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*J Immunol* 2004; 173:4066-4074;doi: 10.4049/jimmunol.173.6.4066
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Hemozoin (Malarial Pigment) Inhibits Differentiation and Maturation of Human Monocyte-Derived Dendritic Cells: A Peroxisome Proliferator-Activated Receptor-γ-Mediated Effect

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Acute and chronic Plasmodium falciparum malaria are accompanied by severe immunodepression possibly related to subversion of dendritic cells (DC) functionality. Phagocytosed hemozoin (malarial pigment) was shown to inhibit monocyte functions related to immunity. Hemozoin-loaded monocytes, frequently found in circulation and adherent to endothelia in malaria, may interfere with DC development and play a role in immunodepression. Hemozoin-loaded and unloaded human monocytes were differentiated in vitro to immature DC (iDC) by treatment with GM-CSF and IL-4, and to mature DC (mDC) by LPS challenge. In a second setting, hemozoin was fed to iDC further cultured to give mDC. In both settings, cells ingested large amounts of hemozoin undegraded during DC maturation. Hemozoin-fed monocytes did not apoptose but their differentiation and maturation to DC was severely impaired as shown by blunted expression of MHC class II and costimulatory molecules CD83, CD80, CD54, CD40, CD1a, and lower levels of CD83-specific mRNA in hemozoin-loaded iDC and mDC compared with unfed or latex-loaded DC. Further studies indicated activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) in hemozoin-loaded iDC and mDC, associated with increased expression of PPAR-γ mRNA, without apparent involvement of NF-κB. Moreover, expression of PPAR-γ was induced and up-regulation of CD83 was inhibited by supplementing iDC and mDC with plausible concentrations of 15(S)-hydroxyeicosatetraenoic acid, a PPAR-γ ligand abundantly produced by hemozoin via heme-catalyzed liperoxidation. The Journal of Immunology, 2004, 173: 4066–4074.

everal studies indicate impairment of immune responses in acute and chronic Plasmodium falciparum malaria. The induction of immunity to a range of vaccines was reduced in acute malaria (1–3). T cells from adults living in endemic areas responded less efficiently to parasite proteins (see Ref. 4 for review). Ab response was short lived and rapidly declining after clearance of parasites, despite large amounts of circulating Ags during the acute phase of the disease. Finally, endemic malaria was associated with a higher incidence of infectious diseases and Bur-ki’s lymphoma (3–6).

Monocytes are a prime source of dendritic cells (DC) in vivo and in vitro (see Ref. 7 for review) that play pivotal roles in adaptive immune responses and innate immunity. DC determine the magnitude and quality of immune response by presenting Ags very efficiently, activating lymphocytes including memory and naive T, B and NK cells, and producing a number of cytokines (see Refs. 8–10 for reviews). In malaria, large numbers of hemozoin-loaded monocytes are observed circulating in peripheral blood or adherent to postvenular endothelia in several organs (11, 12). Hemozoin loading was found to upset several monocyte functions connected with modulation of immunity. For example, hemozoin-loaded monocytes did not up-regulate MHC class II and CD54 (ICAM-1) after IFN-γ stimulation (13), produced increased amounts of TNF-α and IL-1 (14, 15), and showed defective Ag presentation (16). Because of their large numbers and the ready chance to encounter the parasites in peripheral blood, hemozoin-loaded, circulating monocytes are likely to be a source of DC in malaria.

Here we show that isolated hemozoin and trophozoites, similar to the phagocytic meals in vivo, were intensely phagocytosed in vitro either by human monocytes before the initial induction of their differentiation to immature DC (iDC) or by iDC before their final maturation to mature DC (mDC). Expression of MHC class II and costimulatory molecules (surface markers) was studied by cytometry with special attention to CD83. Due to its peculiar functional importance (17, 18), CD83 expression was also analyzed by quantitative real time RT-PCR. Blunted expression of MHC class II and costimulatory molecules indicated that both differentiation and maturation of hemozoin-loaded monocytes to DC were severely impaired. Further studies indicated hemozoin-mediated induction of the peroxisome proliferator-activated receptor-γ (PPAR-γ) (19, 20), while NF-κB, a transcription factor activated during DC maturation (21, 22), was apparently not impaired by hemozoin. Finally, induction of PPAR-γ expression was recapitulated by supplementing differentiating and maturing DC with 15(S)-hydroxyeicosatetraenoic acid (15(S)HETE), a PPAR-γ ligand (23) and effector of the PPAR-γ signaling cascade.

Received for publication January 21, 2004. Accepted for publication June 24, 2004.
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1 This work was supported by Grants Italian Ministry of University (MIUR)-COFIN Projects 2002 and 2003, and Compagnia di San Paolo-Instituto Molembial Italiano (to P.A.); and by Grants Associazione Italiana Ricerca sul Cancro No. 58/2003, Ministero della Sanità RF 2002 No. 183, MIUR-FIRB Project No. RBNE01LNX7-006, and Fondazione Casse Risparmio Provincie Lombardie (to M.A.). The work of M.A. was carried out in the framework of the Italian MIUR Center of Excellence in Physiopathology of Cell Differentiation.

