Phagocytosis of Hemozoin Enhances Matrix Metalloproteinase-9 Activity and TNF-α Production in Human Monocytes: Role of Matrix Metalloproteinases in the Pathogenesis of Falciparum Malaria

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Phagocytosis of Hemozoin Enhances Matrix Metalloproteinase-9 Activity and TNF-α Production in Human Monocytes: Role of Matrix Metalloproteinases in the Pathogenesis of Falciparum Malaria

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Matrix metalloproteinase-9 (MMP-9), secreted by activated monocytes, degrades matrix proteins, disrupts basal lamina, and activates TNF-α from its precursors. In turn, TNF-α enhances synthesis of MMP-9 in monocytes. We show here that trophozoite-parasitized RBCs/hemozoin-fed adherent human monocytes displayed increased MMP-9 activity and protein/mRNA expression, produced TNF-α time-dependently, and showed higher matrix invasion ability. MMP-9 activation was specific for trophozoite/hemozoin-fed monocytes, was dependent on TNF-α production, and abrogated by anti-TNF-α Ab and by a specific inhibitor of MMP-9/MMP-13 activity. Hemozoin-induced enhancement of MMP-9 and TNF-α production would have a 2-fold effect: to start and feed a cyclic reinforcement loop in which hemozoin enhances production of TNF-α, which in turn induces both activation of MMP-9 and shedding of TNF-α into the extracellular compartment; and, second, to disrupt the basal lamina of endothelia. Excess production of TNF-α and disruption of the basal lamina with extravasation of blood cells into perivascular tissues are hallmarks of severe malaria. Pharmacological inhibition of MMP-9 may offer a new chance to control pathogenic mechanisms in malaria. The Journal of Immunology, 2005, 175: 6436–6442.

E xcessive production of TNF-α by monocytes, macrophages, or lymphocytes is considered to be (co)responsible for hallmarks of severe malaria such as hypoglycemia, hyperthermia, and neurologic manifestations, dyserythropoiesis and immunodepression (1–5). Phagocytosed hemozoin (malarial pigment), a specific component of Plasmodium falciparum, was shown to stimulate production of TNF-α and other proinflammatory cytokines in human adherent monocytes by a presently unknown mechanism (6–8). Monocytes and macrophages release matrix metalloproteinase-9 (MMP-9), which degrades matrix proteins (9, 10) and sheds TNF-α from its circulating or cell-bound precursors (11, 12). In turn, TNF-α induces the synthesis of MMP-9 (11, 14). Enhanced production of TNF-α by hemozoin-loaded monocytes (6–8), their extravasation in the brain and other organs (15–18), and the role of MMP-9 in shedding TNF-α from its precursor (11, 12) and degrading matrix proteins (9, 10) led us to explore a possible connection between hemozoin, MMP-9, and malaria pathology. We show here that adherent human monocytes fed with hemozoin or hemozoin-containing trophozoite-parasitized RBC (trophozoites) displayed increased MMP-9 activity and protein/mRNA expression, produced soluble TNF-α time-dependently, and showed higher matrix invasion ability. MMP-9 activation was itself dependent on TNF-α production and abrogated by anti-TNF-α Ab. We also show that a specific inhibitor of MMP-9/MMP-13 activity (Merck’s inhibitor I (N-(4-biphenylcarbonyl)piperazine-2-carboxamide)) remarkably lowered production of TNF-α. Present data suggest that hemozoin phagocytosis may induce activation of MMP-9 and enhance MMP-9-dependent generation of soluble TNF-α with a 2-fold effect: first, to start and feed a positive feedback loop in which hemozoin and MMP-9 cooperate in enhancing production of TNF-α from its precursors; and, second, to disrupt basal lamina and increase extravasation of blood cells and transmigration of hemozoin-loaded monocytes. Both effects may contribute to explain excess production of TNF-α and disruption of basal lamina with extravasation of blood cells into perivascular tissues and offer new therapeutic ways to control excess production of a potentially noxious cytokine through the use of MMP inhibitors.

