 (8, 9); and chemokines, IL-8 and macrophage-inflammatory protein (MIP)-1α/CC chemokine ligand (CCL) 3 (10), have been observed in patients suffering from acute P. falciparum malaria. Furthermore, elevated MIP-1α, monocyte chemoattractant protein (MCP)-1/CCL2, and IL-8 levels were recently found during placental malaria (11).

In addition to data from P. falciparum-infected patients, cytokine (e.g., TNF-α, IFN-γ, IL-6) and chemokine (e.g., MCP-1, IFN-γ-inducible protein-10 (IP-10), RANTES) induction has also been reported in experimental models of murine malaria (12–14), in which it appears to be involved in both protective immunity and pathogenesis. Moreover, in vitro studies have provided evidence of leukocyte activation during malaria infection. In response to Plasmodium, macrophages (Mφ) have been found to secrete TNF-α, IL-1β, IL-6, and IL-8 (15), and dendritic cells to synthesize IL-6 and IL-12 (16).

Even though proinflammatory cytokine and chemokine production during malaria are well documented, the direct contribution of specific parasite components to such induction has not yet been fully unraveled. Because the classic malaria paroxysm characterized by fever and chills is associated with schizogony (3), the ability of parasite metabolites GPI and hemozoin (HZ), released during this process, to stimulate the host immune response has been investigated. In this regard, Schofield and Hackett (17) demonstrated that the P. falciparum GPI moiety induces both TNF-α

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3 Abbreviations used in this paper: CM, cerebral malaria; CCL, CC chemokine ligand; HZ, hemozoin; IP-10, IFN-γ-inducible protein-10; MCP, monocyte chemoattractant protein; mCR, murine chemokine receptor; MIP, macrophage-inflammatory protein; MRP, myeloid-related protein; Mφ, macrophage; Nε, neutrophil, RPA, RNA protection assay; sHZ, synthetic HZ.
and IL-1 in Mφ, and when administered to mice, the parasite GPI leads to cytokine release, transient pyrexia, and hypoglycemia. HZ or malarial pigment is a polymer of heme produced by the parasite during hemoglobin digestion inside the host RBC (18). This pigment is released along with merozoites as the RBC bursts and is avidly phagocytosed by circulating monocytes, neutrophils (Nφ), and resident Mφ (reviewed by Arese and Schwarzer (19)). Formerly, HZ was considered only as a waste product of the parasite resulting from heme detoxification; however, several lines of evidence strongly suggest that it could play an important role in cytokine production and parasite cytoadherence during malaria infection. In vitro studies showed that either Plasmodium HZ or synthetic HZ (sHZ), which is structurally identical with the native pigment (20), induces the release of various proinflammatory mediators, including IL-1β, TNF-α (21), MIP-1α, and MIP-1β/CCL4 (22), in either murine Mφ or human monocytes, as well as adhesion molecule expression and IL-6 production in human endothelial cells (23). These findings have been supported by those of Biswas et al. (24), who reported the presence of Abs against P. falciparum HZ among complicated malaria patients, which had inhibitory effects on HZ-mediated TNF-α and IL-1β production by human monocytes. Moreover, we recently found that both P. falciparum HZ and sHZ increase IFN-γ-mediated Mφ NO generation through specific signaling pathways (25). Finally, in vivo studies revealed that sHZ has the ability to regulate body temperature in rats (22), suggesting that this parasite metabolite could contribute to fever during acute malaria.

In the present study, we sought to investigate the proinflammatory properties of sHZ in vivo. Using the murine air pouch model in BALB/c mice, we demonstrate that sHZ injection causes a significant leukocyte recruitment in the pouch lumen (mainly Nφ and monocytes), which is accompanied by increased levels of various chemokines (MIP-1α, MIP-1β, MIP-2/CXC ligand 2, and MCP-1), CCR and CXCR (CCR1, CCR2, CCR5, CXCR2, and CXCR4), proinflammatory cytokines (IL-1α, IL-1β, and IL-6), and murine MRPs (S100A8, S100A9, and S100A8/A9). In addition, i.v. sHZ inoculation revealed that the malarial pigment leads to a dose-dependent mRNA up-regulation of liver chemokine (MIP-1α, MIP-1β, MIP-2, and MCP-1) and cytokine (IL-1α and IL-12) expression. Taken together, our data suggest that HZ could play an important role in malaria immunopathology related to proinflammatory mediator overproduction.

Materials and Methods

Materials

Hemin chloride and LPS (Escherichia coli, serotype 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO). Isotope [α-32P]UTP (3000 Ci/mmol) was purchased from PerkinElmer (Wellesley, MA). Six- to 8-wk-old female BALB/c mice, 20-30 g body weight, were obtained from Charles River Breeding Laboratories (St. Colomban, Quebec, Canada).