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3 Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; mDC, mature DC; PPAR-γ, peroxisome proliferator-activated receptor-γ; 15(S)HETE, 15(S)-hydroxyeicosatetraenoic acid; OTGP, octylthioglucopyranosid; HODE, hydroxyoctadecadienoic acid; 4-HNE, 4-hydroxynonenal.

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0022-1767/04/$02.00
abundantly produced by hemozoin via nonenzymatic heme catalysis (24).

Materials and Methods

Materials

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture supplements were obtained from Invitrogen Life Technologies (Carlsbad, CA).

Culturing of P. falciparum and isolation of hemozoin

P. falciparum parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described (25). If not stated otherwise, hemoglobin and RBC parasitized with different maturation stages were isolated from cultures during the first 2 days after infection of RBC added to schizonts (multinucleated parasite form). After centrifugation at 5000 × g on a discontinuous Percoll gradient, hemoglobin was collected from the 0–40% interphase and trophozoites/schizonts from the 40–80% interphase. Hemoglobin was washed five times with 10 mM HEPES (pH 8.0) containing 10 mM mannitol at 4°C and once with PBS, and stored at 20% (v/v) in PBS at −20°C. Trophozoites enriched to 95–99% parasitemia were washed twice and resuspended in RPMI 1640. Nonparasitized RBC were treated as parasitized RBC.

Opsonization of hemozoin, trophozoites, nonparasitized RBC, and latex beads

Hemoglobin washed once and finely dispersed at 30% (v/v) in PBS, latex beads (0.114-μm diameter) suspended at 5% (v/v) in RPMI 1640, and trophozoites suspended at 50% hematocrit in RPMI 1640 were added to the same volume of fresh human AB serum and incubated for 30 min at 37°C. Freshly drawn, PBS-washed, nonparasitized RBC suspended at 50% hematocrit in RPMI-glucose were added to half of the volume of human anti-D (Partobulin; Immuno, Vienna, Austria), incubated for 30 min at 37°C, and resuspended in RPMI 1640. Nonparasitized RBC were treated as parasitized RBC.

Modifications of hemozoin

Deltidized hemozoin. Hemoglobin was deltidized as indicated (26). Hemoglobin was collected from the interphase, and the extraction was repeated three times to remove lipids completely, whereby the monosaccharide step was extended to 2 h at room temperature. Deltidized hemoglobin was collected from the interphase, washed five times with excess PBS after evaporation under nitrogen of residual methanol/chloroform, and passed through a needle to obtain a fine dispersion.

GPI-deprived hemozoin. Potentially attached GPI was removed from hemoglobin by treatment with GPI-specific phospholipase D from fresh human serum as described (27). This treatment was shown to remove >90% of membrane-bound GPI (27). Hemoglobin was resuspended 1.1 (v/v) in 25 mM Tris-HCl buffer, pH 7.4, containing 40 mM N-octylglucopyranosid (OTGP) and 5 mM CaCl₂. The suspension was incubated under addition of fresh human AB serum (30%, v/v) for 12 h at 37°C. Thereafter, hemoglobin was washed five times in PBS-glucose at room temperature to remove OTGP. Control phagocytic meal was hemoglobin from the same batch, incubated either in RPMI 1640 or in OTGP-buffer without serum and washed as described before.

Isolation of monocytes and phagocytosis of opsonized hemozoin, trophozoites, nonparasitized RBC, and latex beads

Monocytes were isolated from peripheral blood of healthy human donors by Ficoll centrifugation and lymphocyte-depletion with PanT/PanB-Dynal beads (Dynal, Oslo, Norway) as described (24). Two milliliters of monocytes, resuspended at 10⁵ cells per milliliter of RPMI 1640 supplemented with l-glutamine, sodium pyruvate, penicillin/streptomycin, nonessential amino acids (RS), and 1% (v/v) FCS (Invitrogen Life Technologies), (RS-FCS) were plated in 35-mm diameter culture dishes. After a 30-min incubation at 37°C, dishes were washed three times with RPMI 1640 to remove nonadherent cells. Two milliliters of RS supplemented with 5% (v/v) human AB serum (EuroClone, Wetherby, U.K.) (RS-HS) was added to each dish (1 ml of the suspension by monocytes opsonized hemozoin or modified hemozoin (50 RBC equivalents, in terms of heme content, per monocyte), opsonized trophozoites or nonparasitized RBC (50 cells per monocyte), latex beads (10 μl of a 100-fold dilution of the opsonized latex beads suspension per 10⁶ monocytes). After a 3-h incubation, phagocytosis was stopped by three washings with RPMI 1640. The amount of hemoglobin phagocytosed by monocytes was quantitated by luminescence as described previously (28).