Materials and Methods

Materials

All materials were from Sigma-Aldrich, unless otherwise stated. Cell culture media were as follows: RPMI 1640 with or without red phenol, macrophage-serum-free medium (SFM) and AIM-V macrophage medium were from Invitrogen; TRIZol, Moloney murine leukemia virus reverse transcriptase oligo(dT), sense and antisense primers, Platinum TaqDNA Polymerase were from Invitrogen; Panserin 601 monocyte medium was from PAN Biotech; recombinant human (rh)TNF-α, blocking anti-human (b)TNF-α Ab, and Merck’s inhibitor I (N-hydroxy-1-(4-methoxyphenyl)sulfonyl)-4-(4-biphenylcarbonyl)piperazine-2-carboxamide) and a specific inhibitor of MMP-9/MMP-13 activity, were from Merck; ELISA kit for TNF-α assay was from Cayman; anti-D IgG were from Immuno AG; Percoll was from Pharmacia; Transwell apparatus with Matrigel-coated membrane (12 μm pore diameter) was from BD Biosciences; Dynabeads M-450 CD2 Pan T and M-450 CD19 Pan B were from Dynal; Diff-Quik parasite stain was from Baxter Dade; sterile plastics were from Costar; bicinechonic acid assay was from Pierce; anti-MMP-9 mAb was from Santa Cruz Biotechnology; DNA-free kit was from Ambion; Beacon Designer 2.1 software was from Premier Biosoft International; and dNTPs were from Applied Biosystem.
Cultivation of P. falciparum and isolation of trophozoite-parasitized RBCs and hemozoin

P. falciparum parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described (19). Hemozoin and trophozoite-parasitized RBCs (trophozoites) isolated from cultures during the first 2 days after infection of RBC were added to schizonts (multinucleated parasite form). After centrifugation at 5000 × g on a discontinuous Percoll-mannitol density gradient, hemozoin was collected from the 0–40% interphase and trophozoites/schizonts from the 40–80% interphase (19). Hemozoin 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 95–97% parasitemia were washed twice and resuspended in RPMI 1640. Nonparasitized RBC were treated as parasitized RBC. After isolation of trophozoites, RBC were washed twice and resuspended in RPMI 1640 for 1 h at 37°C before opsonization and phagocytosis.

Preparation and handling of monocytes

Human monocytes were separated by Ficoll centrifugation from freshly collected buffy coats discarded from blood donations by healthy adult donors of both sexes provided by the local blood bank (Associazione Volontari Italiani Sangue, Torino, Italy) (20). Separated lymphomonocytes were resuspended in RPMI 1640 medium and plated on wells of six-well plates. Each well received 2 ml of cell suspension containing 8 × 10^6 cells/ml in RPMI 1640. The plates were incubated in a humidified CO2/air-incubator at 37°C for 60 min. Thereafter, nonadherent cells were removed by three washes with RPMI 1640, and adherent cells were reclustered at 37°C overnight in RPMI 1640. Shortly before starting phagocytosis, wells were washed with RPMI 1640 and macrophage-SFM medium was added (2 ml/well). Adherent cells prepared by this method were detached from the plates by scraping, stained with specific Abb and, analyzed by FACSscan. As an average, monocytes (CD14+/CD16−) were 74.5 ± 8.5%, and lymphocytes were 50.6 ± 8.4% (mean values ± SD; n = 8) of all mononuclear cells. For selected experiments, lymphomonocytes were separated by Ficoll centrifugation from fresh buffy coats (see above), and monocytes were purified by depletion of nonmonocytic cells from lymphomonocytes. Dynabeads M-450 CD2 Pan T and M-450 CD19 Pan B (Dynal) were added to the lymphomonocytes in a 2:1 ratio for 20 min at 4°C. B and T lymphocytes were removed by biomagnetic separation as specified by the manufacturer. The remaining monocytes were washed twice and resuspended in macrophage-SFM medium. By this method monocytes (CD14+ cells) were 74.5 ± 12.7% pure (mean values ± SD; n = 8; range, 63–90.7%).