β-hematin (sHZ) preparation

The method for β-hematin synthesis described by Egan et al. (26) was adapted, as we previously described (25). Briefly, 45 mg of hemin chloride (Sigma-Aldrich) was solubilized in 4.5 ml of 1 N NaOH and neutralized with 450 μl of 1 N HCl. Then 10.2 ml of 1 M sodium acetate, pH 4.8, was added and the suspension was stirred with a magnet for 2-3 h at 60°C. Following addition of 1/100 vol of 10% SDS and 14,000 × g centrifugation for 15 min, the pellet was sonicated at lowest setting in 100 ml of sodium bicarbonate, pH 9.0, 0.5% SDS, and again centrifuged. The pellet was then washed three to four times in 2% SDS and then in water to wash out SDS. The pigment was dried at 37°C overnight, resuspended in endotoxin-free PBS (Life Technologies, Rockville, MD) at a final concentration of 2.5 mg/ml, and kept at −20°C until further use. Absence of contamination by endotoxin was confirmed by performing the Limulus amebocyte lysate test, E-toxate kit (Sigma-Aldrich).

Heme quantitation

Total heme content was determined, as described by Sullivan et al. (27), by depolymerizing heme polymer by adding 20 mM NaOH/10% SDS, incubating the suspension at room temperature for 2 h, and then reading the OD at 400 nm (Beckman DGB UV/visible spectrophotometer (Beckman Coulter, Fullerton, CA)). A total of 25 μg of sHZ equals 26 nmol of heme content.

Air pouch experiments

Air pouches were raised on the dorsum of BALB/c mice by s.c. injection of 3 ml of sterile air on days 0 and 3, as we previously described (28). On day 7, mice were injected with either 5-50 μg/ml sHZ in 1 ml of endotoxin-free PBS for 6 h or 50 μg/ml sHZ over a 24-h period. It should be noted that the amounts of injected sHZ are not solubilized, but are the suspension of insoluble material. Control group mice were treated for 6 h with either 1 ml of endotoxin-free PBS or 10 μg/ml LPS, as negative and positive controls, respectively. At specific times after intrapouch inoculation (0, 1, 6, 12, 24 h), five animals per experimental group were lethally exposed to CO2, and the pouches were washed with a total of 5 ml of endotoxin-free PBS/1 ml of EDTA. Recruited leukocytes in the pouch exudates were counted directly with a hemacytometer following acetic blue staining. Differential counts of leukocyte subpopulations were performed on cytospin preparations stained with Wright-Giemsa (Diff-Quik; Baxter Healthcare, Deerfield, IL). Then exudates from five animals per experimental group were pooled and centrifuged at 1200 rpm × 10 min at room temperature. Total RNA was isolated from recruited cells with TRIZol reagent (Life Technologies), according to the manufacturer’s protocol, for further cytokine, chemokine, and chemokine receptor mRNA analysis. Proteins in the collected supernatants were precipitated with acetone (1:4 v/v) at −20°C overnight. Following centrifugation at 3000 rpm × 10 min at 4°C, pellets were resuspended in 100 μl of PBS and were kept at −20°C for further ELISA analysis.

Intravenous experiments

BALB/c mice were injected i.v. into the tail vein with 200, 750, or 1500 μg of sHZ in 100 μl of endotoxin-free PBS (suspension of insoluble material). Control negative mice were either left untreated or injected with 100 μl of endotoxin-free PBS. As positive controls, mice were inoculated with 10 μg of LPS. After specific times (6 h for dose-response experiments; and 1, 3, 6, 12, or 24 h for kinetic analyses), mice were lethally exposed to CO2. Sections of liver tissue were ground in TRIZol to isolate total RNA for subsequent RNAse protection assays (RPA).

RNase protection assays (RPA)

mRNA expression studies were performed using a RPA kit (Riboquant; BD PharMingen, San Diego, CA), as we described previously (28). One of the various multiprobe templates was labeled with [α-32P]UTP using T7 RNA polymerase. A total of 3 × 106 cpm of labeled probe was allowed to hybridize with 10 μg of total RNA, isolated as described above, for 16 h at 56°C. mRNA probe hybrids were treated with RNase A and phenol-chloroform extracted. Protected hybrids were resolved on a 5% denaturing polyacrylamide sequencing gel and exposed to radiographic film overnight at −80°C. Laser densitometry was performed using an α Imagor 2000 digital imaging and analysis system (Alpha Innotech, San Leandro, CA). The Multiprobe templates (BD PharMingen) used in this study were as follows: mouse chemokine-5, for the murine chemokines lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, and TCA-3; murine chemokine receptor 5 (mCR5), for the murine chemokine receptors CCR1, CCR1b, CCR2, CCR3, CCR4, and CCR5; mCR6, for CXCR2, CXCR4, and BLR-1; and a custom template for murine cytokines IL-4, IL-12p40, IL-10, IL-1α, IL-1β, IL-2, IL-6, and IFN-γ. In addition, the template sets included housekeeping genes mL-32 and/or GAPDH.