Dendritic cells

After phagocytosis of the different meals, monocytes were incubated at 37°C in 1 ml of RS containing 80 ng/ml GM-CSF (human recombinant; Novartis Pharma, Basel, Switzerland) and 50 ng/ml IL-4 (human recombinant; R&D Systems, Minneapolis, MN) or from hydroma clone 31-4 producing human IL-4, kindly provided by G. Casorati, San Raffaele Scientific Institute, Milan, Italy). Half of the culture medium was changed on days 2, 4, 6, and 8. At day 7, cells were considered to be iDC. Two identical dishes were prepared for each condition. Cells were cultured at 10⁶ for each dish of the twin dishes were removed for analysis, the second identical dish was pulsed for maturation by adding LPS (1 μg/ml final concentration; LPS from Escherichia coli, serotype 055:B5) for 2 days, when cells were considered to be mDC (29).

Confocal laser scanning microscopy

mDC adherent to glass coverslips were washed twice with PBS, fixed with 3.7% (v/v) paraformaldehyde, and washed three times with PBS. Thereafter, cells were preincubated in PBS supplemented with 1% (v/v) human AB serum (EuroClone) for 15 min at room temperature and anti-CD83 FITC-conjugated Ab (final dilution, 1/100; BD Pharmingen, San Diego, CA) was added for 30 min at 4°C in the dark. Incubation was terminated by 4 washes with PBS for 5 min. Mowi solution, containing 15% (v/v) Mowiol 40–88, 33% (v/v) glycerol, 2% (v/v) diazobicyclo(2.2.2)-octan, 130 mM Tris-HCl, pH 8.5, was used to mount slides. Confocal laser scanning microscopy was performed with a Zeiss LSM-510 immunofluorescent laser scanning microscope (Zeiss AG, Oberkochen, Germany) using an argon laser 488 nm/514 nm (for green FITC fluorescence) and a helium/neon laser 633 nm/670 nm (for red hemoglobin fluorescence). For cell reconstruction, optical sections were taken at increments of 0.15 μm.

Immunofluorescence labeling, flow cytometry analysis, and cell viability

Labeling and subsequent phenotypic analysis of DC was performed using flow cytometry direct immunofluorescence as described (13). Saturating concentrations of mAbs recognizing the following Ags were used: CD83, CD80, CD1a (BD Pharmingen); CD14 (3C10; American Type Culture Collection, Manassas, VA); CD54 (Serotec, Oxford, U.K.); CD40 (626.1); and MHC class I (W6.32; ATTC); MHC class II (BT-2.9 hybridoma; Ref. 30). Bound Abs were revealed by FITC-conjugated F(ab′)2 goat anti-mouse Ig. Mean fluorescence intensity (MFI) was measured and data analyzed using a FACScan cytofluorograph (BD Biosciences, Sunnyvale, CA) and CellQuest software. Subpopulation quantification of DC was performed, analyzing dot-plot distribution of CD14 negative vs positive, CD83 high positive (high-CD83 expressors) vs negative, and CD1a high positive (high-CD1a expressors) vs negative cells. Cell apoptosis was measured by Annexin V-fluorescent conjugate binding evaluated by cytofluorimetry following the manufacturer’s specifications. Cell viability was evaluated by [³⁵S]methionine incorporation. Cells were washed with l-methionine/cysteine/cysteine-free RPMI 1640 medium supplemented with Tran³⁵S-label (50 μCi/ml; ICN Pharmaceuticals, Costa Mesa, CA) and incubated for 1.5 h at 37°C. Afterward, cells were washed three times with PBS, and proteins were precipitated by adding trichloroacetic acid (20% w/v) for 10 min at 4°C. The washed, precipitated proteins were analyzed for incorporated [³⁵S]methionine by liquid scintillation counting.

RT-PCR and real-time RT-PCR studies

CD83 mRNA expression was analyzed by RT-PCR and real-time RT-PCR. Total cellular RNA was quantitatively isolated from 2 × 10⁶ iDC and mDC using Standard RNA Releaser, a RNA-specific resin and elution solution from a RNA extraction kit (Nurex, Sassari, Italy) following the manufacturer’s specifications. Subsequently, the RNA was reverse-transcribed using M-MLV (7.7 U/μl final concentration; Invitrogen Life Technologies) and oligo(dT) (11.5 μg/μl final concentration; Invitrogen Life Technologies). PCR was performed according to Ref. 31 using cDNA from 50,000 cells with primer pairs specific for CD83. Real-time RT-PCR was performed with iCycler iQ real-time RT-PCR Detection System (Bio-Rad, Hercules, CA) equipment. CD83 intron overlapping primers (GenBank, National Institutes of Health accession number NM_004233) were designed using Beacon designer software (Premier Biosoftware International, Palo Alto, CA): 5′-CAGA GAAACCTAAGTGGCAAG-3′ and 5′-AAATACAAGGAGCCAC AGC-3′. PPAR-γ mRNA expression was analyzed by RT-PCR and real-time RT-PCR. PPAR-γ intron overlapping primers were designed according to Ref. 32. GAPDH intron overlapping primers were from the
Bio-Rad library. For each 50 μl of real-time RT-PCR mix, 2.0 μl of cDNA (corresponding to 50,000 cells), 1.0 μl sense and antisense primer (both 10 μM; Invitrogen Life Technologies), 1.0 μl of dNTP (10 mM; Applied Biosystems, Foster City, CA), 3.0 μl of MgCl2 (50 mM), 2.5 U Platinum Taq DNA Polymerase (Invitrogen Life Technologies), 0.1 μl of buffer (10×), 3.25 μl of SYBR Green (final dilution, 1/10,000), and 33.25 μl of PCR-grade water were mixed. After denaturation for 2 min at 94°C, PCR assays were conducted for 50 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The specified mode of reaction was controlled with the melting curve.