Phagocytosis by adherent monocytes of opsonized trophozoites, hemozoin and nonparasitized RBCs, and latex particles

To each well of a six-well plate with 1–10^6 adherent monocytes, 50 μl of trophozoites (10% hematocrit), hemozoin (120 nmol hemozoin heme, an amount comparable to 50 μl trophozoites on heme content basis), 50 μl of anti-D IgG-opsonized RBCs (10% hematocrit), and 50 μl of amine-modified, red-fluorescent latex particles (2.5% solids; diameter, 0.105 μm) were added. Trophozoites, hemozoin, and latex particles were opsonized with fresh autologous serum, and nonparasitized RBC were opsonized with anti-D IgG as indicated (20). After opsonization, all phagocytic monocytes were suspended in RPMI 1640. The plates were centrifuged at low speed for 5 s to start phagocytosis and incubated in a humidified CO2/air-incubator at 37°C for 3 h. Thereafter, nonadherent cells, hemozoin, and latex particles were removed by washing with RPMI 1640. The plates were then incubated in a humidified CO2/air-incubator at 37°C for the indicated times. In selected experiments, cells were incubated with rhTNF-α (20 ng/ml), blocking anti-hTNF-α Ab (30 ng/ml), or Merck’s inhibitor I, a specific inhibitor of MMP-9/MMP-13 activity (4 ng/ml) for 48 h.

Assay of MMP-9 activity by gelatin zymography

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO2/air-incubator at 37°C for 48 h. Thereafter, cells were washed and lysed at 4°C in lysis buffer containing the following: 300 mM NaCl, 50 mM Tris, 1 mM CaCl2, and 0.018% (v/v) Brij 35 (pH 7.5) with or without 5 mM EDTA to exclude aspecific bands. At the end of the incubation, the gels were stained for 15 min with Coomassie brilliant blue in methanol/acetic acid at a ratio of 30:100.60). The gels were destained in 75% methanol/25% glacial acetic acid. Densitometric analysis of the bands considered to reflect total enzymatic activity of MMP-9, was performed using a computerized densitometer (Chemidoc; Bio-Rad) with activity presented in relative units compared with background.

Assay of MMP-9 protein expression by Western blotting

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO2/air-incubator at 37°C for 48 h. Thereafter, cells were washed and lysed at 4°C in lysis buffer containing the following: 300 mM NaCl, 50 mM Tris, 1% (v/v) Triton X-100; protease and phosphatase inhibitors: 50 ng/ml pepstatin, 50 ng/ml leupeptin, and 10 μg/ml aprotinin. The protein content in the lysate was measured by the bicinchoninic acid assay and 12 μg of protein/lane were added to the loading buffer. The supernatant samples were loaded on 8% polyacrylamide gels containing 0.1% gelatin under denaturing conditions, with addition of Laemmli buffer, blotted on a polivinilidine difluoride membrane, and probed with anti-MMP-9 mAb at 1/1000 final dilution. Bands were visualized by ECL. Densitometric analysis of the bands was performed using a computerized densitometer (Chemidoc; Bio-Rad).