Determination of chemokine and cytokine protein expression

Sandwich ELISAs were established for MIP-1α, MIP-2, MCP-1, IL-1β, and IL-6 to detect these chemokines and proinflammatory cytokines in the supernatant of pouch exudates. Briefly, 96-well plates (Immunoplate; Nunc, Naperville, IL) were coated with anti-MIP-1α, anti-MIP-2, anti-MCP-1, anti-IL-1β, or anti-IL-6 (R&D Systems, Minneapolis, MN) in 100 μl/well of PBS, pH 7.4. Plates were incubated for 16 h at 4°C and were blocked with PBS/5% BSA. Next, 50 μl of PBS/2% BSA was added to each well, followed by the addition of 50 μl/well of standards (R&D Systems) or samples diluted in PBS/2% BSA and incubation for 2 h at room temperature. The biotinylated polyclonal goat anti-chemokine/ cytokine Ab (100 μl/well) was added and incubated for 1 h in PBS/2% BSA.
increasing doses of sHZ (5–50 μg/ml), LPS (10 μg/ml), or endotoxin-free PBS (1 ml) after 6 h. PBS ( ), LPS ( ), sHZ ( ), *, p < 0.05, sHZ or LPS vs PBS. Kinetics of total leukocyte recruitment in response to sHZ (50 μg/ml) over a 24-h period (1, 3, 6, 12, 24 h). Six-hour LPS- and PBS-treated mice were used as positive and negative control groups, respectively, *, p < 0.05, sHZ vs PBS. Total cell counting was performed directly by using a hemacytometer. Results represent mean ± SEM of five mice. Data are representative of one of three independent experiments.

MRP secretion in the air pouch

For murine S100A8, S100A9, and S100A8/A9, ELISAs were performed, as we previously described (29). Briefly, Costar high binding 96-well plates (Corning Glass, Corning, NY) were coated overnight at 4°C with 100 μl/well of purified rabbit IgG against S100A8 or S100A9 (for S100A9 and S100A8/A9) diluted to 1 μg/ml in 0.1 M of carbonate buffer, pH 9.6. The wells were blocked with PBS/0.1% Tween 20/2% BSA (150 μl/well) for 30 min at room temperature. Then the samples and standards (100 μl) were added, and after a 45-min period at room temperature, the plates were incubated with rat IgG (100 μl/well) against S100A8 (for S100A8 and S100A8/A9) or S100A9 diluted in PBS/0.1% Tween 20/2% BSA (1/10,000) for 45 min. To reveal the immune complex, 100 μl/well of peroxidase-conjugated goat anti-rat IgG (H + L) (minimum cross-reaction to rabbit serum proteins) (1/10,000) was added and incubated for 45 min. Next, 100 μl/well of 3,3′,5,5′-tetramethylbenzidine substrate (Research Diagnostics, Flanders, NJ) was added, according to the manufacturer’s instructions, and the ODs were read at 500 nm. The lower limit of quantification was determined as 4 ng/ml for both S100A8 and S100A9, and 10 ng/ml for S100A8/S100A9. All ELISAs were tested using excess amounts of the other S100 proteins and were shown to be specific under the conditions reported in this work.

Statistical analysis

Statistically significant differences between groups were determined by ANOVA module of Statview + SE Software (Abacus Concepts, Berkeley, CA) and the Fisher least significant difference test. Values of p < 0.05 were deemed statistically significant. All data are presented as mean ± SEM.

Results

sHZ induces leukocyte recruitment in the air pouch

To investigate the ability of sHZ to act as a proinflammatory agent in vivo, the air pouch model was chosen. As it has been shown by others (30) and by us (28), this provides a suitable isolated environment to study the proinflammatory properties of a given stimulus. To this end, air pouches were raised on the dorsum of BALB/c mice, and 7 days later they were inoculated with increasing doses of sHZ (5–50 μg/ml), which we previously reported to be effective in vitro (25). As positive and negative control groups, mice were injected with LPS (10 μg/ml) and endotoxin-free PBS, respectively. Following a 6-h treatment, pouch exudates were collected and the total number of leukocytes accumulating in response to the various stimuli was determined. As shown in Fig. 1A, sHZ led to a significant and dose-dependent leukocyte recruitment, reaching up to a 15-fold increase over negative control mice when 50 μg/ml sHZ was inoculated. Of interest, these values were found to be very close to those obtained when LPS was administered, further indicating that the malarial pigment is a strong leukocyte recruiter. In addition, time course experiments revealed that the sHZ-inducible leukocyte accumulation in the air pouches was transient over a 24-h period, being maximal at 6 h, still significant following a 12-h stimulation, and declining thereafter (Fig. 1B). By performing differential cell counts of leukocyte subpopulations, we

![FIGURE 1](image1.png)

**FIGURE 1.** sHZ induces leukocyte recruitment in vivo. A, Number of leukocytes accumulating in the air pouch of BALB/c mice in response to increasing doses of sHZ (5–50 μg/ml), LPS (10 μg/ml), or endotoxin-free PBS (1 ml) after 6 h. PBS ( ), LPS ( ), sHZ ( ), *, p < 0.05, sHZ or LPS vs PBS. B, Kinetics of total leukocyte recruitment in response to sHZ (50 μg/ml) over a 24-h period (1, 3, 6, 12, 24 h). Six-hour LPS- and PBS-treated mice were used as positive and negative control groups, respectively, *, p < 0.05, sHZ vs PBS. Total cell counting was performed directly by using a hemacytometer. Results represent mean ± SEM of five mice. Data are representative of one of three independent experiments.