**PPAR-γ studies**

PPAR-γ mRNA expression was analyzed by real-time RT-PCR as indicated in the preceding section. The effect of PPAR-γ ligand 15(S)HETE, 4-hydroxynonenal (4-HNE) (both Cayman, Ann Arbor, MI), and PPAR-γ activator Ciglitizone (33) was studied on the surface expression of CD83. If not otherwise indicated, 15(S)HETE, 4-HNE, and Ciglitizone were added to the cells on days 0, 2, 4, and 6, at 10, 1, and 20 μM final concentrations, respectively. 15(S)HETE was dispersed in 150 mM HEPES, pH 8.2, by sonication shortly before addition to the cells. The PPAR-γ inhibitor, GW 9662 (33), was added to the cells on days 0, 2, 4, and 6.

**NF-κB studies**

iDC were analyzed for their NF-κB response at 1 and 48 h after stimulation by LPS (1 μg/ml final concentration). Cells were lysed for 30 min on ice in high salt buffer (350 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.1 M EGTA, 1% Nonidet P-40, 20 mM HEPES, 20% glycerol, pH 7.9) containing protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) and DTT (1 mM) to elute nuclear proteins. After centrifugation at 13,000 rpm for 5 min, the supernatant was frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined in each extract by Bradford assay and equal amounts of protein were assayed for DNA-binding sites, as described (34). Briefly, 3.5 μg of protein was incubated for 30 min at 30°C with 32P-labeled H2K probe in binding buffer. Subsequently, the complexes were resolved by electrophoresis on a native 5% polyacrylamide gel (in 0.5× TBE). Western blotting of IκB was done after electrophoretic separation of supernatant proteins of the cell lysate in 11% SDS-PAGE using anti-IκB-α (C-21; Santa Cruz Biotechnology, Santa Cruz, CA).

**Results**

**Phagocytosed hemozoin is not degraded and does not induce apoptosis of immature and mDC**

In DC differentiating and maturing from hemozoin-loaded monocytes, confocal microscopy examination (Fig. 1) and biochemical analysis (data not shown) showed a massive and persistent presence of hemozoin heme inside the cells, confirming stability of ingested hemozoin and no hemozoin shedding as previously shown in monocyte-derived macrophages (35, 36). The confocal pictures show that the hemozoin clumps visualized by the red fluorescence of heme (represented as black spots in Fig. 1) were homogeneously distributed inside the cells and did not adhere to their external surface. Phagocytosis of hemozoin did not induce apoptosis of both iDC and mDC, as indicated by very low and uniform binding of annexin V to hemozoin- or latex-loaded 15(S)HETE-treated or unfed control cells on day 7 or 9 (data not shown). Similar conclusion was provided by measurement of [35S]methionine incorporation, an indicator of de novo protein synthesis. Incorporation was very similar in all conditions listed above (data not shown), confirming premature cell death as unlikely.

**Hemozoin inhibits expression of functionally important DC surface Ags**

The development of mDC from unfed control cells during 7 days of differentiation and additional 2 days of maturation was accompanied by the novel expression of CD83, CD80, and CD1a, increased expression of MHC class II (2.6-fold), CD54 (4.5-fold), CD40 (6.9-fold), and decreased expression of CD14 (4.5-fold).