Assay of MMP-9 mRNA expression by real-time RT-PCR

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO2/air-incubator at 37°C for 15 h. Total cellular RNA from 2 × 10^6 cells was isolated from monocytes by TRizol, according to the manufacturer’s instructions, and eluted in 20 μl of diethyl pyrocarbonate water. To remove any contaminating DNA, RNA was treated with Ambion’s DNA-free kit. Subsequently, 6 μg of RNA was reverse transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (200 U/μl final concentration) and oligo(dT) (20 μg/ml final concentration). Real-time RT-PCR analysis was performed with iCycler iQ real-time RT-PCR Detection System apparatus and Chemidoc software, version 3.0 (Bio-Rad). MMP-9 (GenBank accession no. NM_004994) primers (forward: 5'-CTT GGA GAC CTG AGA ACC AAT C-3'; reverse: 5’-CTC TGC CAC CCG AGT GTA AC-3') were obtained from Invitrogen. Oligonucleotide sequences were identified using Beacon Designer software package and designed to span allowing the differentiation between cDNA and DNA-derived PCR products. A housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. Primer sequences were from the Bio-Rad library: forward, 5’-GAA GTG GAA GGT AGT GA-3’; reverse, 5’-CTG TGG AAT CAT ATT GGA A-3’. For each 25-μl PCR mix: 1 μl of Taq DNA polymerase (Life Technologies, 5 units/μl); 10 μl of sense primer (10 μM); 1 μl of antisense primer (10 μM); 0.5 μl of dNTP (10 mM); 1.5 μl of MgCl2 (50 mM); 1.25 U of Platinum Taq DNA polymerase (2.5 μl of buffer (10X)); 1.7 μl of SYBR Green (stock 1:10,000); and 14.55 μl of PCR-grade water were mixed together. DNA polymerase was preactivated for 2 min at 94°C, and the amplification was performed by 50 cycles (MMP-9) or 35 cycles (GAPDH) with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Relative quantitation for MMP-9 was expressed as fold variation over untreated control cells, was calculated after determination of the difference between Ct of the given gene A (MMP-9) and that of the calibrator gene B (GAPDH) using the 2^-△△Ct method. Ct values are means of triplicate measurements. To validate the use of the 2^-△△Ct method, serial dilutions of cDNA from monocytes, stimulated for 15 h by 20 ng/ml rhTNFα, were tested. Analyzed transcripts exhibited high linearity amplification plots (r > 0.98) and similar PCR efficiency (99.7% for MMP-9 and 92.2% for GAPDH), confirming that the expression of each of these genes can be directly compared with one another. The specificity of PCRs was confirmed by melt curve analysis. The melting temperatures for each amplification product were 85.8°C for MMP-9 and 86.5°C for GAPDH.

Assay of matrix invasion

Adherent monocytes were seeded for 3 h with trophozoites, hemozoin, and control meals (latex particles and nonparasitized RBC). After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO2/air-incubator at 37°C for 48 h.
Thereafter, 2 × 10⁶ monocytes suspended in 50 μl of RPMI 1640 were transferred to the upper chamber of a Transwell apparatus with Matrigel-coated membrane (12-μm pore diameter) and allowed to migrate for 16 h at 37°C. Migrated cells were stained with trypan blue and counted in a Bürker chamber. AIM-V macrophage medium containing 10% FCS was used in the lower chamber as chemotactic stimulus.

**Assay of TNF-α production**

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO₂/air-incubator at 37°C for 0.5, 1, 1.5, and 2 h (short-term incubation), and 19, 32, 48, or 72 h (long-term incubation) in presence (4 ng/ml) or absence of Merck’s inhibitor I, a specific inhibitor of MMP-9/MMP-13 activity. The level of soluble TNF-α was assayed in monocyte supernatants by specific ELISA. A standard calibration curve was generated with rHuTNF-α. The rate of TNF-α production was calculated from the half-hourly/hourly increments for the short-term incubation (time 0–2 h), and from the overall increment for the long-term incubation (time 19–72 h).

**Results**

**Enhancement of MMP-9 activity and protein/mRNA expression in adherent monocytes fed with trophozoites or hemozoin**

If not indicated otherwise, adherent monocytes were allowed to phagocytose trophozoites, hemozoin, and nonparasitized opsonized RBCs and latex particles (control meals) during 3 h. This time period maximized phagocytosis and was not sufficient to induce heme-oxygenase-mediated degradation of ingested heme (23). As an average, each monocyte ingested ~8–10 trophozoites, or hemozoin equivalent to 8–10 trophozoites in terms of ingested heme, or ~8–10 nonparasitized opsonized RBCs, as shown previously (20, 24). After termination of phagocytosis and elimination of noningested phagocytic meals by repeated washings, and further incubation during 48 h, the different parameters were measured in cell supernatants and cell lysates. MMP-9 activity measured by gelatin zymography in cell supernatants and cell lysates (Fig. 1A) and cell lysates (B), was increased 6- or 8-fold after phagocytosis of trophozoites or hemozoin, respectively, whereas phagocytosis of control meals induced distinctly lower increases. MMP-9 activity was also specifically increased after trophozoite and hemozoin phagocytosis when immunopurified monocytes (90.7% purity) were used. In the same experiment, MMP-9 activity increases were abrogated by MMP-9 inhibitor. Immunopurified monocytes of lower purity were also tested with similar results (results not shown). MMP-9 protein expression, evaluated in cell lysates by Western blotting with anti-MMP-9 Ab and densitometry (Fig. 1C), also showed a very remarkable increase after phagocytosis of trophozoite or hemozoin, whereas increase after phagocytosis of control meals was very small. MMP-9 mRNA expression, analyzed by real-time RT-PCR in cell lysates obtained 15 h after termination of phagocytosis was