![FIGURE 2](image2.png)

**FIGURE 2.** Leukocyte subtypes accumulating in the air pouch in response to sHZ. Number of monocytes, neutrophils, and lymphocytes recruited in the pouch exudates in response to sHZ (50 μg/ml) at different time points: 1, 3, 6, 12, and 24 h. Differential cell counts were performed on Wright-Giemsa-stained cytospin preparations. Results represent mean ± SEM of five mice. *, p < 0.05, sHZ 6/12 h vs sHZ 1 h. Data are representative of one of three separate experiments.
found that most of the infiltrated cells in response to sHZ were Nφ, which corresponded to 88 and 73% of the total cell numbers present at 6 and 12 h, respectively. However, following a 12-h treatment, sHZ also induced a significant rise in the number of monocytes, which accounted for 21% of the total recruited leukocytes (Fig. 2).

**sHZ increases cytokine mRNA and protein expression in vivo**

To establish whether sHZ-mediated leukocyte accumulation correlated with proinflammatory cytokine up-regulation, BALB/c mice were inoculated with 50 μg/ml sHZ over a 24-h period, and pouch exudates from each experimental group were pooled. Then total RNA from the recruited leukocytes was extracted, and cytokine mRNA levels were monitored using a cytokine multiprobe RPA system. As shown in Fig. 3A, sHZ led to a rapid and transient accumulation of various cytokine transcripts. Whereas IL-1α mRNA up-regulation was maximal after 6 h (14-fold increase over PBS control mice), both IL-1β and IL-6 gene expression peaked already at 3 h posttreatment, reaching a 9- and 20-fold increase over negative control, respectively. In contrast, sHZ was unable to modulate IL-4, IL-10, IL-2, IL-12, and IFN-γ mRNA levels (data not shown). These data indicate that a Th2 response (IL-4, IL-10) is not favored by the malarial pigment and that sHZ-dependent proinflammatory cytokine expression is not an unselective event. In addition, ELISAs confirmed that the noticed sHZ-inducible cytokine transcription was accompanied by protein expression. As depicted in Fig. 3B, supernatants from pooled exudates of 3- and 6-h-treated mice contained elevated levels of IL-1β and IL-6; however, cytokine secretion was maximal only after 12 h of sHZ inoculation.

**sHZ-mediated chemokine up-regulation in the air pouch**

Chemokines are small peptides leading to activation and recruitment of selected leukocyte populations to the sites of infection. These proteins are known to play important roles in protozoan parasite infections by affecting interactions between parasites and their host cells, as well as by influencing the immune system, thereby further inducing inflammatory diseases (31). Therefore, we sought to examine the induction patterns of chemokine transcripts in leukocytes from pooled exudates of sHZ-stimulated mice (1–12 h). In correlation with the leukocyte populations migrating into the pouches, RPA analyses revealed a significant and time-dependent mRNA up-regulation of Nφ (MIP-2 (32)) and monocyte (MIP-1α, MIP-1β, and MCP-1 (31)) chemoattractants and activators (Fig. 4A). Maximal induction for MIP-1α, MIP-1β, and MIP-2 was observed at 3 h poststimulation, reaching a 16-, 32-, and 188-fold increase over negative control mice, respectively. Even though a slight rise in MCP-1 mRNA levels was detected at 3 h, maximal messenger accumulation (26-fold over PBS control) was found only after 6 h of sHZ inoculation. Even though multiple chemokine induction was observed in sHZ-injected mice, such event was found to be a specific one. In fact, the malarial pigment did not exert any effect on various of the chemokine transcripts evaluated (lymphotactin, RANTES, eotaxin, IP-10, and TCA-3) (data not shown). In line with our data obtained by RPA, maximal MIP-1α, MIP-2, and MCP-1 secretion was detected in supernatants of pooled exudates from 3- and 6-h-treated mice, following a similar kinetics to that of chemokine mRNA induction (Fig. 4B).