**Mean relative MFI values ± SE at time 0 were:**

- CD14: 28.9 ± 6.2, n = 9; CD40: 2.2 ± 0.4, n = 11; CD54: 7.8 ± 2.8, n = 13; MHC class II: 49.3 ± 9.7, n = 12. Data on CD1a, CD80, and CD83 are not given, because they are not expressed in monocytes. In parallel to unfed monocytes, monocytes were allowed to phagocytose hemozoin or latex beads before induction of differentiation and maturation. The changes in Ag expression on day 9 vs monocytes on day 0 are given as relative MFI values (Fig. 2A). Compared with unfed control mDC, hemozoin-loading significantly inhibited the increase in expression of MHC class II (−86%), CD83 (−82%), CD80 (−68%), CD54 (−85%), CD40 (−53%), and CD1a (−62%). CD14 was 9% more expressed than in unfed control mDC. The differences for MHC class II, CD83, CD80, and CD40 between hemozoin-loaded vs latex-loaded mDC were also significant, indicating that changes were hemozoin-specific and not the result of the phagocytic process per se. Expression of MHC class I was unchanged in hemozoin-loaded mDC (data not shown), indicating that membrane loss during phagocytosis was not responsible for decreased Ag expression on the cell surface. Free heme could be excluded as (co)responsible for the hemozoin effects because opsonized nonparasitized RBC fed to monocytes in comparable amounts to hemozoin were degraded rapidly and the liberated heme did not modify the differentiation-dependent Ag expression pattern. The MFI ratios between mDC generated from RBC-loaded and unfed monocytes were 100 ± 41%, n = 9 for MHC class II; 94 ± 34%, n = 10 for MHC class I; 92 ± 21%, n = 10 for CD83; 87 ± 32%, n = 10 for CD54; 101 ± 18%, n = 10 for CD40; 108 ± 17%, n = 9 for CD1a; and 101 ± 41%, n = 9 for CD14. While phagocytosis of nonparasitized RBC did not interfere with DC differentiation and maturation, trophozoite-fed monocytes provoked the same impairment of DC development as hemozoin loading (data not shown).

**Hemozoin impairs DC differentiation**

The effect of hemozoin on differentiation was analyzed by comparing the expression pattern in iDC originating from control unfed or hemozoin- or latex-loaded monocytes on day 7, with
the expression in monocytes on day 0. As shown in Fig. 3, control unfed iDC were characterized by marked up-regulation of MHC class II, CD83, CD80, CD54, CD40, and CD1a, and by a remarkable down-regulation of CD14, a typical monocytic marker. Hemozoin loading markedly counteracted changes observed during the differentiation of monocytes into iDC. Up-regulation of MHC class II, CD83, CD80, CD54, CD40, and CD1a was reduced by 50–80%, while down-regulation of CD14 was not influenced by hemozoin. With the exception of CD80 and CD54, latex loading did not impair the differentiation process as concluded from the very similar Ag expression pattern compared with unfed controls. Thus phagocytosis per se can be ruled out as responsible for changes observed after hemozoin phagocytosis.

**Hemozoin impairs DC maturation**

LPS challenge exerted upon unfed iDC on day 7 markedly enhanced the expression of the maturation-dependent markers MHC class II, CD83, CD54, CD40, and CD1a, measured on mDC on day 9, while CD14 was reduced (Fig. 4). Compared with unfed control iDC, the same LPS challenge exerted upon hemozoin-loaded iDC significantly inhibited the increase in expression of MHC class II (−72%), CD83 (−93%), CD54 (−58%), CD40 (−57%), and CD1a (−54.5%), and, not significantly, that of CD80 (−68%). Reduction in expression of CD14 was not significantly impaired by 20% (Fig. 4). Similarly to differentiation, the expression of MHC class I was not changed in hemozoin-loaded cells during maturation (data not shown). Hemozoin added to unfed iDC on
day 7 was avidly phagocytosed, and impaired LPS-dependent expression of all analyzed Ags (data not shown).

Hemozoin reduces the number of high-CD83/CD1a expressors

We next quantified the number of high-CD83/CD1a expressors related to the whole cohort of cells with physical parameters (forward scatter, side scatter) characteristic for DC. These high expressors can be considered as highly mature and fully functional DC, and can be distinguished by FACS analysis. Hemozoin remarkably reduced the share of high-CD83 expressors (9.3% vs 25% and 55% in control DC on days 7 and 9, respectively), and the share of high-CD1a expressors (6.5% and 15% vs 19% and 30% in controls on days 7 and 9, respectively). The share of CD14-negative cells was not significantly modified by hemozoin (82% vs 89% in controls on day 9). Latex interfered slightly with the expression of CD83, as 18% and 37% of latex-loaded DC were high expressors on days 7 and 9, respectively, and did not modify the high-CD1a expressor subpopulation compared with control (Fig. 5).

Hemozoin decreases the CD83 mRNA levels in DC

CD83 is considered the main maturation marker for developing DC, playing an important role in the regulation of DC-mediated immune response (17, 18). Expression of CD83-specific mRNA was analyzed by RT-PCR (data not shown) and real time RT-PCR. Compared with unfed DC and latex-loaded DC, both techniques clearly indicated impaired increase of CD83-specific mRNA in iDC and mDC developing from hemozoin-loaded monocytes (Fig. 6).

In summary, data shown in the previous sections indicate that hemozoin specifically interfered with differentiation and maturation of DC, irrespective whether hemozoin ingestion occurred at an early or late stage of the differentiation-maturation process. Having
Materials and Methods. Data are presented as fold change of PPAR-γ mRNA expression in DC

FIGURE 5. Hemozoin loading reduces the number of high-CD83 and high-CD1a expressors. DC were generated in vitro from hemozoin-loaded, latex-loaded, and control unfed monocytes as detailed in Materials and Methods. Cells with physical parameters of DC were gated. CD14-negative, CD83-high positive, and CD1a-high positive cells were counted by FACScan after immunostaining as detailed in Materials and Methods. Data are percent shares of each subpopulation in the total DC population on day 7 (iDC, lower section of bar) and day 9 (mDC, full length of bar). Mean values ± SE (n = 7). Significance of differences (p < 0.05) of hemozoin-loaded vs unfed control or latex-loaded cells on day 9 (+) or day 7 (##).

established distinct inhibitory effects of hemozoin loading, we turned our attention to possible mechanisms that may explain how hemozoin impaired DC generation.