**FIGURE 1.** Phagocytosis of trophozoites and hemozoin enhances MMP-9 activity in monocyte supernatants and lysates, and MMP-9 protein and mRNA expression in monocyte lysates. Adherent human monocytes were fed for 3 h with trophozoites, hemozoin, and control meals (latex particles and nonparasitized RBCs). After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO₂/air-incubator at 37°C for 48 h. Thereafter, MMP-9 activity, MMP-9 protein and mRNA expression were assayed in monocyte supernatants and lysates. Lane/column 1, Unfed control monocytes; lane/column 2, nonparasitized RBC-fed monocytes; lane/column 3, trophozoite-fed monocytes; lane/column 4, hemozoin-fed monocytes. A, Gelatin zymography and densitometric quantification of MMP-9 activity in monocyte supernatants. The 82-kDa band in the gel corresponds to MMP-9. Data are means ± SD of four independent experiments. B, Gelatin zymography and densitometric quantification of MMP-9 activity in monocyte lysates. The 92-kDa band in the gel corresponds to pro-MMP-9. Data are means ± SD of four independent experiments. C, Western blot of MMP-9 with anti-MMP-9 Ab and densitometric quantification of MMP-9 protein in monocyte lysates. The 92-kDa band in the gel corresponds to pro-MMP-9. Data are means ± SD of four independent experiments. D, Real-time RT-PCR of MMP-9 mRNA in monocyte lysates. Fifteen hours after termination of phagocytosis, total cellular DNase-treated RNA was reverse-transcribed, and MMP-9 expression was analyzed by real-time RT-PCR. Data are means ± SD of six independent experiments. A–D, All data were analyzed by Student’s t test. The differences between hemozoin/trophozoite-fed monocytes and unfed- or control-meal-fed monocytes were statistically significant with p < 0.001 (A and B), p < 0.02 (C), and p < 0.05 (D).
also remarkably up-modulated after phagocytosis of trophozoites and hemozoin, and almost unmodified after phagocytosis of control meals (Fig. 1D). Finally, the presence of MMP-9 activity was tested in trophozoite lysates obtained under the same conditions as in variously fed monocytes. Zymography of those trophozoite lysates and hemozoin supernatants constantly tested negative for MMP-9 activity in parasitized RBCs and hemozoin (data not shown).

Enhancement of matrix invasion by adherent monocytes fed with trophozoites or hemozoin

The effect of trophozoite and hemozoin phagocytosis on matrix invasion ability of monocytes was assessed by counting the monocytes able to cross a Matrigel layer in response to a chemotactic stimulus. Matrigel layer mimics the extracellular matrix in composition and biological behavior, and its degradation and enhancement of monocyte transfer are typical effects of MMP-9 activation. As shown in Fig. 2, matrix invasion ability was increased in trophozoite- and hemozoin-fed monocytes, and absent in monocytes fed with control meals.

Enhancement of TNF-α production by monocytes fed with hemozoin: abrogation of the effect by inhibition of MMP-9 activity

After a 3-h phagocytic period, the production of TNF-α was assayed time-dependently in the supernatants of hemozoin- or latex-fed monocytes, treated or not with Merck’s inhibitor I. As shown in Fig. 3A, production of TNF-α apparently started immediately after termination of hemozoin phagocytosis and still persisted at the end of the 72-h observational period. Production of TNF-α by hemozoin-fed cells was nonlinear with time, showing a progressively accelerating pace, starting at 25 pg of TNF-α/h/ml of supernatant in the first hour, 150 pg TFN-α/h/ml of supernatant in the second hour, and ~350 pg TNF-α/h/ml of supernatant up to 72 h (Fig. 3A). Phagocytosis of latex induced a very modest TNF-α production that was very close to the production by unfed control monocytes. Supplementation of Merck’s inhibitor I remarkably lowered TNF-α production irrespective of the phagocytic meal (Fig. 3B). TNF-α production, measured time-dependently over 2 h was lower by 25–30% when immunopurified monocytes (90.7% purity) were used, indicating no noticeable interference by contaminating lymphocytes. In the same experiment, TNF-α production was markedly depressed by MMP-9 inhibitor. Immunopurified monocytes of lower purity were also tested with similar results (results not shown).