**FIGURE 3.** sHZ increases cytokine mRNA and protein expression in vivo. A, Kinetic analyses of sHZ-inducible IL-1α, IL-1β, and IL-6 mRNA expression in vivo. BALB/c mice were injected in the air pouches with 50 μg/ml sHZ. At various times (1, 3, 6, 12, and 24 h) following inoculation, exudates from each experimental group were pooled and total RNA was extracted from the leukocytes recruited. Cytokine mRNA levels were monitored using a cytokine multiprobe RPA system (left panel). Densitometric quantification of cytokine mRNA expression over negative control after normalization to GAPDH (right panel). B, IL-1β and IL-6 secretion in the pouches of BALB/c mice following sHZ stimulation (50 μg/ml) over a 24-h period. After sHZ inoculation, exudates from each group of mice were pooled and the resulting supernatants were subjected to ELISA. Negative control mice were treated with PBS for 6 h. PBS (○), sHZ (■). Data represent cytokine mRNA and protein induction in pooled samples (five mice) from each experimental group. Results are representative of one of two independent experiments.
FIGURE 4. sHZ-mediated chemokine up-regulation in the air pouch. A. Time course of chemokine mRNA expression in the air pouch was monitored following sHZ (50 μg/ml) inoculation over a 12-h period. Exudates from each experimental group were pooled, and total RNA was extracted from the leukocytes recruited. Samples were submitted to RPA using the mouse chemokine-5 multiprobe template (left panel). Integrated density values of chemokine mRNA levels normalized to GAPDH (right panel). B. Kinetic analyses of MIP1α, MIP-2, and MCP-1 protein expression in the air pouches of BALB/c mice in response to sHZ (50 μg/ml). Supernatants of pooled exudates from 1- to 24-h-treated mice were subjected to ELISA. As a negative control group, mice were inoculated with PBS for 6 h. PBS (□), sHZ (■). Data represent chemokine mRNA and protein expression in pooled samples (five mice) from each experimental group. Results are representative of one of two separate experiments.

FIGURE 5. sHZ increases chemokine receptor mRNA levels in leukocytes recruited in the air pouch. To monitor gene expression of CCR (A) and CXCR (B), mice were injected with 50 μg/ml sHZ for 1–24 h or 12 h, respectively. Exudates from each experimental group were pooled and total RNA was extracted from the leukocytes recruited. mCR5 and mCR6 multiprobe templates were used to evaluate the induction patterns of the various receptor transcripts by RPA (left panels). Densitometric quantification of CCR and CXCR mRNA expression over negative control after normalization to GAPDH (right panels). Negative control mice were treated with PBS for 6 h. PBS (□), sHZ (■). Data represent CCR and CXCR mRNA levels in pooled samples (five mice) from each experimental group. Results are representative of one of two independent experiments.
sHZ increases chemokine receptor mRNA levels in leukocytes recruited in the air pouch

Chemokines exert their effects by binding to a family of specific seven-transmembrane G protein-coupled receptors (33). Because both CC (MIP-1α, MIP-1β, and MCP-1) and CXC (MIP-2) chemokine production was detected in response to sHZ, we next investigated whether CCR and CXCR gene expression was up-regulated in leukocytes from pooled exudates of sHZ-injected mice. In line with our data regarding chemokine mRNA induction, sHZ was found to selectively modulate CCR and CXCR expression. Although no changes were observed in CCR1b, CCR3, and CCR4 transcripts (data not shown), a rapid and significant increase of CCR1 mRNA levels occurred upon sHZ inoculation (Fig. 5A), reaching maximal messenger accumulation already after 1 h (6-fold increase over negative control mice). To a lesser extent, CCR2 and CCR5 mRNA up-regulation was also detected at 3- to 6-h poststimulation (2-fold increase over PBS control mice). Similarly, CXCR mRNA expression was also enhanced by sHZ. As depicted in Fig. 5B, CXCR2 and CXCR4 transcripts, but not BRL-1 (data not shown), were transiently increased, with the highest mRNA accumulation (3-fold increase) occurring upon a 3-h treatment and a gradual decrease thereafter.

MRP secretion is induced by sHZ in vivo

S100A8 and S100A9 are calcium-binding proteins that belong to a subset of the S100 family known as MRPs because their expression is almost completely restricted to cells of myeloid origin (34). S100A8 and S100A9 associate noncovalently to form homodimers and the heterodimer S100A8/A9 (35). S100A8, S100A9, and S100A8/A9 have been shown by others and by us to induce chemotaxis for Nd as well as Nd adhesion to fibrinogen (36–38), and to play an essential role in Nd migration in response to LPS (29). Importantly, MRP secretion has been documented in various infectious and inflammatory conditions, including malaria (8, 9). Based on this previous evidence and because our first set of experiments indicated that most of the leukocytes recruited in response to sHZ corresponded to a Nd population, we set out to elucidate whether this proinflammatory event was, at least in part, due to the production of MRPs. We found that sHZ induced secretion of three different murine MRPs, S100A8, S100A9, and S100A8/A9, in pooled air pouch exudates of BALB/c mice (Fig. 6). Of interest, and in contrast to our observations regarding another powerful Nd chemotactant, MIP-2, MRP production was sustained up to 12 h following sHZ inoculation, further suggesting an important role for these proteins in the noticed sHZ-inducible Nd accumulation.