Hemozoin increases PPAR-γ mRNA expression in DC

In search of a mechanistic explanation for the hemozoin-mediated inhibition of DC maturation, we examined the effect of hemozoin loading on PPAR-γ mRNA expression. PPAR-γ belongs to a family of ligand-activated transcription factors of the nuclear receptors superfAMILY (see Refs. 19 and 20 for reviews). Our interest was stimulated by recent data connecting induction of PPAR-γ to inhibition of maturation of human DC (37–39), and, second, by the presence of high levels of lipid ligands of PPAR-γ in hemozoin (24). Indeed, hemozoin loading significantly increased (+90 ± 35%, n = 4) the level of PPAR-γ-specific mRNA in iDC compared with latex-loaded and unfed controls (Fig. 7). Hemozoin-loaded mDC also expressed remarkably higher levels of PPAR-γ mRNA, compared with both latex-loaded and unfed controls.

PPAR-γ ligand 15(S)HETE and PPAR-γ inducer 4-HNE are generated by hemozoin and inhibit differentiation and maturation of DC

Various products of lipoperoxidation, such as HETEs and hydroxyoctadecadienoic acid, are ligands of PPAR-γ (40–42). A previous study (24) has shown that hemozoin produces large amounts of HETEs via nonenzymatic heme catalysis of polyunsaturated fatty acids. Supplementation of monocytes with PPAR-γ ligand 15(S)HETE in the same concentration range as in hemozoin-loaded monocytes (24) activated PPAR-γ and induced expression of PPAR-γ mRNA in iDC, while the PPAR-γ inhibitor, GW 9662, inhibited the effects (Fig. 7). In addition, 15(S)HETE, supplemented to mDC at 0.1–20 μM final concentration, dose-dependently inhibited the CD83 surface expression (Fig. 8A). Significant inhibition of CD83 expression was also observed with the PPAR-γ activator Cigliitizone (19, 20). The PPAR-γ inhibitor GW 9662 fully reverted the inhibition of CD83 surface expression in hemozoin-loaded DC but reverted only partially the 15(S)HETE effect (Fig. 8B). Treatment of monocytes with 10 μM 15(S)HETE significantly down-regulated the expression of MHC class II (−45%, −60%), CD54 (−46%, −50%), and CD40 (−49%, −48%) in iDC on day 7 and mDC on day 9, respectively, indicating the inhibitory effect of 15(S)HETE on specific surface Ags and possibly on the whole process of differentiation and maturation to DC. 4-HNE, a terminal hydroxy-aldehyde of lipoperoxidation, found to induce expression of PPAR-γ (43) and generated in significant amounts in hemozoin-loaded monocytes (44), significantly decreased expression of CD83 in maturing DC by −30% (data not shown).

Finally, delipidized hemozoin was used to confirm the role of lipid components in hemozoin inhibitory activity. Compared with

FIGURE 6. Hemozoin loading reduces the level of CD83-specific mRNA in iDC and mDC. DC were generated in vitro from hemozoin-loaded, latex-loaded and control unfed monocytes as detailed in Materials and Methods. mRNA was extracted from monocytes on day 0, iDC on day 7 and mDC on day 9. CD83-specific mRNA was quantified by real-time RT-PCR as detailed in Materials and Methods. Data are presented as fold increase in CD83 mRNA level in iDC (left panel) and mDC (right panel) vs monocytes. Mean values ± SE (n = 4). Significance of differences of hemozoin-loaded vs control unfed or latex-loaded cells: **, p < 0.1.

FIGURE 7. Hemozoin loading and 15(S)HETE increase the level of PPAR-γ-specific mRNA in iDC and mDC. iDC (left panel) and mDC (right panel) were generated in vitro from hemozoin-loaded, latex-loaded, unfed control, and 15(S)HETE-treated monocytes, and mRNA was extracted as detailed in Materials and Methods. 15(S)HETE was supplemented at 10 μM final concentration and cells were pretreated or not with PPAR-γ inhibitor GW 9662 at 10 μM final concentration before adding hemozoin or 15(S)HETE. PPAR-γ-specific mRNA was quantified by quantitative real-time RT-PCR as detailed in Materials and Methods. Data are presented as increases or decreases in PPAR-γ mRNA level in iDC and mDC compared with controls. One representative experiment is shown of four with similar results.
untreated hemozoin, delipidized hemozoin was a less effective inhibitor of the expression of MHC-class II and costimulatory molecules, since it remarkably reduced the expression of MHC class II, CD83, CD54, and CD1a (data not shown). Work by others (45, 46) has indicated malaria-specific GPIs, potent signaling molecules, as responsible for malaria “toxicity” and monocyte impairment. Although there is no direct evidence for the presence of GPIs in untreated hemozoin, we selectively removed possibly adherent GPIs by treating hemozoin with GPI-specific phospholipase D (27). GPI-free hemozoin behaved similarly to untreated hemozoin, thus excluding GPIs as possible mediators of toxicity in our experiments (data not shown).