Enhancement of MMP-9 activity and protein/mRNA expression by rhTNF-α: abrogation of the effect by anti-hTNF-α Ab

rhTNF-α mimicked the enhancement of MMP-9 activity and protein/mRNA expression previously described after phagocytosis of trophozoites and hemozoin. Hemozoin- or latex-fed monocytes were incubated for 48 h after termination of phagocytosis in presence or absence of rhTNF-α and blocking anti-hTNF-α Ab. As shown in Fig. 4, phagocytosis of hemozoin or latex particles increased all MMP-9 parameters considered in accordance with results reported before (see Fig. 1 for comparison). rhTNF-α supplemented to unfed or previously fed monocytes uniformly elicited maximal response of all MMP-9 parameters, whereas this response was fully and consistently abrogated by blocking anti-hTNF-α Ab.

Discussion

Phagocytosis of hemozoin or trophozoites, the hemozoin-containing late parasite forms, impairs the functionality of monocytes and macrophages. For example, trophozoite/hemozoin-fed monocytes were unable to mount oxidative burst and repeat phagocytosis (20), did not kill ingested bacteria (25), did not present Ags correctly (26), and did not differentiate and mature to functioning dendritic cells (19). In addition, trophozoite/hemozoin-fed monocytes shed large amounts of bioactive peroxidation derivatives of polyunsaturated fatty acids (27) and produced TNF-α and other proinflammatory cytokines (6–8) by an unknown mechanism.

We show here that phagocytosis of trophozoite/hemozoin by adherent human monocytes induced remarkable increase in activity, protein, and mRNA expression of intracellular MMP-9, and also a roughly parallel release of MMP-9 activity into the monocyte supernatants. Other phagocytic meals, such as nonparasitized RBCs or inert latex particles, also induced a modest increase in MMP-9 activity and its release into the monocyte supernatants. However, those nonspecific effects were small compared with the trophozoite/hemozoin effects. MMP-9 activity was not contributed by parasite components. In fact, cell extracts of isolated trophozoites and hemozoin supernatants tested negative for MMP-9 activity (data not shown). We further show that hemozoin-fed monocytes produced TNF-α and released it into the supernatant at a progressively accelerating pace, starting at 25 pg TNF-α/h/ml of supernatant in the first hour, and leveling off at a constant rate of ~350 pg TNF-α/h/ml of supernatant from hour 2 until the end of the observational period of 72 h. Activity of MMP-9 and production of TNF-α were mutually correlated because of two reasons. First, abrogation of MMP-9 activity by a specific inhibitor of MMP-9/MMP-13 reduced TNF-α production to control levels. Abrogation was not complete, however, indicating that other TNF-α-shedding proteases, such as TNF-α converting enzyme (TACE) insensitive to the inhibitor used here (28), may play a minor role in TNF-α accumulation. Second, administration of exogenous rhTNF-α was followed by maximal increase in MMP-9 activity and expression, whereas neutralization of TNF-α by blocking Ab...
fully abrogated MMP-9 activation. The bidirectional connection between MMP-9 and TNF-α documented here is in agreement with known characteristics of both molecules. MMP-9 and TACE proteolytically process the TNF-α 26-kDa cell-associated preform to the 17-kDa secreted form. In contrast, TNF-α together with a number of other proinflammatory cytokines have been shown to induce the expression of MMP-9 and other MMPs of the same family (13, 14). Taking into account present data and the above-mentioned molecular characteristics, a likely model to explain accumulation of TNF-α would include a cyclic reinforcement loop in which hemozoin enhances production of TNF-α, which in turn induces activation of MMP-9 and shedding of TNF-α into the extracellular compartment. Such a mechanism could ensure attainment and maintenance over longer periods of time of the high levels of TNF-α observed in severe malaria (1–4, 29), in front of the very short half-life of TNF-α in plasma. A vast number of data indicate that increased systemic levels of TNF-α were associated with manifestations of severe falciparum malaria, including hypoglycemia, cerebral malaria, renal, hepatic, pulmonary damages, circulatory failure, and placental pathology (1–5). Thus, the hemozoin-mediated increase in MMP-9 activity and expression may represent the causal link between enhanced cytokine production and malaria severity. Recently, lymphotixin-α (LT) and not TNF-α was shown to be responsible for development of cerebral malaria in a murine model (30). Although no data on involvement of LT in human cerebral malaria have been published yet, we cannot exclude that LT, an analog of TNF-α with shared receptors and similar cellular effects, may play a role in cerebral malaria pathogenesis.