sHZ induces liver chemokine and cytokine mRNA expression

After having demonstrated that sHZ led to the production of proinflammatory mediators in vivo, we next sought to evaluate the potential role of sHZ as an immunomodulator during malaria infection. To mimic HZ release into the bloodstream at the end of the erythrocytic life cycle of the parasite, increasing doses of sHZ (200–1500 μg) were injected i.v. into the tail vein of BALB/c mice. sHZ effects were monitored in the liver because its accumulation in this organ has been associated with infection chronicity in humans (39), and with cumulative parasite burden and duration of infection in experimental malaria (40). The various concentrations were chosen based on previous studies performed by Sherry et al. (22), who, by reference to standard hematological measurements, estimated that as much as 200 μmol of HZ (~3 μmol/Kg) is released into the circulation of a 70 kg P. falciparum-infected human patient who has a 1% synchronized parasitemia. According to our
measurements, 25 μg of sHZ is equal to 26 nmol of heme content; therefore, the doses used in this set of experiments, 200, 750, and 1500 μg (≈8, ≈30, and ≈60 μmol heme/Kg, respectively), are equivalent to the amounts of HZ, which would be found ranging from mild to severe malaria: ≈3, ≈10, and ≈20% parasitemia. As shown in Fig. 7A, a significant increase of various liver chemokine transcripts was already detectable when 200 μg of sHZ was injected, whereas maximal messenger accumulation was observed upon stimulation with intermediate and high doses of sHZ (750–1500 μg): a 28-fold increase over PBS control was observed for MIP-1α, a 5-fold for MIP-1β, a 26-fold for MIP-2, and a 70-fold for MCP-1. In addition, kinetic analyses performed by injecting the lowest dose of sHZ (200 μg) over a 24-h period revealed that the sHZ-inducible liver chemokine mRNA up-regulation occurred at early times of injection, already detectable after 1 h and maximal at 6 h (data not shown). Of interest, significant mRNA levels of MIP-1α, MIP-1β, and MIP-2 were still found at 24 h posttreatment. In parallel, our data indicating proinflammatory sHZ-mediated chemokine induction in the air pouch prompted us to investigate the ability of sHZ to modulate liver cytokine expression. As depicted in Fig. 7B, 200–1500 μg of i.v. injected sHZ resulted in substantial mRNA accumulation of two strong immunomodulators, IL-1β (41) and IL-12 (42), reaching a 2- and 22-fold increase over negative control mice, respectively. Altogether, this last set of experiments indicates that at doses that could be achievable during malaria infection (≈3% parasitemia), HZ is already able to elicit a proinflammatory response in the host, and suggests that at higher parasitemias HZ is likely to contribute to an exacerbated inflammatory immune response.

**Discussion**

The pathogenic manifestations during a malaria crisis occur after schizogony and have been attributed to proinflammatory cytokines released in response to malaria parasites and their products (7). Several in vitro studies have reported cytokine and chemokine production in response to HZ (21–24), and our data indicated that both *P. falciparum* HZ and sHZ induce mRNA expression of the same chemokines in Mφ (M. Jaramillo and M. Olivier, manuscript in preparation); however, the in vivo proinflammatory properties of the malarial pigment remained unexplored. In the present study, we demonstrate that sHZ leads to leukocyte recruitment in vivo and induces the release of various chemokines, chemokine receptors, proinflammatory cytokines, and MRPs, supporting the idea that this parasite metabolite is likely to play a key role in malaria immunopathology.

Analysis of the inflammatory cells accumulating in response to sHZ revealed a high increase of Nδφ early after stimulation accompanied by a significant rise in monocyte numbers at later times. These findings are in agreement with previous studies reporting a transient increase in the activity and number of circulating phagocytes in response to schizogony (3), and suggest that HZ could be, at least in part, responsible for such augmentation following parasite release into the bloodstream. Even though autopsy studies of *P. falciparum*-infected patients have yielded evidence of parasite clearance by activated Mφ and Nδφ (43, 44), neutrophilia and monocytosis occurring in malaria have also been associated with poor prognosis of the disease (45). Therefore, by inducing leukocyte recruitment into the sites of sequestration of infected RBC,
sHZ could contribute to reduce parasite burden, but could also favor an exacerbated phagocyte response, resulting in tissue damage and microvascular flow disturbance.