**Hemozoin does not affect the NF-κB system**

LPS-induced DC maturation was shown to activate NF-κB signaling (21, 22). Given the lack of induction of CD83 after LPS stimulus in hemozoin-loaded DC, we did not expect activation of NF-κB in those cells. In contrast, hallmarks of NF-κB activation such as strong translocation of NF-κB subunits p65/p50 into the nucleus, phosphorylation of inhibitory subunit IκB-α and degradation of IκB-α, were observed after LPS stimulation in hemozoin- as well as in latex-loaded DC and unfed controls (Fig. 9). These observations, indicating that hemozoin loading did not interfere with NF-κB activation, are compatible with PPAR-γ activation, as discussed below.

**Discussion**

The working hypothesis of this work was that persistent presence of hemozoin in large numbers of circulating and adherent monocytes would result in impairment and derangement of their differentiation and maturation to DC. Our experimental approach used the current in vitro model, in which the process of differentiation and maturation of monocytes to DC was mimicked by stimulating peripheral monocytes by GM-CSF and IL-4. After a few days, monocytes developed into iDC (10, 47) and became mature stimulatory DC after a subsequent challenge with LPS (29). Our results indicate that hemozoin loading inhibited the expression of MHC class II and surface costimulatory molecules typically up-regulated during the differentiation and maturation of DC, such as CD83, CD80, CD54, CD40, and CD1a. Hemozoin also inhibited the down-regulation of CD14, a monocytic marker (47). Hemozoin impaired similarly both differentiation and maturation of monocytes to fully competent mDC (Figs. 3 and 4). The inhibitory effects were specific for hemozoin and not simply the consequence of phagocytosis per se, as shown by lack of effect by phagocytosed inert latex particles. Heme-mediated inhibition could be also excluded, since phagocytosis of nonparasitized opsonized RBCs was ineffective.

The phagocytic meals used here were either crude, native hemozoin collected after spontaneous lysis of schizonts, or intact trophozoites. Both correspond to the “physiological” meals of phagocytes in vivo. Native hemozoin binds remnants of host and parasite membranes rich in unsaturated fatty acids. We have previously shown that large amounts of lipoperoxidation derivatives such as HETEs, hydroxydienoic acids, and the final hydroxyaldehyde, 4-HNE, are produced by close contact of unsaturated fatty acids with hemozoin via nonenzymatic, heme-driven catalysis (24, 46).

**FIGURE 8.** A, PPAR-γ activator 15(S)HETE inhibits dose-dependently the expression of CD83 in mDC generated in vitro from unfed monocytes. 15(S)HETE (0.1–20 μM, final concentration) was supplemented on day 0, 2, 4, and 6 to maturing DC as detailed in Materials and Methods. The relative surface expression of CD83 in mDC vs control mDC was quantified by FACScan after immunostaining as detailed in Materials and Methods. The data represent mean values ± SD, n = 16 (control); n = 4 (0.1 μM); n = 4 (1 μM); n = 7 (10 μM); n = 2 (20 μM) 15(S)HETE. B, PPAR-γ activators 15(S)HETE and Ciglitizone, and hemozoin loading inhibit the relative expression of CD83 in mDC, and PPAR-γ inhibitor GW 9662 reverses those effects. 15(S)HETE and Ciglitizone were supplemented at 10 and 20 μM final concentrations, respectively, on days 0, 2, 4, and 6 to maturing DC as detailed in Materials and Methods. GW 9662 was supplemented at 10 μM final concentration 30 min before adding 15(S)HETE or hemozoin to the cells. The relative surface expression of CD83 in mDC vs control mDC was quantified by FACScan after immunostaining as detailed in Materials and Methods. The data represent mean values ± SD, n = 16 (control); n = 14 (hemozoin), n = 7 (15(S)HETE), n = 3 (Ciglitizone), n = 3 (hemozoin plus GW 9662), n = 3 (15(S)HETE plus GW 9662). Significance of differences of hemozoin-loaded vs unfed control or latex-loaded cells: * p < 0.05; ** p < 0.1.