A second important activity of MMPs is the disruption of the subendothelial basal lamina (9, 10). Of note for malaria, we found here that matrix invasion ability, i.e., crossing Matrigel layers that mimic the basal lamina, was increased in trophozoite- and hemozoin-fed monocytes. Transfer of those in vitro data to the situation in vivo may explain disruption of the basal lamina, extravasation of mononuclear cells and RBCs, and migration into perivascular tissues of monocytes and granulocytes observed in falciparum malaria. A variety of studies have documented mononuclear cell infiltration and microhemorrhages in different organs, such as kidney, lung, and brain, as a consequence of focal lesions of the basal lamina (15, 16). Importantly, cerebral malaria, a diffuse encephalopathy caused by massive sequestration of parasitized RBCs in the brain capillaries, is associated with elevated plasma levels of TNF-α, disruption of endothelial intercellular junctions and basal lamina, mononuclear cell margination, ring hemorrhages, and Dürck’s granulomas infiltrated with macrophages (5, 15–18). Interestingly, an autopsy study in Vietnamese patients with fatal cerebral malaria has shown that endothelial alterations and disruption of the basal lamina were colocalized with sequestered parasites (mostly trophozoites), implying that trophozoite binding may directly affect structure and functional integrity of the endothelial barrier (31).

Finally, extensive abrogation of TNF-α production by Merck’s inhibitor I, a specific MMP-9/MMP-13 inhibitor and a member of a numerous class of anti-MMP drugs already available for human use (32, 33), may open a new, unexpected, and yet-unexplored lane for therapeutic intervention in malaria.
FIGURE 4. rhTNF-α mimics the enhancement of MMP-9 activity and protein/mRNA expression induced by hemozoin phagocytosis. Abrogation of the effect by anti-hTNF-α Ab. Adherent human monocytes were fed for 3 h with hemozoin (A–C, lanes/columns 7–9) and latex particles (A–C, lanes/columns 4–6). After termination of phagocytosis, cells were further incubated at 37°C for 48 h (A and B) and 15 h (C) in presence of rhTNF-α (20 ng/ml) (lanes/columns 2, 5, 8) or blocking anti-hTNF-α Ab (30 ng/ml) (lanes/columns 3, 6, and 9). A, Gelatin zymography and densitometric quantification (■) of MMP-9 activity in monocyte supernatants. Data are means ± SD of four independent experiments. B, Western blot with anti-MMP-9 Ab and densitometric quantification (■) of MMP-9 protein expression in monocyte lysates. Data are means ± SD of four independent experiments. C, Real-time RT-PCR of MMP-9 mRNA expression in monocyte lysates. Data are means ± SD of six independent experiments. See Fig. 1 for details. All data were analyzed by Student’s t test. Numbers refer to lanes/solid columns. Levels of rhTNF-α-stimulated cells (lanes/columns 2, 5, and 8) were similar to those of hemozoin-fed cells (lane/column 7) without significant differences; levels of anti-hTNF-α Ab-stimulated cells (lanes/columns 3, 6, and 9) were similar to those of control cells (lane/column 1) without significant differences. The differences between hemozoin-fed cells/rhTNF-α-stimulated cells and control/anti-hTNF-α Ab-stimulated cells were statistically significant with p < 0.001 (A), p < 0.05 (B), and p < 0.01 (C).

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Disclosures
The authors have no financial conflict of interest.

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