The presence of increased leukocyte numbers in response to sHZ was paralleled by its ability to induce the expression of various proinflammatory mediators with chemoattractant properties. Consistent with elevated Nφ numbers, increased levels of MIP-2, a powerful Nφ chemoattractant and activator (32), were detected in the exudates. Of interest, a significant and dose-dependent up-regulation of MIP-2 transcripts was also observed in liver of i.v. injected mice. Even though the proinflammatory role of MIP-2 has not been explored in murine models of malaria, increased hepatic Nφ accumulation has been reported in mice following systemic administration of high doses of MIP-2 (46), and immunoneutralization of MIP-2 was shown to decrease Nφ influx into the liver and reduce hepatic injury (47). In light of these findings, it is conceivable that by increasing MIP-2 expression, HZ could contribute to favor peripheral Nφ recruitment and subsequent liver injury. In humans, serum levels of IL-8, a potent Nφ chemoattractant and activator (48), increase during severe malaria (49). Interestingly, IL-8 expression was reported only in HZ-laden Mφ in placental malaria (50). Even though no murine homologue has been described for IL-8, this human chemokine most closely resembles murine MIP-2 (51); therefore, HZ-mediated MIP-2 and IL-8 up-regulation may have some common biological effects during malaria.

In addition to MIP-2, sHZ-inducible MRP S100A8, S100A9, and S100A8/A9 synthesis is also likely to account for the noticed Nφ infiltration in the air pouches. Murine S100A8 (36) as well as human S100A8, S100A9, and S100A8/A9 cause Nφ chemotaxis in vivo and induce Nφ adhesion to fibrinogen in vitro (37, 38). Moreover, we recently demonstrated that S100A8 and S100A9 play important roles in Nφ accumulation to extravascular sites (52). In P. falciparum-infected children, serum levels of S100A8/A9 were shown to be significantly related to parasitemia and fever (9) and, during CM, analysis of cellular activation in the brain revealed S100A8 and S100A9 expression in microglial cells (8). In line with these findings, Chen et al. (53) found that Nφ play a critical role in the development of experimental CM. Based on these data and knowing that HZ accumulation also occurs in the brain (3), it is possible that HZ-dependent MRP secretion and subsequent Nφ infiltration contribute to both acute malaria and CM.

In concert with our results showing monocyte infiltration in the air pouches, sHZ induced the expression of MIP-1α, MIP-1β, and MCP-1, three CC chemokines that are potent monocyte chemotactants and activators (31), and the same pattern of chemokine induction was detected in the liver of i.v. sHZ-injected mice. This is in agreement with data from experimental malaria showing increased mRNA levels of MIP-1α and MIP-1β in liver of infected mice (22), as well as MCP-1 mRNA up-regulation in brain and liver of mice suffering of CM (14). In humans, elevated serum concentrations of MIP-1α were detected during acute P. falciparum malaria (10), and more recently, Abrams et al. (11) found a correlation between placental monocyte recruitment and elevated MIP-1α and MCP-1 expression in P. falciparum-infected pregnant women. Of interest, in this study, production of MIP-1β and MCP-1 was observed in some of the HZ-laden maternal Mφ. Because of their biological activities, through the induction of CC chemokines, HZ may be involved in various aspects of malaria immunopathology. On one hand, HZ would contribute to increase monocyte numbers in the sites of parasitisation and, depending on the specific location, exert different effects. In the liver, HZ-mediated CC chemokine expression is likely to be involved in hepatic damage observed both in human (54) and murine (55) malaria. Even though liver chemokine induction has only been reported in murine malaria (14, 22), MIP-1α, MIP-1β, and MCP-1 have been detected during human hepatic disease, and their expression has been associated with monocyte infiltration and hepatic injury (reviewed by Simpson et al. (51)). During CM, HZ-dependent CC chemokine modulation could participate in the observed monocyte infiltration (56) and adhesion (57) in cerebral microvessels, thereby favoring microvascular damage (58). In the case of placental malaria, by increasing CC chemokine levels, HZ might be implicated in poor birth outcomes because monocyte infiltration at this location constitutes a key risk factor for low birth weight (59). Furthermore, given that malaria and HIV-1 coinfections are common in pregnant women in sub-Saharan Africa (60), HZ has the potential to facilitate HIV-1 replication and vertical transmission by creating a reservoir for Mφ-tropic HIV-1. Moreover, by increasing both MIP-1α and MIP-1β, which are potent pyrogenic molecules (61), HZ may play a role in fever episodes occurring during acute malaria. This hypothesis is indeed supported by a previous in vivo study demonstrating the ability of HZ to regulate body temperature in rats (22). In addition to its capacity to evoke fever, MIP-1α also exerts a marked suppressive effect on hemo poetic stem cell proliferation (62); therefore, it can be envisaged that HZ-mediated MIP-1α expression might contribute to the anemia characteristic of malaria infection. Further studies are needed to demonstrate the physiological contribution of HZ to fever episodes and susceptibility to anemia, as well as to the metabolic and morphological alterations in liver, brain, and placenta related to malaria.

