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Malaria pigment stimulates chemokine production by human microvascular endothelium

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ABSTRACT

Severe falciparum malaria is characterized by the sequestration of infected erythrocytes and leukocyte recruitment in the microvasculature, resulting in impaired blood flow and metabolic disturbances. Which parasite products cause chemokine production, thus contributing to the strong host inflammatory response and cellular recruitment are not well characterized.

Here, we studied haemozoin (Hz), the end-product of haem, a ferriprotoporphyrin-IX crystal bound to host and parasite lipids, DNA, and proteins. We found that natural Hz isolated from *Plasmodium falciparum* cultures induces CXCL8 and CCL5 production in human dermal microvascular endothelial cells (HMEC-1) in a time-dependent manner. This up-regulation is not caused by haem but rather by Hz-generated lipoperoxidation products (15-HETE) and fibrinogen associated to Hz, and is, at least in part, triggered by the activation of NF-κB, as it was significantly inhibited by artemisinin and other NF-κB pathway inhibitors.

1. Introduction

Inflammatory cytokines, together with micro-circulatory obstruction, hypoxia and metabolic disturbances, contribute to severe falciparum malaria complications, such as cerebral malaria, which is fatal if not promptly treated (Polimeni and Prato, 2014). Host leukocytes are recruited by chemokines to the site of parasite sequestration in the brain of malaria patients (Hunt and Grau, 2003; Taylor et al., 2004) and contribute to tissue inflammation and disease severity.

The potential, although controversial role of chemokines in the pathogenesis of severe malaria is supported by a series of observations (Ioannidis et al., 2014). In particular, increased levels of CXCL9, a T cell chemoattractant, and of CXCL8 (Interleukin-8, IL-8, a chemoattractant primarily for neutrophils), have been measured in plasma of adults and children with *Plasmodium falciparum* malaria (Ayimba et al., 2011; Berg et al., 2015; Burgmann et al., 1995), while lower levels of other chemokines, such as CCL5 (Chemokine (C-C motif) ligand 5, aka RANTES, a chemoattractant for T cells, monocytes and eosinophils), have been reported in children with *P. falciparum* malaria (Ochiel et al., 2005; Were et al., 2006). However, it should be noted that chemokine concentrations at the site of sequestration may not be reflected by circulating levels. Indeed, increased transcription of CCL5 has been observed in brain samples from patients with cerebral malaria (Sarfo 2004). CCL5 is also up-regulated in the brain of C57BL/6 mice infected with *P. berghei* ANKA (Hanum et al., 2003). Increased presence of leukocytes and platelets, as well as up regulation of different cytokines and chemokines has been shown in murine models of cerebral malaria (Deroost et al., 2016; Hansen et al., 2007). However, the contribution of different host cells and parasite products to the chemokine "storm" generated by the sequestration of parasites at the level of microvessels is still unclear.

Haemozoin (Hz), or malaria pigment, is a parasite product derived from the catabolism of haemoglobin and released into the circulation after schizont rupture at each parasite replication cycle. It accumulates in multiple organs, where its presence has been associated with both activation and inhibition of the host inflammatory responses (Giusti et al., 2011; Olivier et al., 2014; Polimeni and Prato, 2014). In murine malaria models, too, Hz accumulates in different organs and contributes to local increase of oxidative stress response and damage of lung tissue (Deroost et al., 2014; Scaccabarozzi et al., 2015). Moreover, intrave-

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nously injection of *P. falciparum* derived Hz itself has been shown to be able to induce the expression of different cytokines and chemokines in the liver (Deroost et al., 2014).

Natural Hz is a crystal of ferriprotoporphyrin-IX bound to host and parasite lipids, DNA, and plasma proteins like fibrinogen (Barrera et al., 2011; Goldie et al., 1990). Hz can generate peroxidation and hydroxylation products of poly-unsaturated fatty acids (PUFAs) such as 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid (15-HETE) and aldehyde 4-hydroxynonenal (4-HNE) (Schwarzer et al., 2003). In particular, 4-HNE can inhibit erythropoiesis and dendritic cells maturation (Giribaldi et al., 2004; Skorokhod et al., 2004; Skorokhod et al., 2010), while 15-HETE can enhance the expression and secretion of cytokines and chemokines as well as lytic enzymes in human monocytes through NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) and p38 MAPK-dependent mechanisms (Giribaldi et al., 2010; Khadjavi et al., 2014; Polimeni et al., 2013a).

It is not known which of the components of natural Hz contributes to the immune response at the site of parasite sequestration. It has been reported that parasite products, such as DNA, can activate macrophages through the interaction with the immune receptor TLR-9 within the phagolysosome (Parroche et al., 2007). Moreover, serum components, such as fibrinogen, bound to Hz can activate monocytes via TLR-4 and integrins interaction (Barrera et al., 2011). On endothelial cells, Hz can modulate the expression of adhesion molecules and the production of inflammatory and vasoactive mediators (Basilico et al., 2010; Taramelli et al., 1998), and promote the production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Prato et al., 2011; Prato and Giribaldi, 2011).