**FIGURE 9.** Hemozoin loading does not impair activation of NF-κB in DC. iDC were generated in vitro from hemozoin-loaded, latex-loaded, and control unfed monocytes as detailed in Materials and Methods. iDC were stimulated for maturation by LPS. The activation of NF-κB was analyzed immediately before and 1 and 48 h after LPS stimulus by EMSA of the NF-κB subunits p65/p50 (upper panel) and Western blotting of IκB-α and phosphorylated IκB-α (lower panel). One representative experiment of four is shown.
HETEs and 4-HNE were shown to be specific ligands or inducers of PPAR-γ, respectively (41–43). Finally, recent data indicate that stimulation of PPAR-γ negatively interfered with maturation of monocytes to DC (38, 39). For those reasons we focused on PPAR-γ, and were able to show that its mRNA expression was up-regulated by hemozoin, as assessed by real-time PCR, while up-regulation was blocked by the PPAR-γ inhibitor, GW 9662. Importantly, we were also able to up-regulate PPAR-γ expression and consequently inhibit expression of costimulatory molecules by supplementing 15(S)HETE in concentrations similar to those expected in hemozoin-loaded monocytes.

LPS was used here to induce the final maturation of DC. According to current views (see Ref. 48 for review) LPS binds to the membrane complex TLR 4/CD14-accessory protein MD-2, and activates an intracellular signaling pathway ultimately resulting in degradation of IkB, translocation of NF-κB to the nucleus and NF-κB-mediated transcription responses. Present data indicate that LPS stimulus applied to hemozoin-loaded iDC to elicit their full maturation apparently induced activation of NF-κB, as evidenced by the strong translocation of NF-κB subunits p65/p50 into the nucleus and the consistent phosphorylation and degradation of IkB-α. Those data are not necessarily contradictory to the inhibition of expression of genes controlled by NF-κB that consistently follows PPAR-γ up-regulation. In fact, trans-inhibition of NF-κB by ligand-activated PPAR-γ has been described by several authors, whereby PPAR-γ can effectively antagonize the NF-κB signaling pathway by the downstream physical interaction with the p65 subunit through its Rel-homology domain (49, 50).

Present data may be significant in the context of malaria immunodepression in manyfold respect. Reduction in expression of accessory molecules important for Ag presentation to T cells such as CD83, CD80, and CD1a may explain the inhibited response of T and B lymphocytes observed during malaria infection (see Ref. 4 for review). In reduction in expression of the major Ag-presenting molecule, MHC class II, may contribute to insufficient and non-adaptive Ab production (1, 4). Reduction in CD54 (ICAM-1) may explain failure of T cell priming (4) and may also lower the migratory activity of monocytes and DC (37). Reduction in CD40, a member of the TNFR family, may lower production of cytokines, inhibit maturation of DC or other cells, and indirectly contribute to decreased migration of DC (51–53). Finally, maintenance of CD14 (the LPS-receptor complex) can exclude that LPS-receptor blockade is responsible for the observed unresponsiveness to LPS stimulus.

Functionality of DC in human malaria was the object of two recent studies. In the first one, Urban et al. (54) have shown that apposition of intact parasitized RBC to iDC inhibited the maturation of DC, and parasite-exposed DC failed to induce primary and secondary Ag-specific T cell response. Present data basically agree with Urban’s final message, i.e., inhibition of DC maturation and functionality in malaria. As to the latter, data not shown indicate that hemozoin loading inhibited homologue lymphocyte proliferation by 47–54%. However, our results expand Urban’s data to show that massive phagocytosis of both trophozoites and hemozoin by monocytes or iDC was causally connected to the inhibitory effects, and to show that hemozoin loading not only affected maturation but significantly inhibited differentiation of monocytes to DC. In the second study (55), the challenge of iDC with purified hemozoin produced opposite effects to Urban’s and ours, namely hemozoin-mediated up-regulation of DC maturation markers; increase in production of IL-12 and induction of the morphological changes was typical for DC maturation. A plausible explanation of discrepancies may reside in the different characteristics of the extensively purified hemozoin used by Coban et al. (55) and crude hemozoin used here. Their extensive purification procedure may remove attached lipid and proteins, disrupt the general architecture of native hemozoin, and inhibit formation of those lipid derivatives that we consider to be responsible ultimately for hemozoin toxicity in vivo. Blunted responses elicited by phagocytosis of delipidized hemozoin seem to support above interpretation.

In conclusion, we have shown that expression of MHC class II and T cell costimulatory molecules was severely impaired in iDC and mDC developing from hemozoin-loaded monocytes, as well as in mDC developing from hemozoin-loaded iDC. Those phenotypic effects were accompanied by activation and increased expression of PPAR-γ, an antagonist of NF-κB and a down-modulator of DC differentiation. Hemozoin-mediated effects could be mimicked by synthetic and hemozoin-generated ligand and inducer of PPAR-γ, such as 15(S)HETE and 4-HNE, and blocked by PPAR-γ inhibitors. Important phenomena connected with impaired DC functionality, such as malaria immunodepression may thus ultimately depend on the activities of specific lipid derivatives. Most likely, due to the very heterogeneous compounds produced by hemozoin via heme-catalysis (24), other inhibitory molecules await identification in the future.

Acknowledgments
We thank Prof. A. Malgaroli for help with confocal microscopy and Dr. G. Giribaldi for help with real time RT-PCR.

References