Chemokine actions depend on the expression of chemokine receptors that bind and respond to such ligands, bringing about cell activation via intracellular signaling mechanisms (33). Analysis of chemokine receptor up-regulation in cells from air pouch exudates revealed that sHZ increased mRNA levels of CCR1, CCR2, CCR5, CXCR2, and CXCR4, whose induction patterns correlated with the leukocyte populations recruited. Whereas CCR2 and CCR5 are expressed by monocytes, CCR1, CXCR2, and CXCR4 are known to be present in both monocytes and Nφ (63). In addition, a rise in these receptor transcripts in the recruited cells could explain, at least in part, their infiltration in the pouches via their response to sHZ-inducible chemokine secretion. In fact, MCP-1 is a known ligand for CCR2, MIP-2 for CXCR2, and MIP-1α for CCR1 and CCR5, whereas MIP-1β binds exclusively CCR5 (63). Current data indicate the involvement of chemokine receptors in the pathogenesis of a number of relevant acute and chronic inflammatory diseases (33). In CM, an important role for CCR5 has been strongly suggested by Belnoue et al. (64, 65), who recently showed that CCR5 and not CCR2 expression, on both brain-sequestered leukocytes and nonhemopoietic cells, is required for experimental CM development. Such effect has been attributed to increased brain sequestration of cytotoxic CD8+ T cells, a leukocyte subset found to be critical for CM progression (66, 67). Moreover, Tkachuk et al. (68) found that P. falciparum infection enhances CCR5 mRNA expression on placental Mφ of pregnant women. CCR5 is an important coreceptor required for efficient HIV-1 cell entry (69) and has been identified as the main secondary receptor implicated in HIV-1 vertical transmission (70). In addition to CCR5, it was ascertained that CCR2 (71) and CXCR4 (72) could also serve as coreceptors to permit HIV-1 entry. Therefore, the potential of HZ favoring CM through CCR5 induction in the brain, as well as HIV-1 infection and intrauterine transmission during malaria and HIV-1 coinfections via CCR2, CCR5, and CXCR4 up-regulation, deserves further investigation.

In addition to chemokine modulation, proinflammatory cytokine expression in response to sHZ was also observed. In the air
pouches, IL-1α, IL-1β, and IL-6 levels were increased and, in the liver, a significant rise in IL-1β transcripts occurred in a dose-dependent manner. IL-1 and IL-6 share multiple functions, including Nrf2 and p65 activation (73, 74) and the induction of hepatocyte acute-phase responses such as fever and leukocytosis (75, 76). Importantly, IL-1 and IL-6 can stimulate chemokine secretion (30, 77); thus, their release may offer a means of locally amplifying the immunological response. Consistent with these findings, extensive data have documented high IL-1 and IL-6 levels in both human (3–5) and murine malaria (12, 13), which have been implicated in both antiparasitic activity and disease severity. Similarly, in vitro studies have revealed IL-1 and IL-6 induction in response to P. falciparum (15) and to HZ (21, 23, 24). Based on this evidence, our data regarding IL-1 and IL-6 in vivo up-regulation further support a role for HZ in malaria immunopathology related to proinflammatory cytokine overproduction.

Finally, a significant increase of IL-12 mRNA levels was found in liver of HZ i.v. injected mice. IL-12 is a potent immunomodulatory cytokine (42) that seems to play a dual role in malaria. IL-12 has been shown to induce protective immunity against blood stage in the murine model (78); however, it has been proposed that raised concentrations of IL-12 could also be associated with bone marrow dyserythropoiesis (78) and with the acute symptoms of malaria via IFN-γ-mediated IL-1 induction (79). Therefore, if a direct effect of HZ on IL-12 regulation can be established, controlling this pathway of cellular modulation may allow amelioration of morbidity and mortality during malaria infection.

Although the cellular source of HZ-inducible chemokine, cytokine, and MRP expression remains to be determined in vivo, in vitro studies have demonstrated the ability of HZ to increase chemokine and cytokine (21, 22, 24) expression in Mφ, monocytes, and PBMC. Consistent with this, a large proportion of resident Mφ and circulating monocytes of malaria-infected patients has been found to contain granules of malarial pigment, and the presence of HZ in these cells has been associated with cellular activation and disease severity (19). In addition, evidence from autopsy and in vivo histological studies suggests that in P. falciparum malaria, the main sources of cytokines are likely to be activated tissue Mφ in liver (Kupffer cells), spleen, lungs (alveolar Mφ), and brain (microglial cells), as well as PBMC (3). Because erythrocytic schizogony and subsequent HZ release occur in the microvasculature where parasitized erythrocytes are sequestered, and both HZ and leukocyte accumulation have been reported in these organs, it is possible that the HZ-inducible immunological response would be directed against the sites of maximal parasitization, and the intensity and nature of this response may therefore contribute to the pathophysiology of malaria. Given the differences between human and experimental malaria, especially regarding the pathology of CM, careful attention should be paid to the analysis of the potential experimental malaria, especially regarding the pathology of CM, physiology of malaria. Given the differences between human and nature of this response may therefore contribute to the pathophysiology of malaria. Given the differences between human and nature of this response may therefore contribute to the pathophysiology of malaria.