However, little is known about the contribution of Hz to the release of pro-inflammatory chemokines by endothelial cells. This study aimed to assess the effects of Hz in lymphocyte recruitment to target areas, and thus overall contribution to disease severity, by measuring its effects on the production of CXCL8 and CCL5 chemokines by a human microvascular endothelial cell line (HMEC-1). Furthermore, we explored which Hz biochemical components were responsible for these effects.

2. Materials and methods

2.1. Materials

All materials were from Sigma-Aldrich, St Louis, MO, unless otherwise stated. MCDB 131 medium was from Invitrogen, Carlsbad, CA; RPMI 1640 medium was from GIBCO BRL, Grand Island, NY; foetal calf serum was from HyClone, South Logan, UT; epidermal growth factor was from PeproTech, Rocky Hill, NJ. HEPES, L-glutamine, were from EuroClone (Italy).

2.2. HMEC-1 and P. falciparum cultures

A long-term cell line of dermal microvascular endothelial cells (HMEC-1) immortalized by SV 40 large T antigen was kindly provided by the Center for Disease Control, Atlanta, GA. Cells were maintained in MCDB 131 medium supplemented with 10% foetal calf serum, 10 ng/ml of epidermal growth factor, 1 μg/ml of hydrocortisone, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 20 mM Hepes buffer, pH 7.4. *P. falciparum* parasites (D10 and W2 mycoplasma free) were kept in culture as described (Ilboudo et al., 2013) at 5% hematocrit (human type A RBCs) at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated A+ human plasma, 20 mM Hepes buffer, pH 7.4, in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2.

2.3. Preparation of natural and synthetic hz

To isolate Hz, parasitized RBCs (pRBCs, 4–8% parasitemia) were washed twice with serum-free culture medium, resuspended to 25% haematocrit and fractionated on a discontinuous Percoll/4% sorbitol (wt/vol) gradient (0, 40, 80%) (Omodeo-Salè et al., 2003). After centrifugation at 1075 × *g*, Hz was collected at the top of the 0–40% gradient interphase, washed three times with PBS and stored at −20 °C. The haem content of Hz was determined by spectrophotometry (λ = 405 nm) dissolving an aliquot in 1 M NaOH. The concentration of haem was calculated by comparison to a standard curve of haemin. The doses of Hz used in this study are biologically relevant and can be easily reached in vivo (Corbett et al., 2015). Synthetic Hz (Beta-haematin) was prepared in methanol as previously described (Bonifacio et al., 2008).

2.4. HMEC-1 treatment

HMEC-1 were seeded at 10^5 cells/well in 24-well flat bottom tissue culture clusters. After overnight incubation, monolayers were exposed to Hz (10–2.5 μg/ml), either alone or with artemisinin (10 μM) or partenolide (10 μM) or minocycline (100, 50, 25 μM). In some experiments cells were stimulated with IL1β (100U/ml) or TNFα (100 U/ml) in the presence of Hz (10 μg/ml). Cells were also exposed to fibrinogen (FG) (200-20-2 μg/ml), HETE (10-1-0.1 μM), 4-HNE (10 μM) in a humidified CO_2/air-incubator at 37 °C for 72 h. In some experiment, polymyxin B (10 μg/ml) was used in the presence of Hz, FG or lipopolysaccharide (LPS). The concentrations of Hz used are biologically relevant (Corbett et al., 2015). All experiments were performed in serum-free medium. At the end of each treatment, supernatants were collected and frozen for chemokines determinations.

Chemokine determination: CXCL8 and CCL5 were measured in cell supernatants by DuoSet ELISA Kit (R & D System), following the manufacturer's instructions.

2.5. Statistical analyses

All data were obtained from three independent experiments and the results are shown as mean ± standard deviation. Differences between groups were analyzed for statistical significance by using 1-way or 2-way ANOVA test, followed by the must suitable post hoc multiple comparison test (Bonferroni's, Sidak's or Tukey's).

3. Results

3.1. Effect of HZ on chemokine production by HMEC-1

HMEC-1 were left untreated (control) or treated with different doses of natural Hz for different lengths of time. Thereafter, CXCL8 and CCL5 levels were measured in cells supernatants. As shown in Fig. 1, basal production of both chemokines was low. After exposure to Hz the production of both CXCL8 and CCL5 increased in a time- dependent manner. Treatment with 5, 10 and 20 μg/ml of Hz produced a percent increase of 127 ± 9, 176 ± 56, and 197 ± 53 for CXCL8 production, and 142 ± 24, 174 ± 8 and 213 ± 27 for CCL5 (Fig. 1A and B). Cell viability was unaffected: the release of cytoplasmic LDH was comparable in control and treated cells (data not shown). The amounts of both CXCL8 and CCL5 increased over time in control, reflecting chemokines accumulation in the supernatant, and in Hz-treated cells (Fig. 1C and D); the release of both chemokines was approximately two, three-fold higher in Hz-treated cells than in controls. The production of other cytokines/chemokines (TNFα, IL1β, IL6, MIP1β) by HMEC-1 in response to Hz was also investigated, with negative results (data not shown).

3.2. Modulation of hz-dependent release of CCL5 and CXCL8 by pro- and anti-inflammatory agents

Cells were treated with 10 μM (dose chosen based on (Aldieri et al.,